INTERNATIONAL STANDARDS FOR PHYTOSANITARY MEASURES

ISPM 27

DIAGNOSTIC PROTOCOLS FOR REGULATED PESTS

(2006)

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Publication history

This is not an official part of the standard.

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ISPM 27. 2006. *Diagnostic protocols for regulated pests*. Rome, IPPC, FAO.

Publication histories of attachments are included in each attachment

2012-08 Secretariat updated Appendix 2 and the reference section

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CONTENTS

Adoption

This standard was adopted by the First Session of the Commission on Phytosanitary Measures in April 2006. Adoption information for attachments is stated in each attachment, if different from core text.

INTRODUCTION

Scope

This standard provides guidance on the structure and content of the International Plant Protection Convention (IPPC) diagnostic protocols for regulated pests. The protocols describe procedures and methods for the official diagnosis of regulated pests that are relevant for international trade. They provide at least the minimum requirements for reliable diagnosis of regulated pests.

References

IPPC. 1997. *International Plant Protection Convention*. Rome, IPPC, FAO.

ISPM 4. 1995. *Requirements for the establishment of pest free areas*. Rome, IPPC, FAO. [published 1996]

ISPM 5. *Glossary of phytosanitary terms*. Rome, IPPC, FAO.

ISPM 6. 1997. *Guidelines for surveillance*. Rome, IPPC, FAO.

ISPM 7. 1997. *Export certification system*. Rome, IPPC, FAO. [revised; now ISPM 7:2011]

ISPM 8. 1998. *Determination of pest status in an area*. Rome, IPPC, FAO.

ISPM 9. 1998. *Guidelines for pest eradication programmes*. Rome, IPPC, FAO.

ISPM 10. 1999. *Requirements for the establishment of pest free places of production and pest free production sites*. Rome, IPPC, FAO.

ISPM 13. 2001. *Guidelines for the notification of non-compliance and emergency action*. Rome, IPPC, FAO.

ISPM 14. 2002. *The use of integrated measures in a systems approach for pest risk management*. Rome, IPPC, FAO.

ISPM 17. 2002. *Pest reporting*. Rome, IPPC, FAO.

ISPM 20. 2004. *Guidelines for a phytosanitary import regulatory system*. Rome, IPPC, FAO.

ISPM 22. 2005. *Requirements for the establishment of areas of low pest prevalence*. Rome, IPPC, FAO.

ISPM 23. 2005. *Guidelines for inspection*. Rome, IPPC, FAO.

Definitions

Definitions of phytosanitary terms used in the present standard can be found in ISPM 5 (*Glossary of phytosanitary terms*).

Outline of Requirements

This standard sets the framework for the content of diagnostic protocols, their purpose and use, their publication and their development. Diagnostic protocols for specific regulated pests are included as annexes to this standard.

Information relevant for diagnosis is provided in the diagnostic protocol on the specified regulated pest, its taxonomic position, and the methods to detect and identify it. Diagnostic protocols contain the minimum requirements for reliable diagnosis of the specified regulated pests and provide flexibility to

ensure that methods are appropriate for use in the full range of circumstances. The methods included in diagnostic protocols are selected on the basis of their sensitivity, specificity and reproducibility, and information related to these factors is provided for each of these methods.

Detailed information and guidance for the detection of pests is provided on, for example, signs and/or symptoms associated with the pest, illustrations (where appropriate), developmental stages of the pest, and methods for detecting the pest in a commodity, as well as methods for extracting, recovering and collecting the pests from plants. Information and guidance for the identification of pests includes detailed information on morphological and morphometric methods, methods based on biological properties, and methods based on biochemical and molecular properties of the pest. Furthermore detailed guidance is provided on the records that should be kept.

Diagnostic protocols are intended to be used by laboratories performing pest diagnosis as part of phytosanitary measures. They are subject to review and amendment to take into account new developments in pest diagnosis. The standard also provides guidance on how these protocols will be initiated, developed, reviewed and published.

BACKGROUND

Proper pest detection and pest identification are crucial for the appropriate application of phytosanitary measures (see for example ISPM 4:1995, ISPM 6:1997, ISPM 7:1997, ISPM 9:1998 and ISPM 20:2004). In particular, contracting parties need proper diagnostic procedures for determination of pest status and pest reporting (ISPM 8:1998; ISPM 17:2002), and the diagnosis of pests in imported consignments (ISPM 13:2001).

National plant protection organizations (NPPOs) have produced diagnostic protocols for regulated pests in order to adequately fulfil responsibilities according to Article IV of the IPPC (1997), in particular regarding surveillance, import inspections and export certification. In response to the need for regional harmonization, several regional plant protection organizations (RPPOs) have developed a significant number of regional diagnostic standards. This underlines the need for international harmonization, and those national and regional standards may form the basis for international protocols. Subsequently, the ICPM, at its Sixth Session in 2004, recognized that there was a need for international diagnostic protocols within the framework of the IPPC and approved the formation of a Technical Panel on Diagnostic Protocols (TPDP) for that purpose.

PURPOSE AND USE OF DIAGNOSTIC PROTOCOLS

The purpose of harmonized diagnostic protocols is to support efficient phytosanitary measures in a wide range of circumstances and to enhance the mutual recognition of diagnostic results by NPPOs, which may also facilitate trade. Furthermore these protocols should aid the development of expertise and technical cooperation, and they may also be relevant to the accreditation and/or approval of laboratories.

In addition to the methods included in the diagnostic protocols presented in the annexes to this standard, NPPOs may use other methods for diagnosing the same pests (for example based on bilateral agreements). The protocols and their components annexed to this ISPM are considered to have the status of an ISPM or part thereof (see section 3 of this ISPM and Article X of the IPPC). Therefore, contracting parties should take into account, as appropriate, these diagnostic protocols when using or requiring the use of diagnostic methods in particular where other contracting parties may be affected.

Diagnostic protocols describe procedures and methods for the detection and identification of regulated pests that are relevant to international trade.

Diagnostic protocols may be used in different circumstances that may require methods with different characteristics. Examples of such circumstances grouped according to an increased need for high sensitivity, specificity and reliability are:

- routine diagnosis of a pest widely established in a country
- general surveillance for pest status
- testing of material for compliance with certification schemes
- surveillance for latent infection by pests
- surveillance as part of an official control or eradication programme
- pest diagnostic associated with phytosanitary certification
- routine diagnosis for pests found in imported consignments
- detection of a pest in an area where it is not known to occur
- cases where a pest is identified by a laboratory for the first time
- detection of a pest in a consignment originating in a country where the pest is declared to be absent.

For example, in the case of routine diagnosis, the speed and cost of a test method may be more relevant than sensitivity or specificity. However, the identification of a pest by a laboratory or in an area for the first time may require methods with a high level of specificity and reproducibility. The significance of the outcome of a diagnosis is often dependent on proper sampling procedures. Such procedures are addressed by other ISPMs (under preparation).

Diagnostic protocols provide the minimum requirements for reliable diagnosis of regulated pests. This may be achieved by a single method or a combination of methods. Diagnostic protocols also provide additional methods to cover the full range of circumstances for which a diagnostic protocol may be used. The level of sensitivity, specificity and reproducibility of each method is indicated where possible. NPPOs may use these criteria to determine the method or combination of methods that are appropriate for the relevant circumstances.

Diagnostic protocols are intended to be used by laboratories performing pest diagnosis. Such laboratories may be established under or may be authorized by the NPPO to perform these activities in such manner that the results of the pest diagnosis may be considered as part of a phytosanitary measure of the NPPO.

The main elements of the procedure for the development of diagnostic protocols are presented in Appendix 1.

REQUIREMENTS

1. General Requirements for Diagnostic Protocols[1]

Each protocol contains the methods and guidance necessary for the regulated pest(s) to be detected and positively identified by an expert (i.e. an entomologist, mycologist, virologist, bacteriologist, nematologist, weed scientist, molecular biologist) or competent staff who are specifically trained.

The methods included in diagnostic protocols are selected on the basis of their sensitivity, specificity and reproducibility. In addition, the availability of equipment, the expertise required for these methods and their practicability (for example ease of use, speed and cost) are taken into account when selecting methods for inclusion in the diagnostic protocol. Usually these methods and their associated information should also be published. It may be necessary that some methods are validated before inclusion in the protocols. Such validation may include, for example, the use of a set of known samples, including controls, prepared to verify sensitivity, specificity and reproducibility.

Each diagnostic protocol usually describes more than one method to take into account the capabilities of laboratories and the situations for which the methods are applied. Such situations include diagnosis of different developmental stages of organisms, which require different methodologies, the need for an alternative diagnostic technique because of uncertainties of the initial diagnosis, as well as varying requirements for the level of sensitivity, specificity and reliability. For some purposes a single method may be sufficient, for other purposes a combination of methods may be necessary. Each protocol contains introductory information, information on the taxonomic position of the pest, methods for detection and identification of the pest, records to be kept, and references to appropriate scientific publications. In many cases a wide range of supplementary information is available which may

[1] The following general provisions apply to all diagnostic protocols:

- Laboratory tests may involve the use of chemicals or equipment which present a certain hazard. In all cases, national safety procedures should be strictly followed.
- Use of names of chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable.
- Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

support diagnosis, for example geographical distribution of the pest and host lists, but diagnostic protocols focus on the critical methods and procedures for pest diagnosis.

The aspects of quality assurance and in particular the reference materials that are required by diagnostic protocols (such as inclusion of positive and negative controls or collection of specimens) are specifically indicated in the corresponding section of the protocol.

2. Specific Requirements for a Diagnostic Protocol

Diagnostic protocols are arranged according to the following sections:

- Pest information
- Taxonomic information
- Detection
- Identification
- Records
- Contact points for further information
- Acknowledgements
- References.

2.1 Pest information

Brief information is provided on the pest, including, where appropriate, its life cycle, morphology, variation (morphological and/or biological), relationship with other organisms, host range (in general), effects on hosts, present and past geographical distribution (in general), mode of transmission and dissemination (vectors and pathways). When available, reference to a pest data sheet should also be provided.

2.2 Taxonomic information

This section provides information on the taxonomy of the pest involved and includes:

- name (current scientific name, author and year (for fungi, teleomorph name if known))
  - . synonyms (including former names)
  - . accepted common names, anamorph name of fungi (including synonyms)
  - . acronym of viruses and viroids
- taxonomic position (including information on subspecies classifications where relevant).

2.3 Detection

This section of the diagnostic protocol provides information and guidance on:

- the plants, plant products or other articles capable of harbouring the pest
- the signs and/or symptoms associated with the pest (characteristic features, differences or similarities with signs and/or symptoms from other causes), including illustrations, where appropriate
- the part(s) of the plant, plant products or other articles on/in which the pest may be found
- the developmental stages of the pest that may be detected, together with their likely abundance and distribution on/in the plants/plant products or other articles
- the likely occurrence of the pest associated with developmental stages of the host(s), climatic conditions and seasonality
- methods for detecting the pest in the commodity (e.g. visual, hand lens)

- methods for extracting, recovering and collecting the pest from the plants, plant products or other articles, or for demonstrating the presence of the pest in the plants, plant products or other articles
- methods for indicating the presence of the pest in asymptomatic plant material or other materials (e.g. soil or water), such as ELISA[2] tests or culturing on selective media
- viability of the pest.

For all the methods included in this section, information is provided on their sensitivity, specificity and reproducibility, where relevant. Where appropriate, guidance is provided on positive and negative controls and reference material to be included in tests. Guidance is also provided on resolving possible confusion with similar signs and/or symptoms due to other causes.

2.4 Identification

This section provides information and guidance on methods that either used alone or in combination lead to the identification of the pest. When several methods are mentioned, their advantages/disadvantages are given as well as the extent to which the methods or combinations of methods are equivalent. A flow diagram may be presented if several methods are needed to identify the pest or many alternative methods are included.

Main types of methodologies used in diagnostic protocols include those based on morphological and morphometric characteristics, biological properties such as virulence or host range of a pest, and those based on biochemical and molecular properties. Morphological characteristics may be investigated directly or after culturing or isolation of the pest. Culturing and/or isolation may also be required for biochemical and/or molecular assays. Details are provided when culturing or isolation procedures are necessary components of methods.

For morphological and morphometric identifications, details are provided, as appropriate, on:

- methods to prepare, mount and examine the pest (such as for light microscopy, electron microscopy and measurement techniques)
- identification keys (to family, genus, species)
- descriptions of the morphology of the pest or of its colonies, including illustrations of morphological diagnostic characteristics, and an indication of any difficulties in seeing particular structures
- comparison with similar or related species
- relevant reference specimens or cultures.

For biochemical or molecular identifications, each method (e.g. serological methods, electrophoresis, PCR[3], DNA barcoding, RFLP[4], DNA sequencing) is described separately in sufficient detail (including equipment, reagents and consumables) to perform the test. Where appropriate, reference may be made to methodology described in other diagnostic protocols annexed to this standard.

In cases where more than one method can be used reliably, other appropriate methods may be presented as alternative or supplementary methods, e.g. where morphological methods can be used reliably and appropriate molecular methods are also available.

Where appropriate, methods for isolation of pests from asymptomatic plants or plant products (such as tests for latent infection) are given, as well as methods for extraction, recovery and collection of pests from plant or other material. In these cases, methods may also be provided for direct identification of pests using biochemical or molecular tests on asymptomatic material.

[2] Enzyme-linked immunosorbent assay.

[3] Polymerase chain reaction.

[4] Restriction fragment length polymorphism.

For all the methods included in this section, information is provided on their sensitivity, specificity and reproducibility, where relevant. Where appropriate, guidance is provided on positive and negative controls and reference material to be included in tests. Guidance is also provided on removing possible confusion with similar and related species or taxa.

