**Diagnostics**
**Diagnostic**

# *Ceratitis capitata*

## Specific scope

This standard describes a diagnostic protocol for *Ceratitis capitata*.[1]

## Specific approval and amendment

Approved in 2011–09.

## Introduction

*Ceratitis capitata* is the most serious pest for citrus and many other fruits in the majority of countries with a warm, Mediterranean, tropical or subtropical climate (EPPO/CABI, 1997).

Native to sub-Saharan Africa, *Ceratitis capitata* has spread to Mauritius, Reunion, Seychelles, North Africa, Southern Europe, the Middle East, Western Australia and to parts of Central South and North America. Details on its current geographical distribution are available in the EPPO Database Plant Quarantine data Retrieval system (EPPO, 2011).

*Ceratitis capitata* is a highly polyphagous species whose larvae develop in a very wide range of unrelated fruits. In the EPPO region, important hosts include apples (*Malus pumila*), avocados (*Persea americana*), *Citrus* spp, figs (*Ficus carica*), kiwifruits (*Actinidia deliciosa*), mangoes (*Mangifera indica*), medlars (*Mespilus germanica*), pears (*Pyrus communis*), and *Prunus* spp. (especially peaches, *P. persica*). *Ceratitis capitata* affects practically all the tree fruit crops, but has also been recorded on wild hosts belonging to a large number of families; White & Elson-Harris (1992) give a more detailed host list by region. Additional information on the biology of the pest can also be found in EPPO/CABI (1997).

## Identity

**Name**: *Ceratitis capitata* (Wiedemann, 1824).
**Synomyms**: *Ceratitis citriperda* MacLeay, *Ceratitis hispanica* De Breme, *Pardalaspis asparagi* Bezzi, *Tephritis capitata* Wiedemann.

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

**Taxonomic position**: Diptera Brachycera Tephritidae.
**EPPO code:** CERTCA.
**Phytosanitary categorization**: EPPO: A2 no. 105.

## Detection

Fruit flies may be detected as eggs or larvae in fruits or as adults caught in traps.

### Detection on fruits

Attacked fruit will often have puncture marks made by the female's ovipositor. Sometimes there may be some tissue decay or secondary rot around these marks, and some fruits with a very high sugar content (e.g. *Prunus persicae*) exude globules of sugar which are usually visible surrounding the oviposition puncture (White and Elson-Harris, 1992). Rotting of the underlying tissue causes a depression on the surface.

A primary method of collecting larvae is by cutting infested fruit. When the surrounding air temperature is warm, fully grown larvae flex and 'jump' repeatedly up to 25 mm when removed from fruit. Larval identification is extremely difficult, so that when feasible it is best to rear them to adults for identification. Infested fruits should be placed in a container that has a gauze or muslin top and dry medium at its base, such as sterilized sawdust or sand, in which emerging larvae can pupate. Samples should be checked every 2 days for puparia and fruit from which larvae have emerged should be discarded. When all the larvae have emerged from the fruit or if any sign of mould appears the sawdust should be sieved and the puparia collected. Puparia can then be transferred to petri dishes and covered with a thin layer of moist heat-sterilized sawdust and then placed in a small emergence cage. It is important to provide sugar solution as food for the emerging adults and to keep the adults alive for at least 4 days after emergence, so that the flies develop their full body


 

colouration and normal shape. Failure to feed the flies will result in specimens that have shrivelled abdomens and dull colours making identification problematic (White & Elson-Harris, 1992).

When larvae are to be preserved, they should be placed in boiling water for a few seconds and then transferred to 70% ethanol. Other procedures can also be used.

### Detection of adults

Adult males of *C. capitata* can be monitored by traps baited with Tri-Med-Lure, but both females and males can be monitored by Bio-Lure or by sticky traps. Additional information on trapping is available in EPPO/CABI (1997).

## Identification

Morphological identification is only reliable based on characteristics of the adult specimen. A molecular test is also described that can be performed on adults or larvae.

### Morphological identification

Please note that morphological terminology follows White & Elson-Harris (1992).

Morphological identification with a binocular microscope is the recommended diagnostic method. Magnification × 10 for adult to × 200 for larvae.

Note that a reliable morphological identification can only be performed on an adult specimen. Although larvae are described below, identification based on this stage is not recommended.

*Description for egg, larva and pupa stage*

• Egg: (after Weems, 1981)

Very slender curved, 1 mm long, smooth and shiny white. Micropylar region distinctly tubercular.

• Larva: (after Weems, 1981)

Elongate and pointed at head end. Length of 1st instar larva 1 mm or less, body mostly transparent; 2nd instar body partially transparent; fully grown 3rd instar 6.8–8.2 mm, body fully opaque white or colour of ingested food. Exact size and colour of larva depends on diet (Fig. 1). Head with accessory teeth near oral hooks.

Anterior spiracles in characteristic, almost parallel, pattern not on raised surface, and without rings or semicircles; typically bears 7–10 lobes or digits in a simple arc or nearly straight line.

Caudal spiracles in characteristic, almost parallel, pattern not on raised surface and without black ring or semicircles. Distinct low ridge connecting two tubercles or posterior swellings (observed on dry larval surface). Primary larval identification characters used: mouth hooks and cephalo-pharyngeal skeleton, anterior spiracles (Fig. 2), rear view maggot (posterior view and lateral view of posterior end), and shape and arrangement of caudal spiracles.

Larval identification is based primarily on characteristics of mature 3rd instar larvae. However, this identification has a high level of uncertainty. For identification of the family Tephritidae, see Stehr (1991); for identification of the genera and species *Ceratitis capitata* larvae, see White & Elson-Harris (1992), but it should be noted that this key is based on old and inadequate descriptions and does not include all *Ceratitis* spp. of economic importance.

• Pupa: (after Weems, 1981)

Cylindrical 4–4.3 mm long, dark reddish brown, resembling swollen grain of wheat.

*Description and specific determination for adult stage (after Weems, 1981)*

Length 3.5–5 mm, yellowish with brown tinge, especially on abdomen, legs, and some markings on wings (Fig. 3). Wing markings very characteristic (Fig. 4). Lower half of occiput with white setae. Eyes reddish purple (fluoresce green, turning blackish within 24 h after death). Ocellar bristles present (Fig. 5). Male has pair of modified bristles with apical end dark and diamond-shaped, next to inner margins of eyes (Fig. 6). Postpronotum (humerus) white, with distinct black spot. Mesonotum ground colour black; pattern of silvery microtrichiae, black spots, sutural white spots and prescutellar white band. Humeral bristles present. Dorsocentral bristles slightly posterior to anterior supra-alar. Scutellum yellow-white basally, apically with three merged black spots (Fig. 7). Abdomen oval, yellow, with silverish bands on posterior margins