Diagnostic protocols provide guidance on the criteria for the determination of a positive or negative result for each method or information necessary to determine if an alternative method be applied.

Those cases where the use of appropriate controls for a specific technique, including where relevant reference material, is essential are clearly indicated in the protocol. When appropriate controls are not available, other tests, preferably based on different identification principles, may increase the certainty of the identification. Alternatively, a sample, specimen or, where appropriate, an image should be sent to another laboratory with experience in diagnosis of the suspected pest and possessing the required control or reference materials. Specimen(s) or material for reference purposes should be properly preserved.

Methods for quick, preliminary indications of identity (which will later need to be confirmed) may also be included in diagnostic protocols.

2.5 Records

This section provides information on the records that should be kept:

- scientific name of pest identified
- code or reference number of the sample (for traceability)
- nature of the infested material including scientific name of host where applicable
- origin (including the geographic location if known) of the infested material, and location of interception or detection
- description of signs or symptoms (including photographs where relevant), or their absence
- methods, including controls, used in the diagnosis and the results obtained with each method
- for morphological or morphometric methods, measurements, drawings or photographs of the diagnostic features (where relevant) and, if applicable, an indication of the developmental stage(s)
- for biochemical and molecular methods, documentation of test results such as photographs of diagnostic gels or ELISA printouts of results on which the diagnosis was based
- where appropriate, the magnitude of any infestation (how many individual pests found, how much damaged tissue)
- the name of the laboratory and, where appropriate, the name of the person(s) responsible for and/or who performed the diagnosis
- dates of collection of the sample, and of detection and identification of the pest
- where appropriate, state of the pest, alive or dead, or viability of its development stages.

Evidence such as culture(s) of the pest, nucleic acid of the pest, preserved/mounted specimens or test materials (e.g. photograph of gels, ELISA plate printout results) should be retained, in particular in cases of non-compliance (ISPM 13:2001) and where pests are found for the first time (ISPM 17:2002). Additional items may be required under other ISPMs such as ISPM 8:1998.

The period for which records should be kept depends on the purpose for which a diagnosis is made. In cases where other contracting parties may be adversely affected by the results of the diagnosis, records and evidence of the results of the diagnosis should be retained for at least one year.

2.6 Contact points for further information

Contact details of organizations or individuals with particular expertise on the pest(s) are provided; they may be consulted regarding details on the diagnostic protocol.

2.7 Acknowledgements

The name and address of the experts who wrote the first draft of the diagnostic protocol are given, together with those of any others who made major contributions.

2.8 References

References to accessible scientific publications and/or published laboratory manuals are given that may provide further guidance on the methods and procedures contained in the diagnostic protocol.

3. Publication of Diagnostic Protocols

Diagnostic protocols are published as annexes to this ISPM and thus are individual publications under the IPPC framework with a specific publication and/or revision date. If appropriate, they may also form part of other ISPMs. The process of their adoption includes stringent review by internationally acknowledged scientists/experts for the relevant discipline.

An index to the annexes is provided as Appendix 2.

This appendix is for reference purposes only and is not a prescriptive part of the standard.

APPENDIX 1: Main elements of procedures for diagnostic protocols

1. Development of Diagnostic Protocols

The TPDP will commission an expert to lead the development of a diagnostic protocol by adapting, as appropriate, protocols that have already been approved by RPPOs, or other international or national organizations, or by developing a new diagnostic protocol. The diagnostic protocol will be developed further by a small group of experts selected by the TPDP and will then be submitted, in cooperation with the IPPC Secretariat, to the TPDP which, when satisfied with the content, will submit it to the Standards Committee.

2. Review of Existing Diagnostic Protocols

TPDP members will review the diagnostic protocols in their discipline on an annual basis or as determined by the TPDP. A request for a revision to a diagnostic protocol may also be submitted by NPPOs, RPPOs or CPM subsidiary bodies through the IPPC Secretariat (ippc@fao.org), which will in turn forward it to the TPDP.

The TPDP will evaluate the request, identify those diagnostic protocols that require revision and oversee their revision. New methods should be at least equivalent to existing methods or provide a significant advantage for their worldwide application such as costs, sensitivity or specificity. Appropriate evidence should be provided to support any claims.

3. Requests for New Diagnostic Protocols

Requests for new diagnostic protocols, in addition to those identified in the work programme of the TPDP, should be sent by NPPOs, RPPOs or CPM subsidiary bodies through the IPPC Secretariat using a form for topics and priorities for standards, by 31 July of each year.

Appendix 2 is for reference purposes only and is not an official part of the standard.
This appendix was updated by the Secretariat in August 2012.

APPENDIX 2: List of adopted diagnostic protocols

The following diagnostic protocols have been adopted by the Commission of Phytosanitary Measures as annexes to ISPM 27:2006. Diagnostic protocols are published separately and are available on the International Phytosanitary Portal (https://www.ippc.int).

Annex no.	Title of diagnostic protocol	Adoption year
DP 1:2010	*Thrips palmi* Karny	2010
DP 2:2012	*Plum pox virus*	2012
DP 3:2012	*Trogoderma granarium* Everts	2012

This diagnostic protocol was adopted by the Fifth Session of the Commission on Phytosanitary Measures in March 2010.
The annex is a prescriptive part of ISPM 27:2006.

INTERNATIONAL STANDARDS FOR PHYTOSANITARY MEASURES

ISPM 27 DIAGNOSTIC PROTOCOLS

DP 1: *Thrips palmi* Karny

(2010)

CONTENTS

1. Pest Information

Thrips palmi Karny (Thysanoptera: Thripidae) is a polyphagous plant pest, especially of species in the Cucurbitaceae and Solanaceae. It appears to have originated in Southern Asia and to have spread from there during the latter part of the twentieth century. It has been recorded throughout Asia and is widespread throughout the Pacific and the Caribbean. It has been recorded locally in North, Central and South America and Africa. For more general information about *T. palmi*, see EPPO/CABI (1997) or Murai (2002); online pest data sheets are also available from the Pests and Diseases Image Library (PaDIL, 2007) and EPPO (EPPO, 2008).

The species causes economic damage to plant crops both as a direct result of its feeding activity and from its ability to vector tospoviruses such as *Groundnut bud necrosis virus*, *Melon yellow spot virus* and *Watermelon silver mottle virus*. It is extremely polyphagous, and has been recorded from more than 36 plant families. It is an outdoor pest of, amongst others, *Benincasa hispida*, *Capsicum annuum*, *Citrullus lanatus*, *Cucumis melo*, *Cucumis sativus*, *Cucurbita* spp., *Glycine max*, *Gossypium* spp., *Helianthus annuus*, *Nicotiana tabacum*, *Phaseolus vulgaris*, *Pisum sativum*, *Sesamum indicum*, *Solanum melongena*, *Solanum tuberosum* and *Vigna unguiculat*a. In glasshouses, economically important hosts are *Capsicum annuum*, *Chrysanthemum* spp., *Cucumis sativus*, *Cyclamen* spp., *Ficus* spp., Orchidaceae and *Solanum melongena*. The thrips may be carried on plants for planting, cut flowers and fruits of host species, as well as on or associated with packing material, and in soil.

Thrips palmi is almost entirely yellow in coloration (Figures 1–3), and its identification is hampered by both its small size (1.0–1.3 mm) and its great similarity to certain other yellow or predominantly yellow species of *Thrips*.

Figure 1: *Thrips palmi*, female (left) and male (photo: A. J. M. Loomans, PPS, Wageningen, the Netherlands; scale bar = 500 µm = 0.5 mm)

Figure 2: *Thrips palmi*, female
Figure 3: *Thrips palmi*, male
(Photos: W. Zijlstra, PPS, Wageningen, the Netherlands; scale bars: 300 µm)

2. Taxonomic Information

Name:	*Thrips palmi* Karny, 1925
Synonyms:	*Thrips clarus* Moulton, 1928
	Thrips leucadophilus Priesner, 1936
	Thrips gossypicola Ramakrishna & Margabandhu, 1939
	Chloethrips aureus Ananthakrishnan & Jagadish, 1967
	*Thrips gracili*s Ananthakrishnan & Jagadish, 1968
Taxonomic position:	Insecta, Thysanoptera, Terebrantia, Thripidae
Common name:	melon thrips

3. Detection

Thrips palmi may be found in different locations depending on the life stages present.

eggs	in the leaf, flower and fruit tissue
larva I	on the leaves, flowers and fruits
larva II	on the leaves, flowers and fruits
pupa I	in the soil, packing cases and growing medium
pupa II	in the soil, packing cases and growing medium
adult	on the leaves, flowers and fruits

On plant material, *T. palmi* may potentially be found on most above-ground parts of the plant; the parts of the plant infested can differ according to variables such as the host and the characteristics of each separate *T. palmi* population.

During visual examination of plant material for the presence of *T. palmi*, attention must be paid to silvery feeding scars on the leaf surfaces of host plants, especially alongside the midrib and the veins. Heavily infested plants are often characterized by a silvered or bronzed appearance of the leaves, stunted leaves and terminals, or scarred and deformed fruits. Detection may be hampered in circumstances such as:

- low-level infestation, which may produce little or no detectable symptoms
- the presence of the eggs within the plant tissue only (for example after external treatment which may have removed visible life stages).

Specimens for morphological examination are best collected in a fluid called AGA, which is a mixture of 10 parts of 60% ethanol with 1 part of glycerine and 1 part of acetic acid. If the specimens are to be stored, they should be transferred to 60% ethanol and kept in the dark, preferably in a freezer to prevent loss of colour. However, several laboratories have reported that AGA may act to denature the DNA of the thrips thereby hindering any subsequent molecular work. An alternative is to use 80–95% ethanol as the collecting fluid as any unmounted specimens may then be used for molecular studies. However, in this case specimens must be stored in the freezer until used, or they may prove difficult to slide mount.

Several methods can be used to collect thrips specimens (Mantel and Vierbergen, 1996; modified):

- Thrips may be individually removed from the plant (leaves, flowers or fruit), and transferred into microtubes containing AGA, using a moist, fine brush.
- Thrips may be beaten from plant parts onto a small plastic tray (e.g. a white tray for dark-coloured specimens or a black tray for light-coloured specimens). In cooler conditions, the thrips usually start walking across the tray rather than flying off, allowing time for the thrips to be picked off with a moist fine brush, whereas in warmer conditions collection has to be done more rapidly as the thrips are likely to fly off much more quickly. The thrips are easily seen on the tray using just a hand lens, but an experienced observer can also see them easily with the naked eye.
- Plant parts may be sealed in a plastic bag for 24 hours, with a piece of filter paper enclosed to absorb condensation. Most thrips will leave the plant parts and can then be collected from the inside of the bag.
- A Berlese funnel can be used to process plant material such as bulbs, flowers, turf, leaf litter, moss and even dead branches of trees. The funnel contains a sieve on which the plant material is deposited. Beneath the sieve, the bottom of the funnel leads into a receptacle containing 70–96% ethanol. An alternative is to use 10% ethanol plus wetting agent as some workers find that this makes the preparation of good quality microscope slide mounts easier. The funnel is placed under an electric lamp (60 W), and the heat and light will drive most of the thrips present in the plants down towards the receptacle. After an appropriate period (e.g. 8 hours for cut flowers), the content of the receptacle can then be checked under a stereomicroscope.
- Thrips may be monitored (winged adults only) using coloured sticky traps or other appropriate methods. The ability of a colour to attract thrips varies for different thrips species, but blue or white traps are good for *T. palmi*, though yellow traps will also work. For microscope slide preparation and identification, the thrips will have to be removed from the traps using glue-removing fluids such as those based on citrus oils, dichloromethane or a turpentine substitute.

There are no recognized methods for extracting thrips pupae from the soil in a quarantine context.

4. Identification

Identification of thrips species by morphological examination is restricted to adult specimens because there are no adequate keys for the identification of eggs, larvae or pupae. However, the presence of larvae in samples can give important additional information such as confirming their development on the host plants. The primary method of identification of adult material is from morphological

characters. In order to achieve species identification, these must be examined using a high-power microscope (e.g. x400). Using this protocol with good-quality slide preparations should allow adult *T. palmi* to be identified with certainty by morphological examination alone.

Molecular assays can be applied to all life stages including the immature stages for which morphological identification to species is not possible. Additionally, in cases where adult specimens are atypical or damaged, molecular assays may provide further relevant information about their identity. However specificity of molecular assays is limited as they have been developed for specific purposes and evaluated against a restricted number of species, using samples from different geographic regions; therefore, such information needs to be carefully interpreted.

4.1 Morphological identification of the adult thrips

4.1.1 Preparation of thrips for microscopic examination

For high-power microscopic examination, adult thrips must be mounted on microscope slides. Specimens to be kept in a reference collection are best macerated, dehydrated and mounted in Canada balsam; Mound and Kibby (1998) provide a full description of this process. However, the full slide preparation protocol for archival mounts takes 3 days to complete.

For routine identifications, a water-soluble mountant such as Hoyer's medium (50 ml water, 30 g gum arabic, 200 g chloral hydrate, 20 ml glycerine) is more rapid and relatively inexpensive. One popular method of routine slide preparation is given by Mound and Kibby (1998) and described below (different laboratories may find that other variants work equally well):

Transfer the specimens from the collecting fluid into clean 70% ethanol; if the specimens are reasonably flexible, attempt to spread the legs, wings and antennae using micropins; transfer a single thrips, ventral side uppermost, to a drop of Hoyer's medium on a 13 mm diameter cover slip and use micropins to rearrange the thrips if necessary; gently lower a microscope slide onto the mountant so that the cover slip and mountant adhere to the middle of the slide; invert the slide as soon as the mountant has spread to the edges of the cover slip; label the slide with details including locality, date of collection and host plant; place the slide, cover slip up, into a drying oven at 35–40 °C and leave for 6 hours before attempting study; leave in the oven for approximately 3 weeks to dry the mountant, before sealing the cover slip with resin or nail varnish.