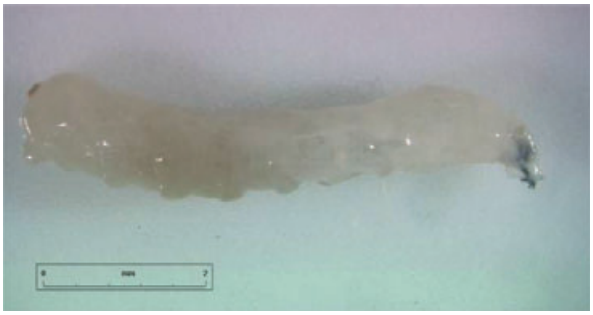

**Fig. 1** *Ceratitis capitata* larvae.

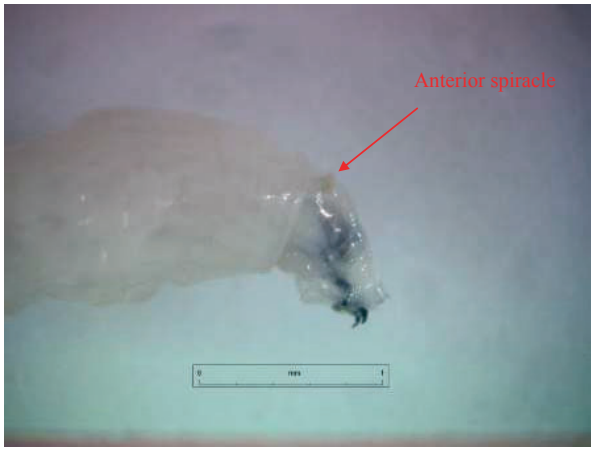


**Fig. 2** Head of larvae *Ceratitis capitata*.

**Fig. 3** *Ceratitis capitata.*

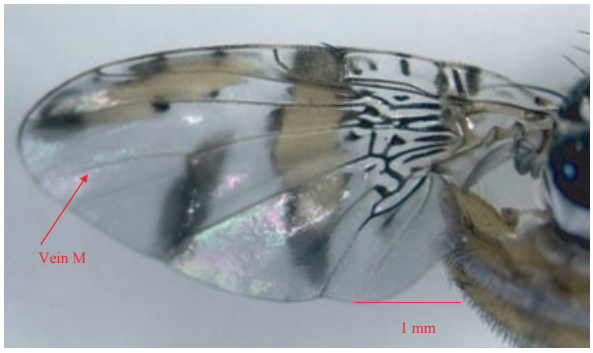


**Fig. 4** Wing of *Ceratitis capitata.*

of terga two and four and with fine black bristles scattered on dorsal surface. Extended ovipositor 1.2 mm long. Wings usually held in a drooping position on live flies, are broad and hyaline. Bands on wing well developed, predominantly yellow. Basal part with characteristic pattern of streaks and spots typical of genus. Basal and marginal bands brownish yellow, usually not touching. Cubital band black, straight and free. Apex of anal cell elongate.

Males of *C. capitata* are easily distinguished from all other species of this family (see Appendix 1) by the characteristic diamond-shaped (capitate) expansion at the apex of the anterior pair of orbital bristles (Fig. 6). Females can be distinguished by the characteristic wing pattern (Fig. 4) and by the apical half of the scutellum being entirely black (Fig. 7).

Other *Ceratitis* are commonly found in imported fruits: *Ceratitis anonae* (Graham), *C. cosyra* (Walker) and *C. rosa* Karsch. However, they can be easily distinguished by the pattern on the scutellum (Figs 8 and 9).


**Fig. 5** Head of female *Ceratitis capitata.*

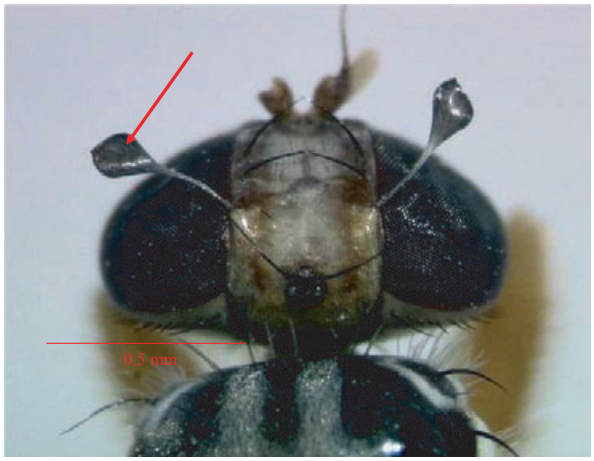


**Fig. 6** Head of male *Ceratitis capitata.*


**Fig. 7** Scutellum of *Ceratitis capitata.*

## Molecular methods

Molecular biological identification is performed by using a PCR-RFLP method based on ITS1 primers described by Douglas and Haymer (2001), and can be used to confirm morphological

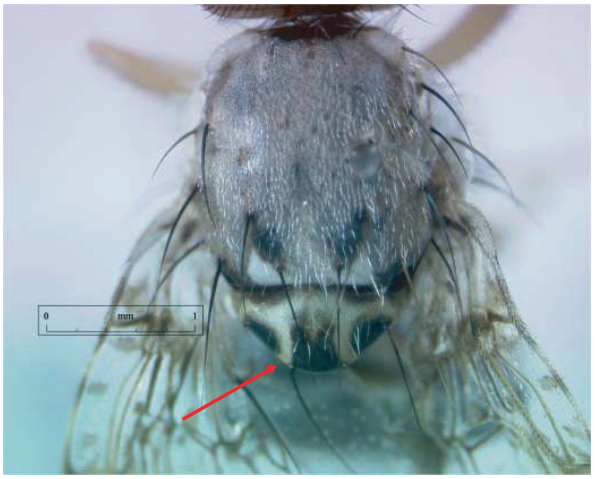

**Fig. 8** Scutellum of *Ceratitis rosa*.

**Fig. 9** Scutellum of *Ceratitis cosyra*.

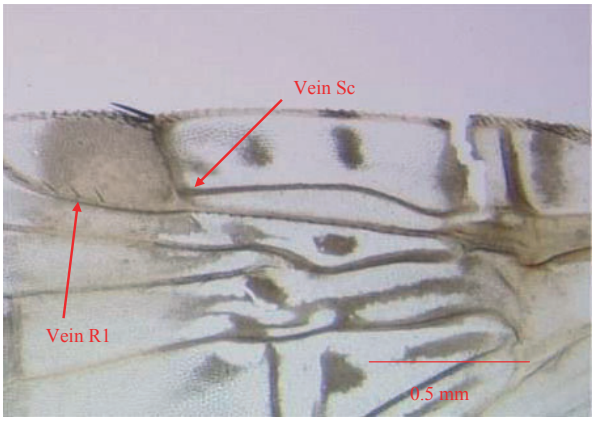


**Fig. 10** *Ceratitis capitata* Vein Sc and Vein R1.

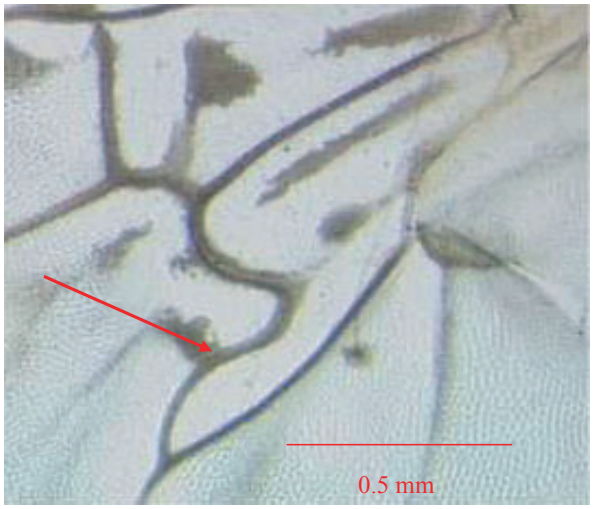


**Fig. 11** *Ceratitis capitata* extension of cell cup.

examination of adults and larvae. It is likely that the test will work equally well on eggs and pupae, but no validation data is available to support this. For technical details see Appendix 2.

## Reference material

Specimens are available in many laboratories in the EPPO region.

Positive Amplification Controls (*C. capitata* WGA product) for the ITS1 PCR-RFLP assay can be obtained from BTLH van de Vossenberg, National Reference Laboratory, Wageningen, The Netherlands (for address see below).

## Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM7/77 (1) *Documentation and reporting on a diagnosis.*

## Further information

Further information on this organism can be obtained from:

V Balmès, France. E-mail: valerie.balmes@anses.fr.

BTLH van de Vossenberg, the Netherlands. E-mail: b.t.l.h.van.de.vossenberg@minlnv.nl.

## Acknowledgements

This protocol was originally drafted by Ms V. Balmès, Laboratoire de la Santé des Végétaux, unité entomologie et plantes invasives- CBGP – Campus internationnnal de Baillarguet CS30016- 34988 Montferrier sur Lez, France. E-mail: valerie.balmes@anses.fr.