4.1.2 Identification of the family Thripidae

Thrips palmi belongs to the family Thripidae, which includes more than 2000 species in 276 genera. Species share the characteristics outlined in Table 1.

Table 1: Family Thripidae – shared characteristics

Body part	Characteristic
Antennae	seven or eight segments (occasionally six or nine)
	segments III–IV have emergent sense cones (sensoria)
Forewings (if fully developed)	usually slender, with two longitudinal veins each bearing a series of setae
Abdomen – female	with a serrated ovipositor, which is turned downwards at the apex
Median sternites – male	with or without glandular areas

4.1.3 Identification of the genus *Thrips*

The genus *Thrips* contains more than 280 species from all parts of the world, though the genus is primarily from the Holarctic region and the Old World tropics. Members of the genus share the characteristics outlined in Table 2.

Table 2: Genus *Thrips* – shared characteristics, adult specimens

Body part	Characteristic
Body form (female)	macropterous or micropterous
Antennae	seven or eight segments
	segments III–IV with forked emergent sense cones
Ocellar setae	only two pairs present (pair I absent)
	pair II shorter (at least no longer) than pair III
Pronotum	two pairs (rarely one or none) of major posteroangular setae
	usually three, sometimes four, pairs of posteromarginal setae
Prosternal basantra	no setae present
Forewings	the first vein with variably spaced setal row, second vein with complete setal row
	clavus with five veinal setae (rarely six)
Metascutum	median pair of setae at or behind the anterior margin
	striate or reticulate sculpturing
	campaniform sensilla (metanotal pores) present or absent
Metasternal furca	without a spinula
Fore tibia	apical claw absent
Tarsi	two-segmented
Abdominal tergites and sternites	without posteromarginal craspeda (flanges)
Abdominal tergites	tergites V–VIII with paired ctenidia laterally (combs – each comprising a submarginal row of microtrichia) (occasionally also on IV)
	tergite VIII: ctenidia posteromesad to the spiracles
Abdominal sternites and pleurotergites	with or without discal (accessory) setae
Abdominal sternites (male)	abdominal sterna III–VII, or less, each with a glandular area

(A simplified summary of the main characteristics is given in Table 4 and is accompanied by illustrative line drawings and photomicrographs (Figures 4 to 5.12).)

Identification of the adults can be carried out with keys. Mound and Kibby (1998) provided a key to 14 *Thrips* species of economic importance including *T. palmi*. In addition, a CD-ROM identification aid for thrips is available which includes an identification system to 100 pest species from around the world based on photomicrographs (Moritz *et al.*, 2004).

More comprehensive keys to the genus are available, produced on a regional basis (no such key has been produced for the Afrotropical region):

Asia: Bhatti (1980) and Palmer (1992) provide keys for the identification of species of *Thrips* occurring in the Asian tropics. Mound & Azidah (2009) provide a key to the species of Peninsular Malaysia.

Europe: zur Strassen (2003) has produced the most recent comprehensive key to the species of Europe including *Thrips* (in German).

North, Central and South America: Nakahara (1994) provides a key for *Thrips* species from the New World. A key to the species of *Thrips* found in Central and South America is given by Mound and Marullo (1996) though only one of these species is native to the region.

Oceania: Mound and Masumoto (2005) provide a key to the *Thrips* species of Oceania. (The authors of the paper are aware of the error inadvertently introduced on p. 42 in the section "Relationships" whereby a characteristic of *T. flavus* Schrank – ocellar setae III close together behind the first ocellus – is attributed to *T. palmi*. The correct information is provided in the *T. palmi* species description immediately above and is illustrated in Figure 72.)

4.1.4 Identification of *Thrips palmi*

4.1.4.1 Morphological characteristics of *Thrips palmi*

Bhatti (1980), Bournier (1983), Sakimura *et al.* (1986), zur Strassen (1989), Nakahara (1994) and Mound and Masumoto (2005) all provide detailed descriptions of *T. palmi*. Sakimura *et al.* (1986) gave a list of major diagnostic characters to distinguish *T. palmi* from the other known species of the genus *Thrips*; a modified version is presented in Table 3.

Thrips palmi can be reliably separated from all other species of the genus *Thrips* by the possession of all the characters listed in Table 3. Nevertheless, thrips morphology is subject to variation even within a single species and some characters listed here may be subject to occasional slight variation. For instance antennal coloration or the number of distal setae on the forewing can vary from the most commonly observed states. If the specimen differs with respect to one or more of these character states, then the identification should be checked by reference to an appropriate regional key such as those listed in section 4.1.3.

Table 3: A list of morphological characteristics that collectively distinguish *Thrips palmi* from other species in the genus *Thrips*

	Morphological character
1.	A clear yellow body with no dark areas on the head, thorax or abdomen (slightly thickened blackish body setae); antennal segments I and II pale, III yellow with apex shaded, IV to VII brown but usually with base of IV–V yellow; forewings uniformly slightly shaded, prominent setae dark
2.	Antennae always seven-segmented
3.	Postocular setae II and IV much smaller than remaining setae
4.	Ocellar setae III standing either just outside of the ocellar triangle, or touching the tangent lines connecting the anterior ocellus and each of the posterior ocelli
5.	Metascutum with sculpture converging posteriorly; median pair of setae behind anterior margin; paired campaniform sensilla present
6.	Forewing first vein with three (occasionally two) distal setae
7.	Abdominal tergite II with four lateral marginal setae
8.	Abdominal tergites III to IV with setae S2 dark and subequal to S3
9.	Abdominal tergite VIII with posteromarginal comb in female complete, in male broadly developed posteriorly
10.	Abdominal tergite IX usually with two pairs of campaniform sensilla (pores)
11.	Abdominal sternites without discal setae or ciliate microtrichia
12.	Abdominal pleurotergites without discal setae
13.	Male: sternites III–VII each with a narrow transverse glandular area

A simplified summary of the main characteristics is given in Table 4 and is accompanied by illustrative line drawings and photomicrographs (Figures 4 to 5.12).

4.1.4.2 Comparison with similar species (species that are yellow without darker body markings, or predominantly yellow, or sometimes yellow)

For each species listed here, the main character differences by which they may be separated from *Thrips palmi* are given. If in any doubt, refer to an appropriate regional key such as those listed in section 4.1.3. These also give details of other *Thrips* species that are not listed below.

Two Indian species (*T. alatus* Bhatti and *T. pallidulus* Bagnall) are very similar to *T. palmi*, although little is known about their biology.

Thrips alatus

- antennal segment V uniformly brown
- abdominal tergites III and IV with setae S2 paler and much weaker than S3 in both sexes
- the striate sculpture on the metascutum usually not converging posteriorly
- distribution: India, Malaysia, Nepal.

Thrips pallidulus

- antennal segment IV pale
- sculpture on the metascutum medially reticulate, not striate
- distribution: India.

Three common Palearctic species (but also with wider distributions) that may be confused with *T. palmi* are *T. flavus*, *T. nigropilosus* Uzel and *T. tabaci* Lindeman.

Thrips flavus

- ocellar setae pair III inside the ocellar triangle, just behind the anterior ocellus
- length of antennal segment VI, 54–60 μm (42–48 μm in *T. palmi*)
- lines of sculpture on the metascutum not converging posteriorly
- distribution: common flower thrips throughout Asia, Europe.

Thrips nigropilosus

- usually with dark markings on the thorax and abdomen
- metascutum with irregular reticulations medially (longitudinal striae in *T. palmi*) and no campaniform sensilla
- abdominal tergite II with three lateral marginal setae
- abdominal tergites IV–V with median pair of setae (S1) more than 0.5 times as long as the median length of their tergites (less than 0.3 times in *T. palmi*)
- distribution: common leaf-feeding species, sometimes a pest of plants in the family Compositae; Asia, East Africa, Europe, North America, Oceania.

Thrips tabaci

- highly variable in coloration, but usually with more or less brown or greyish markings
- all postocular setae subequal in length
- metascutum with irregular longitudinal reticulations, usually with small internal wrinkles medially, and no campaniform sensilla
- forewing first vein usually with four (occasionally between two or six) distal setae
- abdominal tergite II with three lateral marginal setae
- abdominal tergite IX with posterior pair of campaniform sensilla only
- abdominal pleurotergites with numerous ciliate microtrichia arising from lines of sculpture
- male: narrow transverse glandular area on abdominal sternites III–V only
- distribution: polyphagous pest with a worldwide distribution.

Two further species, one Palearctic (*T. alni* Uzel) and one European (*T. urticae* Fabricius), are less commonly encountered but may be confused with *T. palmi*. Females of *T. alni* are particularly similar in morphology to those of *T. palmi*.

Thrips alni

- antennal segment V uniformly brown
- abdominal tergites II–V with setae S2 pale
- abdominal tergite V with seta S2 much weaker than seta S3 (these setae are subequal in *T. palmi*)
- abdominal tergite VIII with seta S1 subequal to seta S2 (S1 is much weaker than S2 in *T. palmi*)
- male: abdominal sternites III–VI each with a small oval glandular area
- distribution: restricted to the leaves of *Alnus*, *Betula*, *Salix*; Europe, Siberia, Mongolia.

Thrips urticae

- pronotum with a pair of setae on the anterior margin almost twice as long as any of the discal setae (usually more than 30 µm; not so in *T. palmi*, all less than 25 µm)
- metascutum with longitudinal reticulations medially
- abdominal tergites usually with a grey area medially
- abdominal tergite IX with posterior pair of campaniform sensilla only
- distribution: restricted to *Urtica dioica*; Europe.

Table 4: Simplified checklists of the diagnostic features for quick recognition: (a) the genus *Thrips*; (b) *Thrips palmi* (See Figure 4 for the location of the various features.)

(a) Specimens can be recognized as *Thrips* by the following combination of characters		
Antenna	with seven or eight distinct segments; segments III and IV with forked sense cones	Figs 5.1, 5.2
Head	with two pairs of ocellar setae (II and III); pair I missing, pair II shorter than pair III	Fig. 5.3
Forewing	1st vein – setal row on the first vein continuous or interrupted	Fig. 5.5
Abdominal tergites V to VIII	with paired ctenidia	Fig. 5.6
Abdominal tergite VIII	with ctenidia posteromesad to the spiracles	Fig. 5.6
(b) Specimens can be identified as *Thrips palmi* by the presence of the following characters		
Body colour	clear yellow body with no dark areas on the head, thorax or abdomen; antennal segments I and II are pale	Figs 1–3
Antennal segment V	usually yellowish in basal ⅓ to ½	Fig. 5.1
Antennal segment VI	length = 42–48 µm	Fig. 5.1
Head: ocellar setae pair III	with their bases sited outside of the ocellar triangle or touching the tangent lines connecting the anterior ocellus to each of the posterior ocelli	Fig. 5.3
Pronotum	with two pairs of major posteroangular setae	Fig. 5.4
Forewing: 1st vein	with three (occasionally two) distal setae	Fig. 5.5
Metascutum	with median pair of setae behind the anterior margin and a pair of campaniform sensilla; with striate sculpture converging posteriorly	Fig. 5.7
Abdominal pleurotergites	discal setae absent; lines of sculpture without ciliate microtrichia	Fig. 5.8
Abdominal tergite II	with four lateral marginal setae	Fig. 5.9
Abdominal tergites III and IV	S2 almost equal to S3	Fig. 5.10

Table 4 continued

Abdominal tergite VIII	female with complete posteromarginal comb; male with posteromarginal comb broadly developed medially	Fig. 5.6
Abdominal tergite IX	with anterior and posterior pairs of campaniform sensilla (pores)	Fig. 5.11
Male: sternites	transverse glandular areas on sternites III to VII	Fig. 5.12

Figure 4. Location of general characters of *Thrips* (♀ – dorsal view)