The molecular part was drafted by Mr B.T.L.H. van de Vossenberg, National Reference Laboratory, PO Box 9102, 6700HC Wageningen, the Netherlands. E-mail: b.t.l.h.van.de.vossenberg@minlnv.nl.

## 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.fr.

## References

Douglas L & Haymer D (2001) Ribosomal ITS1 polymorphisms in *Ceratitis capitata* and *Ceratitis rosa* (Diptera:tephritidae). *Annals of the Entomological Society of America* **94**, 726–731.

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Papp L & Darvas B. (2000) Contribution to a Manual of Palaearctic Diptera, Volume 1. Science Herald, Bupapest. pp. 978.

Sambrook J, Fritsch EF & Maniatis T (1989) Molecular Cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor (US).

Stehr FW (1991) Immature Insects, Volume 2. Kendall /Hunt Publishing Company, Dubuque, Iowa (US), pp. 975.

Weems Jr HV (1981) Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) *Florida Department of Agriculture, Consumer Services, Plant Industry,* Entomology Circular n° 230 September 1981, pp. 8.

White IM & Elson-Harris M (1992) Fruit Flies of Economic Significance: their identification and bionomics. *International Institute of Entomology*. CAB International, Wallingford (GB).

## Appendix 1

For identification of the Family Tephritidae, see Papp & Darvas (2000).

### Identification of adult *Ceratitis capitata*

(Note that this key adapted from White & Elson-Harris (1992) is not exhaustive. It will only separate *Ceratitis* from the four other major pest genera, and *C. capitata* from a few other *Ceratitis* spp. Users should ensure that the specimens match the species description given).

| | | |
|---|---|---|
| 1 | Subcostal vein abruptly bent and dorsal side of vein R1 with setulae (Fig. 10) | Tephritidae 2 |
| | Subcostal vein not abruptly bent or dorsal side of vein R1 lacks setulae | Other families |
| 2 | Cell cup with sinuous extension (as shown in Fig. 11) | *Ceratitis* 3 |
| | Cell cup with extension of another shape | Other genera |
| 3 | Scutellum yellow-white basally, apically with three merged black spots (Fig. 7) | 4 |
| | Scutellum different (Figs 8 and 9) | Other species |
| 4 | Wing with apex of vein M not covered by a diagonal coloured band (Fig. 4). Apical end of male anterior pair of orbital bristles dark and diamond-shaped | *Ceratitis capitata* (Wiedemann) 5 |
| | Wing with apex of vein M covered by a diagonal crossband. Male anterior pair of orbital bristles different (Fig. 6) | Other species |
| 5 | Anterior pair of orbital bristles modified with apical end dark and diamond-shaped | *Ceratitis capitata* (male) |
| | Anterior pair of orbital bristles not modified (Fig. 5). Sclerotized oviscape at apex of abdomen. | *Ceratitis capitata* (female) |

## Appendix 2 – ITS1-based PCR-RFLP test for the identification of *Ceratitis capitata* adults and larvae

### 1. General information

1.1 Protocol developed by the Plant Protection Service, the Netherlands (2004, Data not published).

1.2 Adults and/or larvae serve as input for DNA extraction. *Ceratitis capitata* eggs and pupae have not been tested.

1.3 The assay is designed to internal transcribed spacer (ITS) 1 sequences of *C. capitata*.

1.4 Primers described by Douglas and Haymer (2001) located in the 18S and 5.8S regions spanning the ITS1 region: forward primer ITS1-F5 (5′- CAC GGT TGT TTC GCA AAA GTT G – 3′) and reverse primer ITS1-B9 (5′- TGC AGT TCA CAC GAT GAC GCA C – 3′), each used at a final concentration of 0.4 μM.

1.5 ITS1 primers ITS1-F5/ITS1-B9 amplify 1020 bp of *C. capitata* DNA. Primers are not specific for *C. capitata* and have been found to amplify the ITS1 region from species belonging to the genera *Anastrepha, Bactrocera, Ceratitis, Delia* and *Rhagoletis*.

1.6 Platinum®Taq DNA Polymerase (5 U $\mu L^{-1}$, Invitrogen) used for PCR amplification at a final amount of 1 Unit.

1.7 *Dra*I, *Hinf*I, *Ssp*I and *Taq*I (10 U $\mu L^{-1}$, Promega) used for amplicon digestion at a final amount of five Units.

1.8 A *C. capitata* specimen is identified when the PCR product is digested as follows: *Dra*I: 400, 350, 170 and 100 bp, *Hinf*I: 900 and 120 bp, *Ssp*I: 510, 230, 150, 100 and 30 bp and *Taq*I: 480, 470 and 70 bp.

1.9 Nucleotides are used at a final concentration of 0.2 mM each.

1.10 10 × PCR Buffer, Minus Mg (Invitrogen), used at a final concentration of 1 ×.

1.11 10 × restriction enzyme buffers (Promega), used at a final concentration of 1 ×.

1.12 $MgCl_2$ (50 mM, Invitrogen) used at a final concentration of 4.0 mM.

1.13 Molecular grade water (MGW) is used to make up reaction mixes; this should be purified (deionised or distilled), sterile (autoclaved or 0.45 μM filtered) and nuclease free.

1.14 Amplification is performed in a Peltier-type thermocycler with heated lid, e.g. PTC-200 (MJ-Research).

### 2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 Larvae and/or (parts of) adults (e.g. a single leg) serve as input for DNA extraction.

2.1.2 DNA is extracted using the High Pure PCR Template Preparation Kit (Roche) according to the mammalian tissue protocol. Alternatively, the Blood & Tissue Kit (Qiagen) according to the animal tissue protocol can be used.

2.1.3 Crushing of the insect in a lysis buffer (provided by manufacturer) prior to DNA extraction is required.
2.1.4 For both the Roche-Kit and the Qiagen-Kit, DNA is eluted in 50 µL preheated elution buffer (provided).
2.1.5 After DNA extraction, no DNA clean-up is required.
2.1.6 Either use extracted DNA immediately or store it at −20°C until use.

2.2 Polymerase Chain reaction
2.2.1 Total reaction volume of a single PCR reaction is 50 µL
2.2.2 34.8 µL MGW.
2.2.3 5.0 µL 10 × reaction buffer, minus Mg (Invitrogen) final concentration 1 ×.
2.2.4 4.0 µL $MgCl_2$ (50 mM, Invitrogen), final concentration 4.0 mM.
2.2.5 1.0 µL dNTPs (10 mM each), final concentration 0.2 mM.
2.2.6 2.0 µL forward primer ITS1-F5 (10 µM), final concentration 0.4 µM.
2.2.7 2.0 µL reverse primer ITS1-B9 (10 µM), final concentration 0.4 µM.
2.2.8 0.2 µL Platinum®Taq DNA Polymerase (Invitrogen, 5 U $\mu L^{-1}$), final amount 1 Unit.
2.2.9 1.0 µL extracted DNA obtained as described above.
2.2.10 PCR cycling parameters. Two minutes at 94°C, 35 cycles of 1 min at 94°C, 30 s at 63°C, and 1 min at 72°C, followed by a final extension for 10 min at 72°C and quickly cooled to room temperature.
2.2.11 After amplification, 5 µL of the PCR products are subjected to electrophoresis on a 1.5% agarose gel by standard methods (Sambrook et al., 1989) along with a 1 kb-plus DNA ladder (Invitrogen) to size fragments. PCR products are viewed and photographed under UV light.