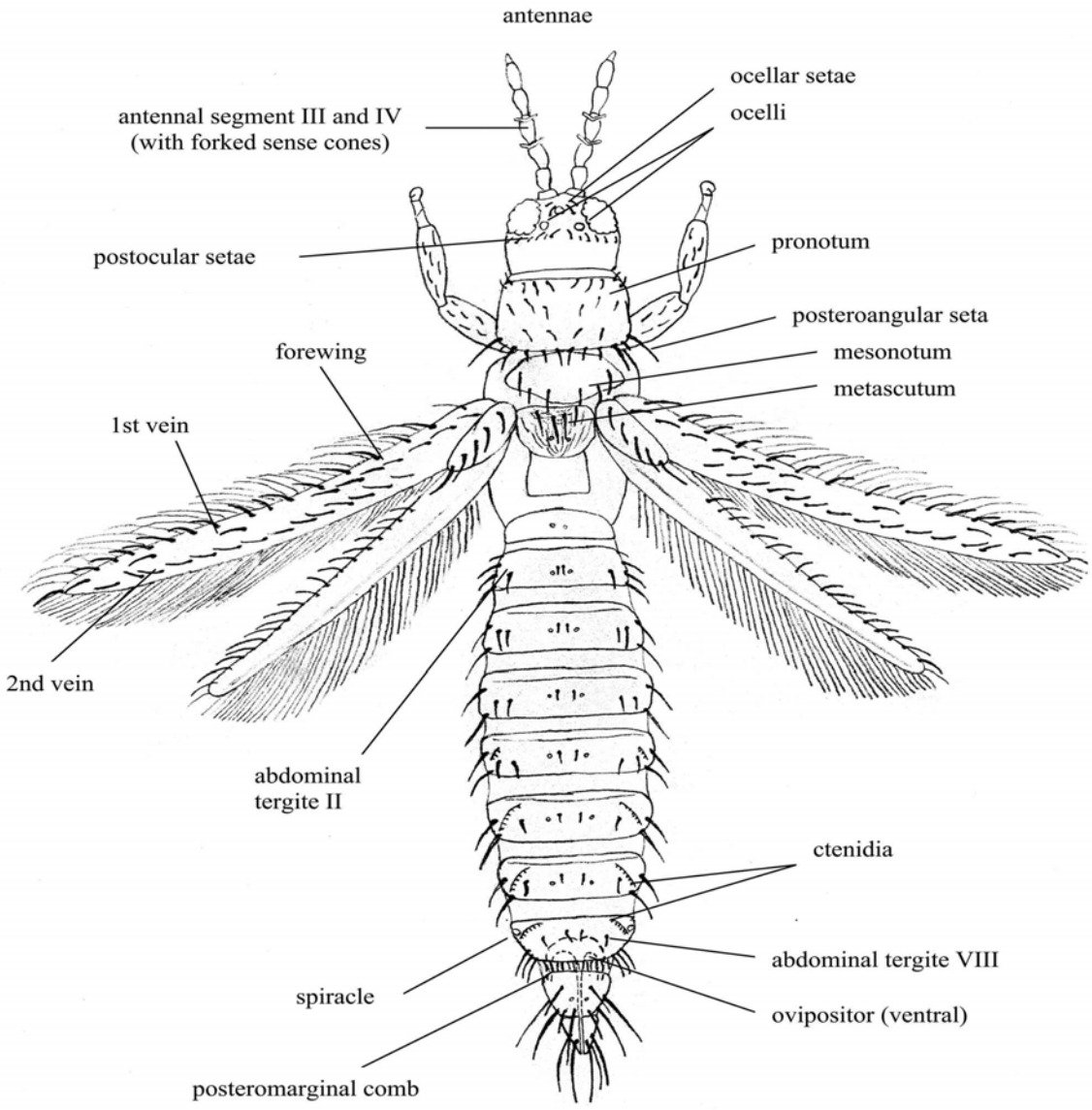


Figure 5 (Figs 5.1 to 5.12): Characters of *Thrips palmi* (photos: G. Vierbergen, PPS, Netherlands; figures drawn by S. Kobro, Norwegian Crop Protection Institute, Norway)

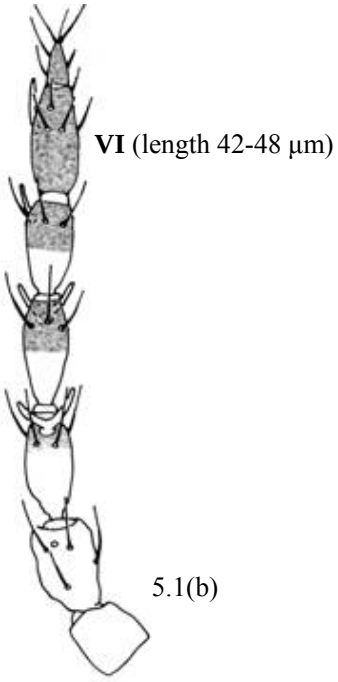
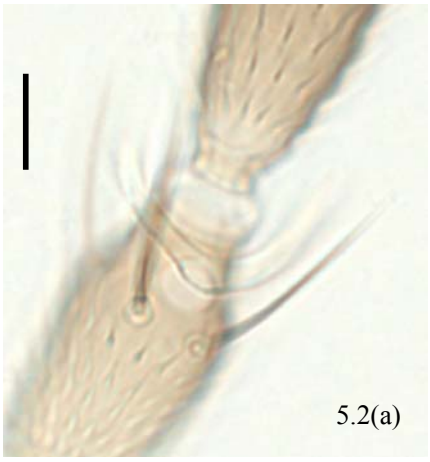
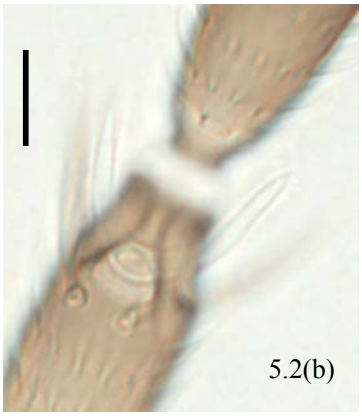


Fig. 5.1(a), (b): Antenna: seven segments (scale bar: 100 µm)





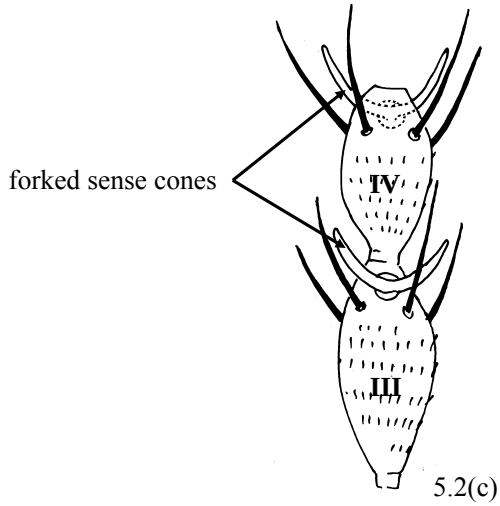


Fig. 5.2(a)–(c): Antenna, forked sense cones; (a) segment III, dorsal; (b) segment IV, ventral; (c) segment III and IV, dorsal (scale bars: 10 µm)

Fig. 5 continued.

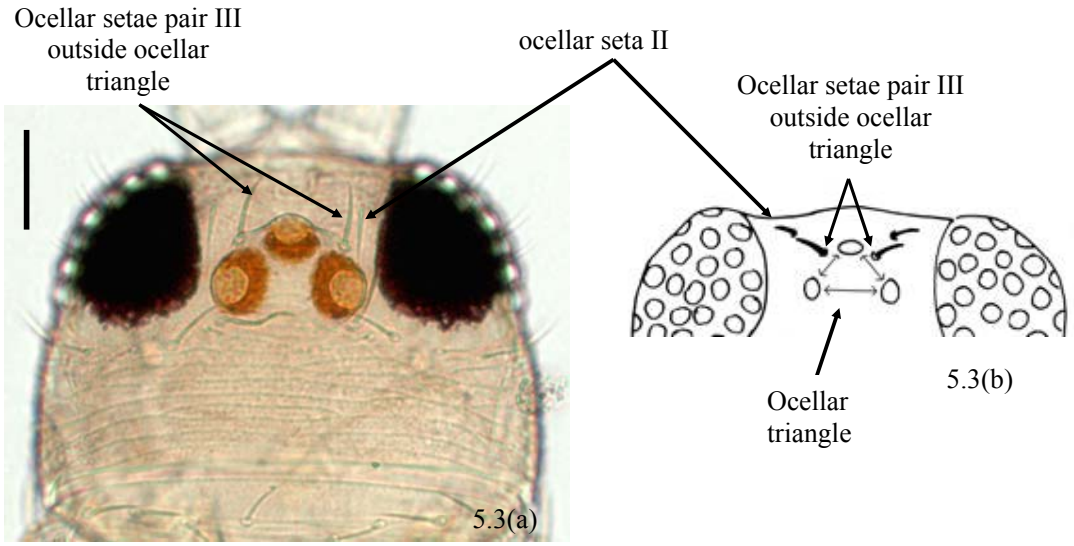


Fig. 5.3(a), (b): Head: with two pairs of ocellar setae (pair I missing). Ocellar setae pair III situated outside of ocellar triangle (scale bar: 30 µm)

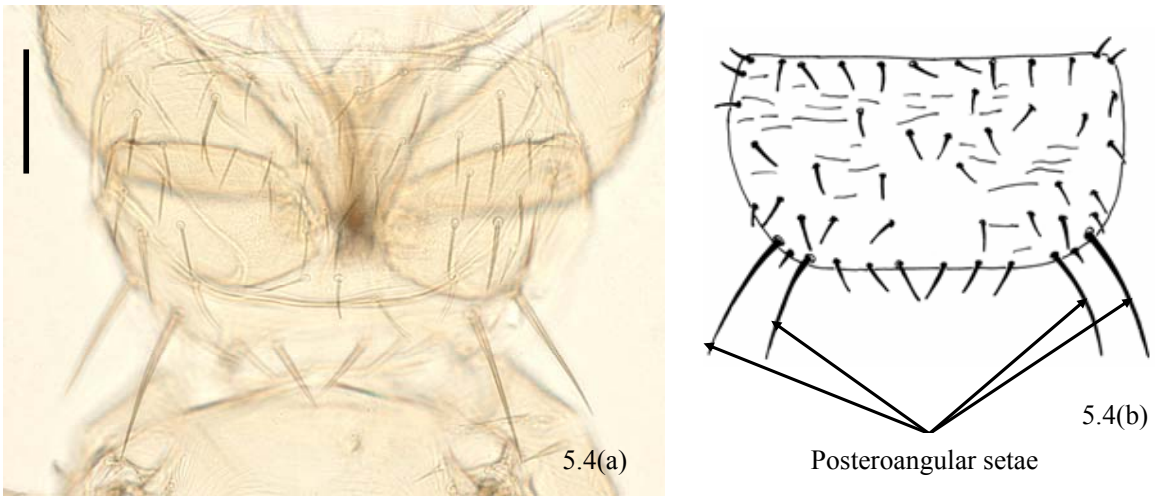


Fig. 5.4(a), (b): Pronotum, two pairs of major posteroangular setae (scale bar = 50 µm)

Fig. 5 continued

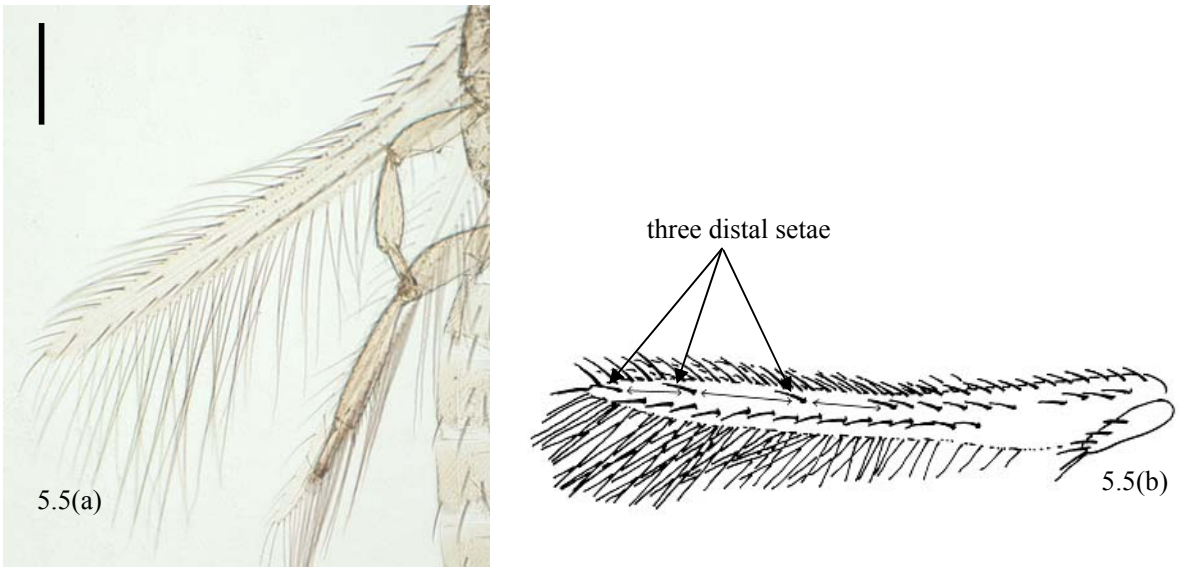


Fig. 5.5(a), (b): Forewing, first vein – three setae with gaps in distal half (scale bar: 100 µm)

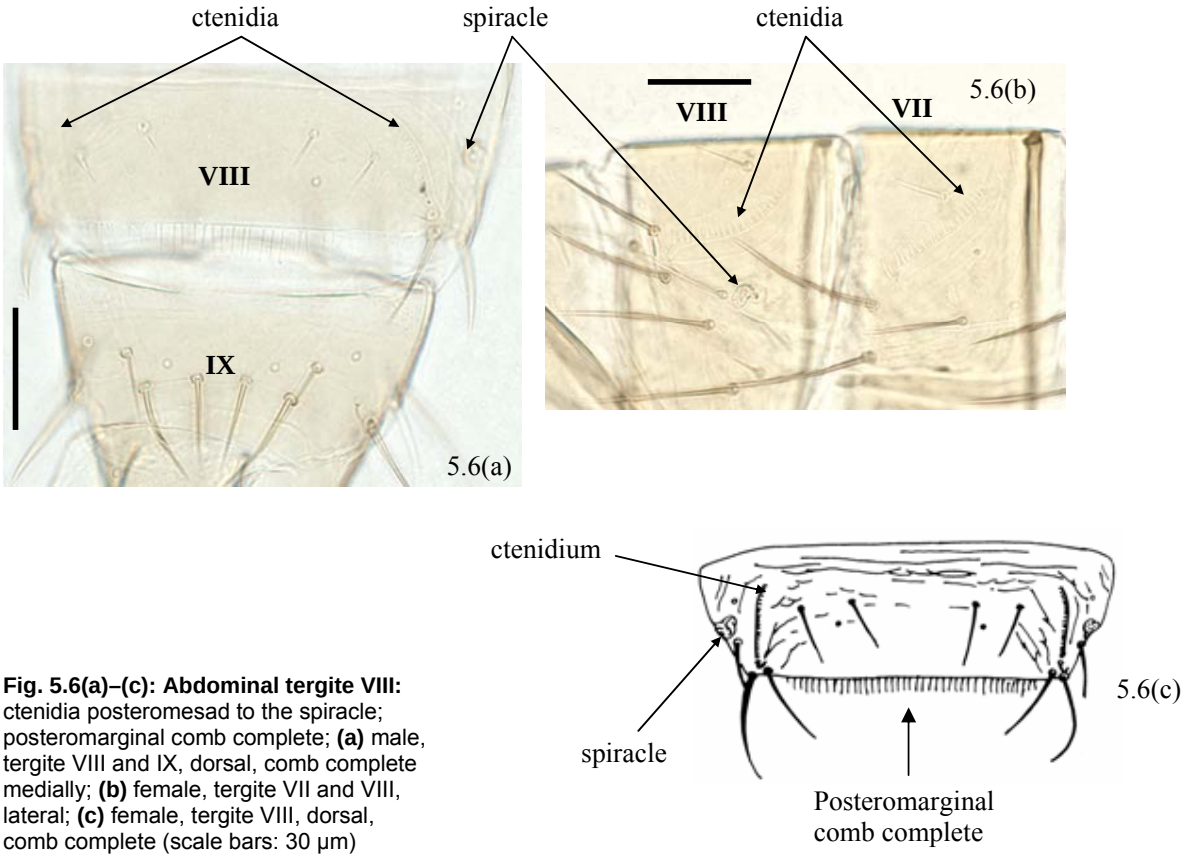


Fig. 5.6(a)–(c): Abdominal tergite VIII: ctenidia posteromesad to the spiracle; posteromarginal comb complete; **(a)** male, tergite VIII and IX, dorsal, comb complete medially; **(b)** female, tergite VII and VIII, lateral; **(c)** female, tergite VIII, dorsal, comb complete (scale bars: 30 µm)

Fig. 5 continued.

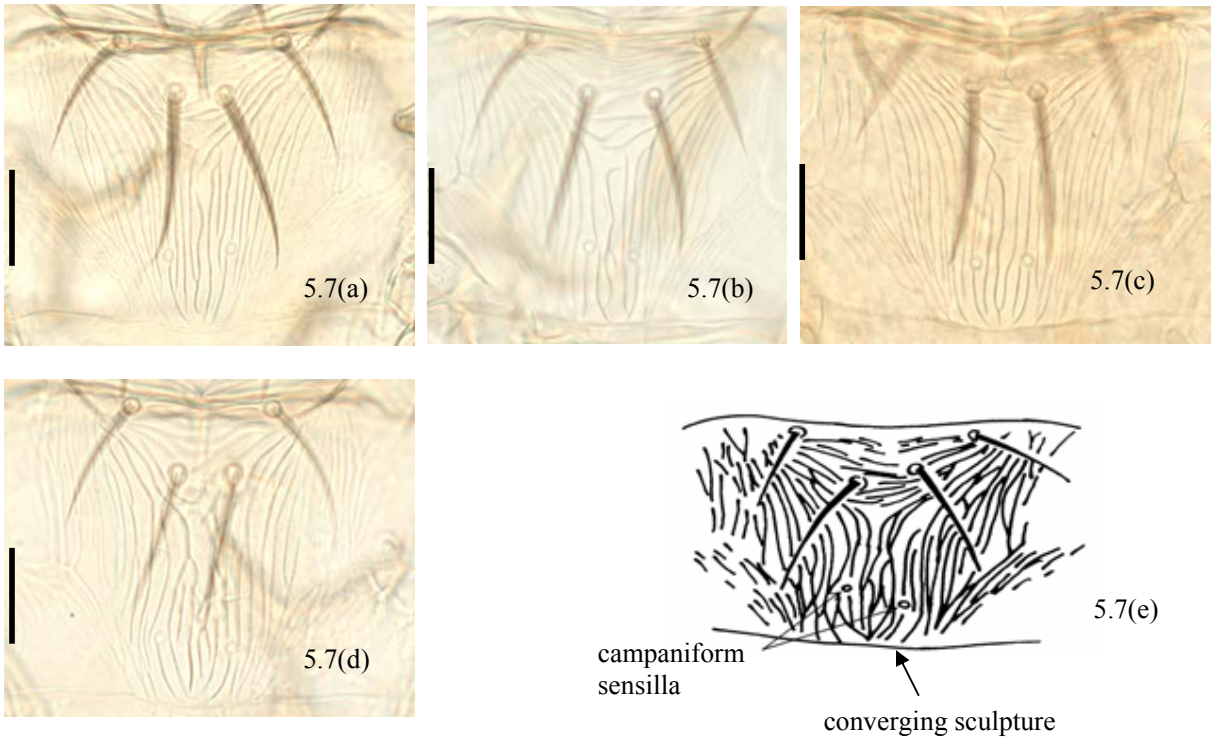


Fig. 5.7(a)–(e): Metascutum, variation in sculpture; campaniform sensilla (scale bars: 20 µm)

Fig. 5.8(a)–(c): Abdominal pleurotergites IV and V, ciliate microtrichia and discal setae absent; **(a)** bright field; **(b)** phase contrast; **(c)** complete tergite (scale bars: 20 µm)

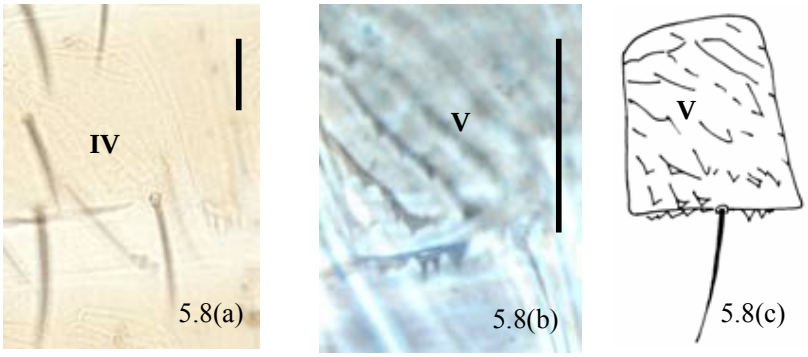


Fig. 5.9(a), (b): Abdominal tergite II, four lateral marginal setae (scale bar: 20 µm)

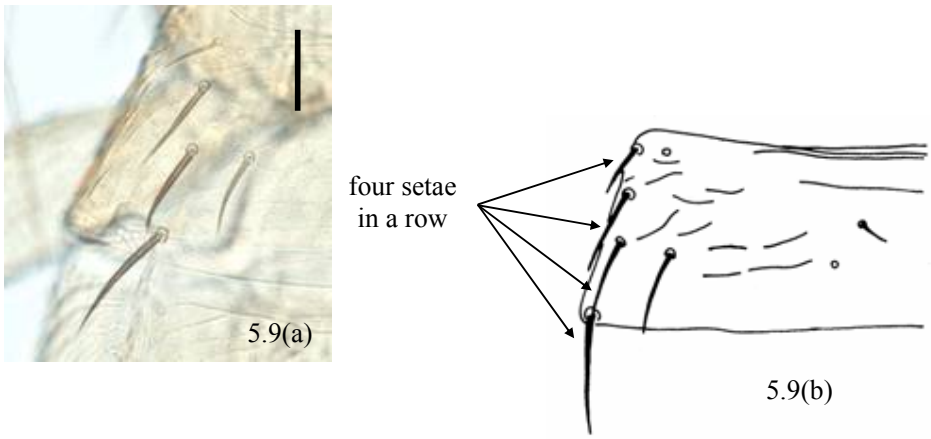
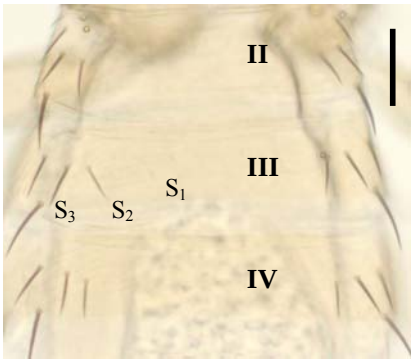
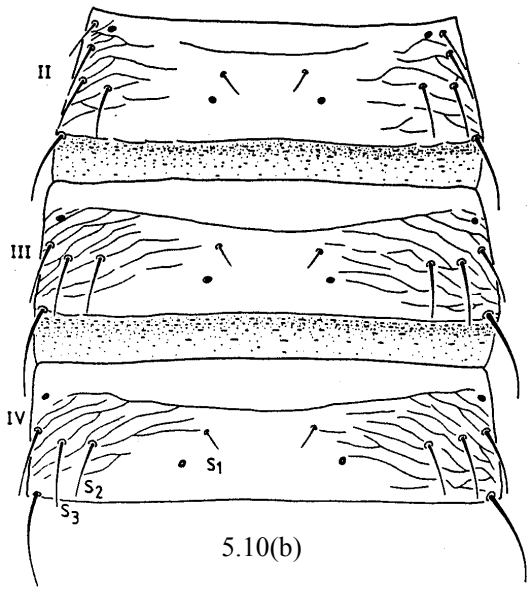


Fig. 5 continued.

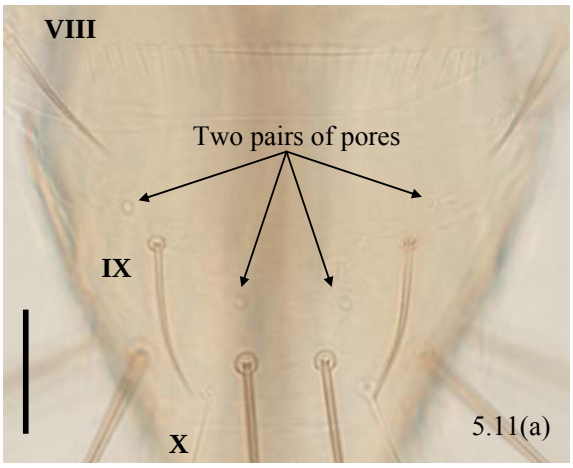


5.10(a)

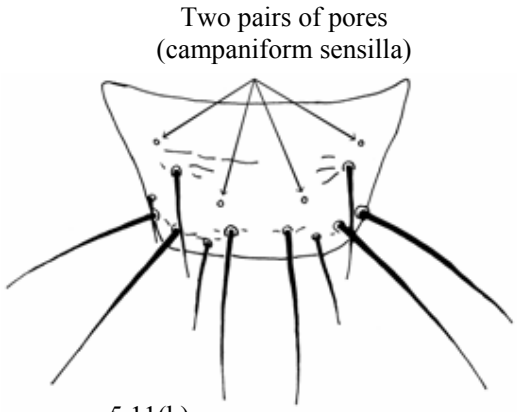


5.10(b)

Fig. 5.10(a), (b): Tergites II–IV, female, setae S2 about same size as setae S3 (5.10b from zur Strassen, 1989) (scale bar: 50 µm)

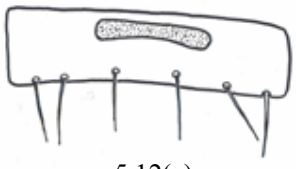


5.11(a)

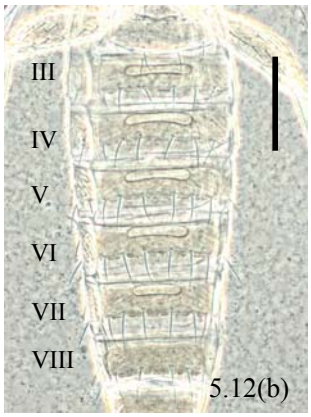


5.11(b)

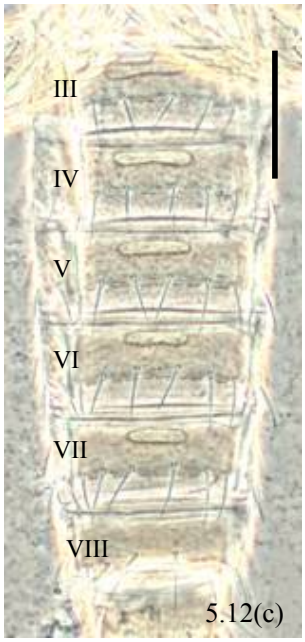
Fig. 5.11(a), (b): Abdominal tergite IX (dorsal), two pairs of campaniform sensilla (scale bar: 30 µm)



5.12(a)



5.12(b)



5.12(c)

Fig. 5.12(a)–(c): Male glandular areas (showing variation); **(a)** sternite V; **(b)-(c)** sternites III–VIII, phase contrast (scale bars: 100 µm)

4.2 Molecular assays for identifying *Thrips palmi*

Four molecular assays have been published that can be used to support a morphological identification of *T. palmi* and these are described below. The specificity of each assay is also described. This indicates the thrips species against which each assay was evaluated and the original use for which the assay was designed. A CD-ROM identification system is also available that includes molecular data for thrips species (Moritz *et al.*, 2004). Considering the specific limitations of molecular methods a negative molecular test result does not exclude the possibility of positive identification by morphological methods.

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these define the original level of sensitivity, specificity and/or reproducibility achieved.

Requirements for controls

With all molecular methods the use of appropriate controls is essential; a validated *T. palmi*-positive extract must be included as an additional sample to ensure that amplification has been successful. PCR amplification, either for real-time PCR or PCR-RFLP, must also be performed on a sample with no DNA. This negative control indicates possible reagent contamination and false positives.

DNA extraction

DNA may be extracted from single eggs, adults, pupae or larvae. For each of the assays described below refer to the source paper for the original specific DNA extraction technique used. Laboratories may find that alternative extraction techniques work equally well; DNA may be extracted using any DNA extraction methods suitable for insects. For example:

- The thrips may be ground in a lysis buffer in a microtube using a micropestle, and the homogenate taken through a proteinase-K-based DNA extraction kit according to the appropriate manufacturer's instructions.
- Alternatively, a thrips may be ground in 50 µl nuclease-free water before the addition of 50 µl of a 1:1 (volume to volume) slurry of Chelex 100 resin, and nuclease-free water, heated to 95 ºC for 5 min and centrifuged at 11,000 *g* for 5 min. The supernatant is transferred to a new microtube and stored at −20 ºC.