2.3 Restriction Fragment Length Polymorphism (RFLP) analysis.
2.3.1 PCR products are (without prior purification) digested using four different restriction enzymes in four separate reactions: *Dra*I (Promega), *Hinf*I (Promega), *Ssp*I (Promega) and *Taq*I (Promega).
2.3.2 Total reaction volume of a single RFLP-reaction is 10 µL.
2.3.3 3.5 µL MGW.
2.3.4 1.0 µL 10 × reaction buffer (Promega), final concentration 1 ×.
2.3.5 0.5 µL 10 Units restriction enzyme, final amount 5 Units.
2.3.6 5.0 µL PCR product.
2.3.7 Reaction mixes containing *Dra*I, *Hinf*I and *Ssp*I are incubated for 1 h or overnight at 37°C.
2.3.8 Reaction mixes containing *Taq*I are incubated for 1 h or overnight at 65°C.
2.3.9 After digestion, 10 µL of the digested PCR products are subjected to electrophoresis on a 3% agarose gel by standard methods (Sambrook *et al*., 1989) along with a 1 kb-plus DNA ladder (Invitrogen) to size fragments. PCR products are viewed and photographed under UV light.

## 3. Essential procedural information

3.1 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.
3.1.1 Negative Isolation Control (NIC) to monitor contamination during sample preparation and DNA extraction: empty tube processed as if it was a real sample.
3.1.2 Negative Amplification Control (NAC) to monitor contamination during reaction mix preparation: amplification of MGW that was used to prepare the reaction mix.
3.1.3 Positive Amplification Control (PAC) to monitor efficiency of the amplification: amplification of nucleic acid of *C. capitata* [genomic DNA or Whole Genome Amplicon (WGA)] with a concentration of 10 ng $\mu L^{-1}$.

3.2 A specimen is identified as *C. capitata* when the PCR product is digested as follows: *Dra*I: 400, 350, 170 and 100 bp, *Hinf*I: 900 and 120 bp, *Ssp*I: 510, 230, 150, 100 and 30 bp and *Taq*I: 480, 470 and 70 bp, providing that the contamination controls are negative. The test is considered negative if the expected bands following digestion are not produced as described. Tests should be repeated if contradictory or unclear results are obtained.

## 4. Performance criteria

4.1 Method validation of the ITS1 PCR-RFLP assay for the identification of adults and larvae of *Ceratitis capitata* has been performed according to the Dutch national guideline for the validation of detection and identification methods for plant pathogens and pests (Version 2, March 2010) which is based on EPPO standard PM7/98. The following performance criteria have been determined: analytical sensitivity, analytical specificity, repeatability, reproducibility and robustness.

4.2 The analytical sensitivity was determined using five *C. capitata* larvae. Dilutions of DNA extracts were prepared and tested according to the ITS1 PCR-RFLP assay. The average values of the detection limits per larvae (expressed in DNA mass) plus three times standard deviation was calculated. An analytical sensitivity of 1.1 ng *C. capitata* DNA was found.

4.3 The analytical specificity was determined using 13 *C. capitata* specimens from different localities, and 89 specimens from 24 species belonging to the genera *Anastrepha*, *Bactrocera*, *Ceratitis*, *Delia* and *Rhagoletis*. Starting material used for the DNA-extraction consisted of legs of adult specimens. The ITS1 PCR-RFLP results obtained using the *C. capitata* specimens were consistent and unique to target species. No false-positive results were observed.

4.4 The repeatability and reproducibility were determined using six *C. capitata* larvae cut in three equal parts. Two parts

were analysed at the same moment, the third part was analysed by another technician using different equipment on a different day. The repeatability and reproducibility tests were spread over the course of 2 weeks to mimic a day-to-day variation in testing conditions. The ITS1 PCR-RFLP assay was found to be 100% repeatable and reproducible.

4.5 The robustness of the ITS1 PCR-RFLP assay was tested by using two *C. capitata* larvae cut in half. DNA was extracted using an alternative DNA-extraction kit: Blood & Tissue Kit (Qiagen). Also different incubation times (45 min, 1 h, 24 h) were used for the digestion with restriction enzymes. The ITS1 PCR-RFLP assay yielded the same results for the samples analysed with the different DNA-extraction Kits and alternative incubation times.