Several recent papers have described non-destructive techniques for extracting DNA from thrips, which have the advantage that after DNA extraction has been completed a cleared specimen remains available for slide mounting (e.g., Rugman-Jones *et al.*, 2006; Mound and Morris, 2007).

4.2.1 SCAR marker-generated sequence-based real-time PCR assay for *Thrips palmi*

This assay of Walsh *et al.* (2005) was designed as a species-specific assay against *T. palmi* for use by the phytosanitary authorities in England and Wales. It was evaluated by screening it against 21 other species of Thysanoptera, including ten belonging to the genus *Thrips* (*T. flavus*, *T. major* Uzel, *T. minutissimus* L., *T. nigropilosus*, *T. sambuci* Heeger, *T. tabaci*, *T. trehernei* Priesner or *T. physapus* L., *T. urticae*, *T. validus* Uzel, *T. vulgatissimus* Haliday). These were predominantly, but not exclusively, European species.

Methodology

The *T. palmi*-specific PCR primers and TaqMan probe used in this assay were as follows:

PCR primer: P4E8-362F (5′-CCGACAAAATCGGTCTCATGA-3′)

PCR primer: P4E8-439R (5′-GAAAAGTCTCAGGTACAACCCAGTTC-3′)

TaqMan probe: P4E8-385T (FAM 5′-AGACGGATTGACTTAGACGGGAACGGTT-3′ TAMRA).

Real-time PCR reactions were set up using the TaqMan PCR core reagent kit (Applied Biosystems)[1], with 1 µl (10–20 ng) of DNA extract, 7.5 pmol of each primer and 2.5 pmol probe in a total volume of 25 µl. Plates were cycled at generic system conditions (10 min at 95 °C and 40 cycles of 1 min at 60 °C, 15 s at 95 °C) on either of the ABI Prism 7700 or ABI 7900HT Sequence Detection Systems (Applied Biosystems)[2], using real-time data collection. Ct values lower than 40 indicated the presence of *T. palmi* DNA.

4.2.2 COI sequence-based real-time PCR assay for *Thrips palmi*

This assay of Kox *et al.* (2005) was designed as a species-specific assay against *T. palmi* for use by the phytosanitary authorities in the Netherlands. It was evaluated by screening the assay against 23 other species of thrips, including 11 belonging to the genus *Thrips* (*T. alliorum* (Priesner), *T. alni*, *T. angusticeps* Uzel, *T. fuscipennis* Haliday, *T. latiareus* Vierbergen, *T. major*, *T. minutissimus*, *T. parvispinus* (Karny), *T. tabaci*, *T. urticae*, *T. vulgatissimus*). These were predominantly, but not exclusively, European species.

Methodology

The *Thrips palmi*-specific PCR primers and TaqMan probe used in this assay are as follows:

PCR primer: Tpalmi 139F* (5′-TCA TGC TGG AAT TTC AGT AGA TTT AAC-3′)

PCR primer: Tpalmi 286R* (5′-TCA CAC RAA TAA TCT TAG TTT TTC TCT TG-3′)

TaqMan probe: TpP (6-FAM 5′-TAG CTG GGG TAT CCT CAA-3′ MGB).

* Primers have been adjusted for greater sensitivity since original publication.

(COI sequences that mismatch with the TaqMan probe in this assay have been deposited on GenBank from a number of specimens from India identified as *T. palmi* on the basis of their morphology (Asokan *et al.*, 2007). These sequences would not produce a positive result using this assay. The taxonomic or phylogenetic significance of this sequence differentiation currently remains unclear.)

The 25 µl reaction mixture contained 12.5 µl of 2x TaqMan Universal Master Mix (Applied Biosystems)[3], 0.9 µM each primer, 0.1 µM TaqMan probe, 1.0 µl DNA. The real-time PCR was performed on either of the ABI Prism 7700 or ABI 7900HT Sequence Detection Systems (Applied Biosystems)[4] using the following conditions: 10 min at 95 °C; then 40 cycles of 1 min at 60 °C and 15 s at 94 °C. Ct values lower than 40 indicated the presence of *T. palmi* DNA.

4.2.3 ITS2 sequence-based PCR-RFLP assay for nine species of thrips including *Thrips palmi*

This assay (Toda and Komazaki, 2002) was designed to separate nine species of thrips, including *T. palmi*, that are found in fruit trees in Japan: *Frankliniella occidentalis* (Pergande), *F. intonsa* (Trybom), *T. hawaiiensis* Morgan, *T. coloratus* Schmutz, *T. flavus*, *T. tabaci*, *T. palmi*, *T. setosus* Moulton, *Scirtothrips dorsalis* Hood.

[1, 2] The use of the brand Applied Biosystems for the TaqMan PCR core reagent kit and the ABI Prism 7700 or ABI 7900HT Sequence Detection Systems in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

[3, 4] The use the brand Applied Biosystems for the TaqMan Universal Master Mix and ABI Prism 7700 or ABI 7900HT Sequence Detection Systems in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute and endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

Methodology

The PCR primers (located in the 5.8 S and 28 S regions flanking the ITS2 region of ribosomal DNA) used in this assay were as follows:

5′-TGTGAACTGCAGGACACATGA-3′

5′-GGTAATCTCACCTGAACTGAGGTC-3′.

T. palmi generated a 588-base-pair (bp) PCR product (longer or shorter fragments were produced from the other species). The 20 µl reaction mixture was composed as follows: 1 µM each primer, 250 µM dNTPs, 1 Unit of AmpliTaq Gold DNA polymerase (Applied Biosystems)[5], 2 µl 10x reaction buffer [with 25 mM $MgCl_2$], 0.5 µl DNA. The PCR was performed in a 9600 DNA thermocycler (Applied Biosytems)[6], with the following conditions: 9 min at 95 °C, 35 cycles of 1 min at 94 °C, 30 s at 50 °C, and 1 min at 72 °C, followed by a final extension for 7 min at 72 °C and quickly cooled to room temperature. The PCR products were analysed by agarose gel electrophoresis.

5 µl of PCR product (without purification) was digested with the enzyme *Rsa*I according to the manufacturer's instructions. Digested PCR products were separated by 2.0% agarose gel electrophoresis.

Restriction fragment sizes produced by *T. palmi* when the ITS2 fragment is digested with *Rsa*I were as follows: 371, 98, 61 and 58 bp.

4.2.4 COI sequence-based PCR-RFLP assay for ten species of thrips including *Thrips palmi*

This assay of Brunner *et al.* (2002) was designed to separate ten species of thrips, including *T. palmi*, which are mostly, but not exclusively, pest species found in Europe: *Anaphothrips obscurus* (Müller), *Echinothrips americanus* Morgan, *Frankliniella occidentalis*, *Heliothrips haemorrhoidalis* (Bouché), *Hercinothrips femoralis* (Reuter), *Parthenothrips dracaenae* (Heeger), *Taeniothrips picipes* (Zetterstedt), *Thrips angusticeps* Uzel, *T. palmi*, *T. tabaci*.

Methodology

The PCR primers (located in the mitochondrial COI gene sequence) used in this assay are as follows:

mtD-7.2F (5′-ATTAGGAGCHCCHGAYATAGCATT-3′)

mtD9.2R (5′-CAGGCAAGATTAAAATATAAACTTCTG-3′).

These primers amplified a 433-bp fragment in all the species separated by this assay. The 50 µl reaction mixture was composed as follows: 0.76 µM each primer, 200 µM dNTPs, 1 Unit Taq DNA polymerase, 5 µl 10X reaction buffer [with 15 mM $MgCl_2$], 1 µl DNA. The PCR was performed in a standard thermocycler with the following conditions: 1 min 94 °C, 40 cycles of 15 s at 94 °C, 30 s at 55 °C, and 45 s at 72 °C, followed by a final extension for 10 min at 72 °C and quickly cooled to room temperature. To gauge the fragment size produced after amplification, 5 µl of the PCR products were analysed by 1.0–2.0% agarose gel electrophoresis.

5 µl of PCR product (without purification) was digested with the enzymes *Alu*I and *Sau*3AI in separate reactions according to the manufacturer's instructions. Digested PCR products were separated by agarose gel electrophoresis.

[5, 6] The use of the brand Applied Biosystems AmpliTaq Gold DNA polymerase and 9600 DNA thermocycler in this diagnostic protocol implies no approval of them to the exclusion of others that may be suitable. This information is given for the convenience of users of this protocol and does not constitute and endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

Restriction fragment sizes produced by *T. palmi* when the COI fragment is digested with *Alu*I and *Sau*3AI are as follows:

*Alu*I:	291 and 194 bp
*Sau*3AI:	293, 104, 70 and 18 bp.

5. Records

Records and evidence should be retained as described in section 2.5 of ISPM 27:2006.

In cases where other contracting parties may be adversely affected by the diagnosis, the records and evidence (in particular, preserved or slide-mounted specimens, photographs of distinctive taxonomic structures, DNA extracts and photographs of gels, as appropriate), should be kept for at least one year.

6. Contact points for further information

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8. References

Asokan, R., Krishna Kumar, N.K., Kumar, V. & Ranganath, H.R. 2007. Molecular differences in the mitochondrial cytochrome oxidase I (mtCOI) gene and development of a species-specific marker for onion thrips, *Thrips tabaci* Lindeman, and melon thrips, *T. palmi* Karny (Thysanoptera: Thripidae), vectors of tospoviruses (Bunyaviridae). *Bulletin of Entomological Research*, 97: 461–470.

Bhatti, J.S. 1980. Species of the genus *Thrips* from India (Thysanoptera). *Systematic Entomology*, 5: 109–166.

Bournier, J.P. 1983. Un insecte polyphage: *Thrips palmi* (Karny), important ravageur du cotonnier aux Philippines. *Cotonnier et Fibres Tropicales*, 38: 286–288.

Brunner, P.C., Fleming, C. & Frey, J.E. 2002. A molecular identification key for economically important thrips species (Thysanoptera: Thripidae) using direct sequencing and a PCR-RFLP-based approach. *Agricultural and Forest Entomology*, 4: 127–136.

EPPO. 2008. URL: http://www.eppo.org/. Accessed 17 June 2008.

EPPO/CABI. 1997. *Thrips palmi*. *In* I.M. Smith, D.G. McNamara, P.R. Scott & M. Holderness, eds. *Quarantine pests for Europe*, 2nd edition. Wallingford, UK, CAB International. 1425 pp.

Kox, L.F.F., van den Beld, H.E., Zijlstra, C. & Vierbergen, G. 2005. Real-time PCR assay for the identification of *Thrips palmi*. *Bulletin OEPP/EPPO Bulletin*, 35: 141–148.

Mantel, W.P. & Vierbergen, G. 1996. Additional species to the Dutch list of Thysanoptera and new intercepted Thysanoptera on imported plant material. *Folia Entomologica Hungarica*, 57 (Suppl.): 91–96.

Moritz, G., Mound, L.A., Morris, D.C. & Goldarazena, A. 2004. Pest thrips of the world: visual and molecular identification of pest thrips (CD-ROM), Centre for Biological Information Technology (CBIT), University of Brisbane. ISBN 1-86499-781-8.

Mound, L.A. & Azidah, A.A. 2009. Species of the genus *Thrips* (Thysanoptera) from Peninsular Malaysia, with a checklist of recorded Thripidae. *Zootaxa*, 2023: 55–68.

Mound, L.A. & Kibby, G. 1998. *Thysanoptera. An Identification Guide*. 2nd edition. Wallingford, UK, CAB International. 70 pp.

Mound, L.A. & Marullo, R. 1996. The thrips of Central and South America: an introduction (Insecta: Thysanoptera). *Memoirs on Entomology, International*, 6: 1–488.

Mound, L.A. & Masumoto, M. 2005. The genus *Thrips* (Thysanoptera, Thripidae) in Australia, New Caledonia and New Zealand. *Zootaxa*, 1020: 1–64.

Mound, L.A. & Morris, D.C. 2007. A new thrips pest of *Myoporum* cultivars in California, in a new genus of leaf-galling Australian Phlaeothripidae (Thysanoptera). *Zootaxa*, 1495: 3545.

Murai, T. 2002. The pest and vector from the East: *Thrips palmi*. *In* R. Marullo, & L.A. Mound, eds. *Thrips and Tospoviruses: Proceedings of the 7th International Symposium on Thysanoptera*. Italy, 2–7 July 2001, pp. 19–32. Canberra, Australian National Insect Collection.

Nakahara, S. 1994. The genus *Thrips* Linnaeus (Thysanoptera: Thripidae) of the New World. USDA Technical Bulletin No. 1822. 183 pp.

PaDIL. 2007. Pests and Diseases Image Library. URL: http://www.padil.gov.au. Accessed 18 Oct 2007.

Palmer, J.M. 1992. Thrips (Thysanoptera) from Pakistan to the Pacific: a review. *Bulletin of the British Museum (Natural History). Entomology Series*, 61: 1–76.

Rugman-Jones, P.F., Hoddle, M.S., Mound, L.A. & Stouthamer, R. 2006. Molecular identification key for pest species of *Scirtothrips* (Thysanoptera: Thripidae). *Journal of Economic Entomology*, 99 (5): 1813–1819.

Sakimura, K., Nakahara, L.M. & Denmark, H.A. 1986. A thrips, *Thrips palmi* Karny (Thysanoptera: Thripidae). Entomology Circular No. 280. Division of Plant Industry, Florida; Dept. of Agriculture and Consumer Services. 4 pp.

Toda, S. & Komazaki, S. 2002. Identification of thrips species (Thysanoptera: Thripidae) on Japanese fruit trees by polymerase chain reaction and restriction fragment length polymorphism of the ribosomal ITS2 region. *Bulletin of Entomological Research*, 92: 359–363.

Walsh, K., Boonham, N., Barker, I. & Collins, D.W. 2005. Development of a sequence-specific real-time PCR to the melon thrips *Thrips palmi* (Thysan., Thripidae). *Journal of Applied Entomology*, 129 (5): 272–279.

zur Strassen, R. 1989. Was ist *Thrips palmi*? Ein neuer Quarantäne-Schädling in Europa. *Gesunde Pflanzen*, 41: 63–67.

zur Strassen, R. 2003. Die terebranten Thysanopteren Europas und des Mittelmeer-Gebietes. *In Die Tierwelt Deutschlands. Begründet 1925 von Friedrich Dahl, 74*: 5–277. Keltern, Goecke & Evers.

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INTERNATIONAL STANDARDS FOR PHYTOSANITARY MEASURES

ISPM 27 DIAGNOSTIC PROTOCOLS

DP 2: *Plum pox virus*

(2012)

CONTENTS

1. Pest Information

Sharka (plum pox) is one of the most serious diseases of stone fruit. The disease, caused by *Plum pox virus* (PPV), affects plants of the genus *Prunus*. It is particularly detrimental in *P. armeniaca*, *P. domestica*, *P. persica* and *P. salicina* because it reduces quality and causes premature fruit drop. It is estimated that the costs of managing sharka worldwide since the 1970s exceed 10 000 million euros (Cambra *et al*., 2006b).

Sharka was first reported in *P. domestica* in Bulgaria in 1917–1918, and was described as a viral disease in 1932. Since then, the virus has spread progressively to a large part of Europe, around the Mediterranean basin and the Near and Middle East. It has been found with a restricted distribution in South and North America and Asia (EPPO, 2006; CABI, 2011).

PPV is a member of the genus *Potyvirus* in the family *Potyviridae*. The virus particles are flexuous rods of approximately 700 nm × 11 nm, and are composed of a single-stranded RNA molecule consisting of almost 10 000 nucleotides coated by up to 2 000 subunits of a single coat protein (García and Cambra, 2007). PPV is transmitted in the field by aphids in a non-persistent manner, but movement of infected propagative plant material is the main way in which PPV is spread over long distances.

PPV isolates can be classified currently into seven types or strains: D (Dideron), M (Marcus), C (Cherry), EA (El Amar), W (Winona), Rec (Recombinant) and T (Turkish) (Candresse and Cambra, 2006; James and Glasa, 2006; Ulubaş Serçe *et al.*, 2009). Most PPV isolates belong to the D and M types. PPV D and M strains can easily infect *P. armeniaca* and *P. domestica* but differ in their ability to infect *P. persica* cultivars. The strains vary in the pathogenicity; for example, M isolates generally cause faster epidemics and more severe symptoms than D isolates in *P. armeniaca*, *P. domestica*, *P. persica* and *P. salicina*. EA isolates are geographically restricted to Egypt and little information is available about their epidemiology and biological properties. PPV isolates infecting *P. avium* and *P. cerasus* have been identified in several European countries recently. These isolates form a distinct type that has been defined as PPV-C. An atypical PPV was isolated from *P. domestica* in Canada (PPV-W) representing a distinct PPV type. In addition, natural recombinants between the D and M types of PPV have been described as PPV-Rec showing an epidemiological behaviour similar to the D type. Recently a second type of recombinant isolate has been reported in Turkey (T type).

Further information about PPV, including illustrations of disease symptoms, can be found in Barba *et al*. (2011), CABI (2011), EPPO (2004), EPPO (2006), García and Cambra (2007) and PaDIL (2011).

2. Taxonomic Information

Name:	*Plum pox virus* (acronym PPV)
Synonym:	Sharka virus
Taxonomic position:	*Potyviridae*, *Potyvirus*
Common names:	Sharka, plum pox.

3. Detection and Identification

Under natural conditions, PPV readily infects fruit trees of the genus *Prunus* used as commercial varieties or rootstocks: *P. armeniaca*, *P. cerasifera*, *P. davidiana*, *P. domestica*, *P. mahaleb*, *P. marianna*, *P. mume*, *P. persica*, *P. salicina*, and interspecific hybrids between these species. *Prunus avium*, *P. cerasus* and *P. dulcis* may be infected occasionally. The virus also infects many wild and ornamental *Prunus* species such as *P. besseyi*, *P. cistena*, *P. glandulosa*, *P. insititia*, *P. laurocerasus*, *P. spinosa*, *P. tomentosa* and *P. triloba*. Under experimental conditions, PPV can be transmitted mechanically to numerous *Prunus* spp. and several herbaceous plants (*Arabidopsis thaliana*, *Chenopodium foetidum*, *Nicotiana benthamiana*, *N. clevelandii, N. glutinosa* and *Pisum sativum*).

PPV symptoms may appear on leaves, shoots, bark, petals, fruits and stones in the field. They are usually distinct on leaves early in the growing season and include mild light-green discoloration; chlorotic spots, bands or rings; vein clearing or yellowing; or leaf deformation. Some of these leaf symptoms are similar to those caused by other viruses, such as *American plum line pattern virus*. *Prunus cerasifera* cv. GF 31 shows rusty-brown corking and cracking of the bark. Flower symptoms can occur on petals (discoloration) of some *P. persica* cultivars when infected with PPV-M or in *P. glandulosa* infected with PPV-D. Infected fruits show chlorotic spots or lightly pigmented yellow rings or line patterns. Fruits may become deformed or irregular in shape and develop brown or necrotic areas under the discoloured rings. Some fruit deformations, especially in *P. armeniaca* and *P. domestica*, are similar to those caused by *Apple chlorotic leaf spot virus*. Diseased fruits may show internal browning and gummosis of the flesh and reduced quality. In severe cases the diseased fruits drop prematurely from the tree. In general the fruits of early maturing cultivars show more marked symptoms than those of late maturing cultivars. Stones from diseased fruits of *P. armeniaca* show typical pale rings or spots. The alcohol or spirits produced from diseased fruits are unmarketable owing to an undesirable flavour. Symptom development and intensity depend strongly on the host plant and climatic conditions; for example the virus may be latent for several years in cold climates.

General guidance on sampling methodologies is described in ISPM 31:2008 (*Methodologies for sampling of consignments*). Appropriate sample selection is critical for PPV detection. Sampling should take into account virus biology and local climatic conditions, in particular the weather conditions during the growing season. If typical symptoms are present, collect flowers, leaves or fruits showing symptoms. In symptomless plants, samples should be taken from at least one-year-old shoots with mature leaves or fully expanded leaves collected from the middle of each of the main branches (detection is not reliable in shoots less than one year old). Samples should be collected from at least four different sites (e.g. four branches or four leaves) in each plant; this is critical because of the uneven distribution of PPV. Sampling should not be done during months with the highest temperatures. Tests on samples collected in the autumn are less reliable than tests done on samples collected earlier in the spring. Plant material should preferably be collected from the internal parts of the tree canopy. In springtime, samples can be flowers, shoots with fully expanded leaves or fruits. In summer and autumn, mature leaves and the skin of mature fruits collected from the field or packing houses can be used for analysis. Flowers, leaves, shoots and fruit skin can be stored at 4 °C for not more than 10 days before processing. Fruits can be stored for one month at 4 °C before processing. In winter dormant buds or bark tissues from the basal part of twigs, shoots, or branches, or complete spurs can be selected.

Detection of PPV can be achieved using a biological, serological or molecular test; identification requires either a serological or molecular test. A serological or molecular test is the minimum requirement to detect and identify PPV (e.g. during routine diagnosis of a pest widely established in a country). In instances where the national plant protection organization (NPPO) requires additional confidence in the identification of PPV (e.g. detection in an area where the virus is not known to occur or detection in a consignment originating in a country where the pest is declared to be absent), further tests may be done. Where the initial identification was done using a molecular method, subsequent tests should use serological techniques and vice versa. Further tests may also be done to identify the strain of PPV present. In all cases, positive and negative controls must be included in the tests. The recommended techniques are described in the following sections.

In some circumstances (e.g. during the routine diagnosis of a pest widely established in a country) multiple plants may be tested simultaneously using a bulked sample derived from a number of plants. The decision to test individual or multiple plants depends on the virus concentration in the plants and the level of confidence required by the NPPO.

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these define the original level of sensitivity, specificity and/or reproducibility achieved. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

3.1 Biological detection

The main indicator plants used for PPV indexing are seedlings of *P. cerasifera* cv. GF31, *P. persica* cv. GF305, *P. persica* × *P. davidiana* cv. Nemaguard, or *P. tomentosa*. Indicator plants are raised from seed, planted in a well-drained soil mixture and maintained in an insect-proof greenhouse between 18 °C and 25 °C until they are large enough to graft (usually 25–30 cm high with a diameter of 3–4 mm). Alternatively seedlings of other *Prunus* species may be grafted with indicator plant scions. The indicators must be graft-inoculated according to conventional methods such as bud grafting (Desvignes, 1999), using at least four replicates per indicator plant. The grafted indicator plants are maintained in the same conditions and, after 3 weeks, are pruned to a few centimetres above the top graft (Gentit, 2006). The grafted plants should be inspected for symptoms for at least 6 weeks. Symptoms, in particular chlorotic banding and patterns, are observed on the new growth after 3–4 weeks and must be compared with positive and healthy controls. Illustrations of symptoms caused by PPV on indicator plants can be found in Damsteegt *et al*. (1997; 2007) and Gentit (2006).

There are no quantitative data published on the specificity, sensitivity or reliability of grafting. The method is used widely in certification schemes and is considered a sensitive method of detection. However, it is not a rapid test (symptom development requires several weeks post-inoculation), it can only be used to test budwood, it requires dedicated facilities such as temperature-controlled greenhouse space, and the symptoms observed may be confused with those of other graft-transmissible agents. Moreover, there are asymptomatic strains that do not induce symptoms and thus are not detectable on indicator plants.

3.2 Serological detection and identification

Enzyme-linked immunosorbent assays (ELISA) are highly recommended for screening large numbers of samples.

For sample processing, approximately 0.2–0.5 g of fresh plant material is cut into small pieces and placed in a suitable tube or plastic bag. The sample is homogenized in approximately 4–10 ml (1:20 w/v) of extraction buffer using an electrical tissue homogenizer, or a manual roller, hammer or similar tool. The extraction buffer is phosphate-buffered saline (PBS) pH 7.2–7.4, containing 2% polyvinylpyrrolidone and 0.2% sodium diethyl dithiocarbamate (Cambra *et al.*, 1994), or an alternative suitably validated buffer. Plant material should be homogenized thoroughly and used fresh.

3.2.1 Double-antibody sandwich indirect enzyme-linked immunosorbent assay

Double-antibody sandwich indirect enzyme-linked immunosorbent assay (DASI)-ELISA, also called triple-antibody sandwich (TAS)-ELISA, should be performed according to Cambra *et al*. (1994) using a specific monoclonal antibody such as 5B-IVIA, following the manufacturer's instructions.

5B-IVIA is currently the only monoclonal antibody demonstrated to detect all strains of PPV with high reliability, specificity and sensitivity (Cambra *et al.*, 2006a). In a DIAGPRO ring-test done by 17 laboratories using a panel of 10 samples, PPV-infected (PPV-D, PPV-M and PPV-D+M) and healthy samples from France and Spain, DASI-ELISA using the 5B-IVIA monoclonal antibody was 95% accurate (number of true negatives and true positives diagnosed by the technique/number of samples tested). This accuracy was greater than that achieved with either immunocapture reverse transcription-polymerase chain reaction (IC-RT-PCR) which was 82% accurate, or co-operational RT-PCR (Co-RT-PCR) which was 94% accurate (Cambra *et al*., 2006c; Olmos *et al*., 2007). The proportion of true negatives (number of true negatives diagnosed by the technique/number of healthy plants) identified by DASI-ELISA using the 5B-IVIA monoclonal antibody was 99.0%, compared with real-time RT-PCR using purified nucleic acid (89.2%) or spotted samples (98.0%), or IC-RT-PCR (96.1%). Capote *et al.* (2009) also reported that there is a 98.8% probability that a positive result obtained in winter with DASI-ELISA using the 5B-IVIA monoclonal antibody was a true positive.

3.2.2 Double-antibody sandwich enzyme-linked immunosorbent assay

The conventional or biotin/streptavidin system of double-antibody sandwich (DAS)-ELISA should be performed using kits based on the specific monoclonal antibody 5B-IVIA or on polyclonal antibodies that have been demonstrated to detect all strains of PPV without cross-reacting with other viruses or healthy plant material (Cambra *et al*., 2006a; Capote *et al*., 2009). The test should be done according to the manufacturer's instructions.

Whereas the 5B-IVIA monoclonal antibody detects all PPV strains specifically, sensitively and reliably, some polyclonal antibodies are not specific and have limited sensitivity (Cambra *et al.*, 1994; Cambra *et al.*, 2006a). Therefore the use of additional methods is recommended in situations where polyclonal antibodies have been used in an assay and the NPPO requires additional confidence in the identification of PPV.

3.3 Molecular detection and identification

Molecular methods using reverse transcription-polymerase chain reaction (RT-PCR) may be more expensive and/or time consuming than serological techniques, especially for large-scale testing. However, molecular methods, especially real-time RT-PCR, are generally more sensitive than serological techniques. The use of real-time RT-PCR also avoids the need for any post-amplification processing (e.g. gel electrophoresis) and is therefore quicker with less opportunity for contamination than conventional PCR.

With the exception of immunocapture (IC)-RT-PCR (for which RNA isolation is not required), RNA extraction should be done using appropriately validated protocols. The samples should be placed in individual plastic bags to avoid cross-contamination during extraction. Alternatively for real-time RT-PCR, spotted plant extracts, printed tissue sections or squashes of plant material can be immobilized on blotting paper or nylon membranes and analysed by real-time RT-PCR (Olmos *et al.*, 2005; Osman and Rowhani, 2006; Capote *et al.*, 2009). It is not recommended to use spotted or tissue-printed samples in conventional PCR because of the lower sensitivity compared with real-time RT-PCR.

Each method describes the volume of extracted sample that should be used as a template. Depending on the sensitivity of the method the minimum concentration of template required to detect PPV varies as follows: RT-PCR, 100 fg RNA template ml-1; Co-RT-PCR, 1 fg RNA template ml-1; and real-time RT-PCR, 2 fg RNA template ml-1.

3.3.1 Reverse transcription-polymerase chain reaction

The RT-PCR primers used in this assay are either the primers of Wetzel *et al.* (1991):

P1 (5′-ACC GAG ACC ACT ACA CTC CC-3′)

P2 (5′-CAG ACT ACA GCC TCG CCA GA-3′)

or the primers of Levy and Hadidi (1994):

3′NCR sense (5′-GTA GTG GTC TCG GTA TCT ATC ATA-3′)

3′NCR antisense (5′-GTC TCT TGC ACA AGA ACT ATA ACC-3′).

The 25 µl reaction mixture is composed as follows: 1 µM of each primer (P1/P2 or the 3′NCR primer pair), 250 µM dNTPs, 1 unit AMV reverse transcriptase, 0.5 units Taq DNA polymerase, 2.5 µl 10 × Taq polymerase buffer, 1.5 mM $MgCl_2$, 0.3% Triton X-100 and 5 µl RNA template. The reaction is performed under the following thermocycling conditions: 45 min at 42 °C, 2 min at 94 ºC, 40 cycles of 30 s at 94 °C, 30 s at either 60 °C (P1/P2 primers) or 62 °C (3′NCR primers), and 1 min at 72 °C, followed by a final extension for 10 min at 72 °C. The PCR products are analysed by gel electrophoresis. The P1/P2 and 3′NCR primers produce a 243 base pair (bp) and 220 bp amplicon, respectively.

The method of Wetzel *et al.* (1991) was evaluated by testing PPV isolates from Mediterranean areas (Cyprus, Egypt, France, Greece, Spain and Turkey). The assay was able to detect 10 fg of viral RNA, corresponding to 2 000 viral particles (Wetzel *et al.*, 1991). The method of Levy and Hadidi (1994) was evaluated using PPV isolates from Egypt, France, Germany, Greece, Hungary, Italy, Spain and Romania.

3.3.2 Immunocapture reverse transcription-polymerase chain reaction

The immunocapture phase should be performed according to Wetzel *et al.* (1992), using plant sap extracted as in section 3.2 using individual tubes or plastic bags to avoid contamination.

Prepare a dilution (1 µg ml^{-1}) of polyclonal antibodies or PPV-specific monoclonal antibody (5B-IVIA) in carbonate buffer pH 9.6. Add 100 µl of the diluted antibodies into PCR tubes and incubate at 37 °C for 3 h. Wash the tubes twice with 150 µl of sterile PBS-Tween (washing buffer). Rinse the tubes twice with RNase-free water. Clarify 100 µl of plant extract (see section 3.2) by centrifugation (5 min at 15 500 × g), and add the supernatant to the coated PCR tubes. Incubate for 2 h on ice or at 37 °C. Wash the tubes three times with 150 µl of sterile PBS-Tween. Prepare the RT-PCR reaction mixture as described in section 3.3.1 using the primers of Wetzel *et al.* (1992), and add directly to the coated PCR tubes. Perform the amplification as described in section 3.3.1.

IC-RT-PCR generally requires the use of specific antibodies, although direct-binding methods may eliminate this requirement. IC-RT-PCR using the 5B-IVIA monoclonal antibody has been validated in a DIAGPRO ring-test showing an accuracy of 82% for PPV detection (Cambra *et al.*, 2006c; Olmos *et al.*, 2007). Capote *et al.* (2009) reported that there is a 95.8% probability that a positive result obtained in winter with IC-RT-PCR using the 5B-IVIA monoclonal antibody was a true positive.

3.3.3 Co-operational reverse transcription-polymerase chain reaction

The RT-PCR primers used in this co-operational (Co)-RT-PCR assay are the primers of Olmos, Bertolini and Cambra (2002):

Internal primer P1 (5′-ACC GAG ACC ACT ACA CTC CC-3′)

Internal primer P2 (5′-CAG ACT ACA GCC TCG CCA GA-3′)

External primer P10 (5′-GAG AAA AGG ATG CTA ACA GGA-3′)

External primer P20 (5′-AAA GCA TAC ATG CCA AGG TA-3′).

The 25 µl reaction mixture is composed as follows: 0.1 µM of P1 and P2 primers, 0.05 µM of P10 and P20 primers, 400 µM dNTPs, 2 units AMV reverse transcriptase, 1 unit Taq DNA polymerase, 2 µl 10 × reaction buffer, 3 mM $MgCl_2$, 5% DMSO, 0.3% Triton X-100 and 5 µl RNA template. The RT-PCR is performed under the following thermocycling conditions: 45 min at 42 °C, 2 min at 94 °C, 60 cycles of 15 s at 94 °C, 15 s at 50 °C, and 30 s at 72 °C, followed by a final extension for 10 min at 72 °C.

The RT-PCR reaction is coupled to a colorimetric detection of amplicons using a 3′digoxigenin (DIG)-labelled PPV universal probe (5′-TCG TTT ATT TGG CTT GGA TGG AA-DIG-3′) as follows. Denature the amplified cDNA at 95 °C for 5 min and immediately place on ice. Place 1 µl of sample on a nylon membrane. Dry the membrane at room temperature and UV cross-link in a transilluminator for 4 min at 254 nm. For pre-hybridization, place the membrane in a hybridization tube at 60 °C for 1 h using a standard hybridization buffer. Discard the solution and perform the hybridization by mixing the 3′DIG-labelled probe with standard hybridization buffer at a final concentration of 10 pmol ml^{-1}, before incubating for 2 h at 60 °C. Wash the membrane twice for 15 min at room temperature with 2 × washing solution, and twice for 15 min at room temperature with 0.5 × washing solution. Equilibrate the membrane for 2 min in washing buffer before soaking for 30 min in sterilized 1%blocking solution (1 g blocking reagent dissolved in 100 ml maleic acid buffer). Incubate the membrane at room temperature with anti-DIG-alkaline phosphatase conjugate antibodies at a working concentration of 1:5 000 (150 units $litre^{-1}$) in 1% blocking solution (w/v) for

30 min. Wash the membrane twice for 15 min with washing buffer, and equilibrate for 2 min with detection buffer (100 mM Tris-HCl, 100 mM NaCl, pH 9.5). The substrate solution is prepared by mixing 45 µl NBT solution (75 mg ml^{-1} nitro blue tetrazolium salt in 70% (v/v) dimethylformamide) and 35 µl BCIP solution (50 mg ml^{-1} 5-bromo-4chloro-3indolyl phosphate toluidinium salt in 100% dimethylformamide) in 10 ml of detection buffer. After incubation with the substrate stop the reaction by washing with water.

This method was 100 times more sensitive than RT-PCR using the assay of Wetzel *et al.* (1991) (Olmos, Bertolini and Cambra, 2002). The method was validated in the DIAGPRO ring-test and had an accuracy of 94% (Cambra *et al.*, 2006c; Olmos *et al.*, 2007).

3.3.4 Real-time reverse transcription-polymerase chain reaction

Real-time RT-PCR can be performed using either TaqMan or SYBR Green I. Two TaqMan methods have been described for universal detection of PPV (Schneider *et al.*, 2004; Olmos *et al.*, 2005). The primers and TaqMan probe used in the first assay are those reported by Schneider *et al.* (2004):

Forward primer (5′-CCA ATA AAG CCA TTG TTG GAT C-3′)

Reverse primer (5′-TGA ATT CCA TAC CTT GGC ATG T-3′)

TaqMan probe (5′-FAM-CTT CAG CCA CGT TAC TGA AAT GTG CCA-TAMRA-3′).

The 25 µl reaction mixture is composed as follows: 1 × reaction mix (0.2 mM of each dNTP and 1.2 mM $MgSO_4$), 200 nM of forward and reverse primers, 100 nM TaqMan probe, 4.8 mM $MgSO_4$, 0.5 µl RT/Platinum® Taq mix (Superscript™ One-Step RT-PCR with Platinum® Taq kit; Invitrogen)[1] and 5 µl RNA template. The RT-PCR is performed under the following thermocycling conditions: 15 min at 52 °C, 5 min at 95 °C, 60 cycles of 15 s at 95 °C, and 30 s at 60 °C. The PCR products are analysed in real-time according to the equipment manufacturer's instructions.

The method of Schneider *et al.* (2004) was evaluated by testing PPV isolates from the United States, strains PPV-C, PPV-D, PPV-EA and PPV-M, and eight other viral species. The method was specific and able to detect consistently 10–20 fg of viral RNA (Schneider *et al.*, 2004). The method could also detect PPV in a number of hosts and in the leaves, stems, buds and roots of *P. persica*.

The primers and TaqMan probe used in the second assay are those reported by Olmos *et al.* (2005):

P241 primer (5′-CGT TTA TTT GGC TTG GAT GGA A-3′)

P316D primer (5′-GAT TAA CAT CAC CAG CGG TGT G-3′)

P316M primer (5′-GAT TCA CGT CAC CAG CGG TGT G-3′)

PPV-DM probe (5′-FAM-CGT CGG AAC ACA AGA AGA GGA CAC AGA-TAMRA-3′).

The 25 µl reaction mixture is composed as follows: 1 µM of P241 primer, 0.5 µM each of P316D and P316M primers, 200 nM TaqMan probe, 1 × TaqMan Universal PCR Master Mix (Applied Biosystems)[2], 1 × MultiScribe and RNase Inhibitor Mix (Applied Biosystems)[3] and 5 µl RNA template. The RT-PCR is performed under the following thermocycling conditions: 30 min at 48 °C,

[1] The use of the brand Invitrogen for the Superscript™ One-Step RT-PCR with Platinum® Taq kit in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

[2] The use of the brand Applied Biosystems for the TaqMan Universal PCR Master Mix and the MultiScribe and RNase Inhibitor Mix in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

[3] See footnote 2.

10 min at 95 °C, 40 cycles of 15 s at 95 °C, and 60 s at 60 °C. The PCR products are analysed in real-time according to the equipment manufacturer's instructions.

The method of Olmos *et al.* (2005) was evaluated using three isolates each of PPV-D and PPV-M, and was 1 000 times more sensitive than DASI-ELISA using the 5B-IVIA monoclonal antibody. The proportion of true positives (number of true positives diagnosed by the technique/number of PPV-infected plants) identified correctly by real-time RT-PCR using TaqMan (Olmos *et al.*, 2005) and purified nucleic acid was 97.5%, compared with real-time RT-PCR using spotted samples (93.6%), immunocapture RT-PCR (91.5%) or DASI-ELISA using the 5B-IVIA monoclonal antibody (86.6%) (Capote *et al.*, 2009).

Varga and James (2005) described a SYBR Green I method for the simultaneous detection of PPV and identification of D and M strains:

P1 (5′-ACC GAG ACC ACT ACA CTC CC-3′)

PPV-U (5′-TGA AGG CAG CAG CAT TGA GA-3′)

PPV-FD (5′-TCA ACG ACA CCC GTA CGG GC-3′)

PPV-FM (5′-GGT GCA TCG AAA ACG GAA CG-3′)

PPV-RR (5′-CTC TTC TTG TGT TCC GAC GTT TC-3′).

The following internal control primers may be included to ensure the correct performance of the assay:

Nad5-F (5′-GAT GCT TCT TGG GGC TTC TTG TT-3′)

Nad5-R (5′-CTC CAG TCA CCA ACA TTG GCA TAA-3′).

A two-step RT-PCR protocol is used. The RT reaction is composed as follows: 2 µl of 10 µM P1 primer, 2 µl of 10 µM Nad5-R primer, 4 µg total RNA and 5 µl water. Incubate at 72 °C for 5 min, place on ice. Add 4 µl 5 × first strand buffer (Invitrogen)[4], 2 µl 0.1 M DTT, 1 µl 10 mM dNTPs, 0.5 µl RNaseOUT™ (40 units μl^{-1}) (Invitrogen)[5], 1 µl Superscript™ II (Invitrogen)[6] and 2.5 µl water. Incubate at 42 °C for 60 min followed by 99 °C for 5 min. The 24 µl PCR reaction mixture is composed as follows: 400 nM PPV-U primer, 350 nM PPV-FM primer, 150 nM PPV-FD primer, 200 nM PPV-RR primer, 100 nM Nad5-F primer, 100 nM Nad5-R primer, 200 µM dNTPs, 2mM $MgCl_2$, 1 × Karsai buffer (Karsai *et al.*, 2002), 1:42 000 SYBR Green I (Sigma)[7] and 0.1 µl Platinum® Taq DNA high fidelity polymerase (Invitrogen)[8]. The reaction mixture and 1 µl of diluted cDNA (1:4) are added to a sterile PCR tube. The PCR is performed under the following thermocycling conditions: 2 min at 95 °C, 39 cycles of 15 s at 95 °C, and 60 s at 60 °C. Melting curve analysis is done by incubation at 60 °C to 95 °C at 0.1 °C s^{-1} with a smooth curve setting averaging 1 point. Following the conditions of Varga and James (2005), the melting temperatures for each product are:

Universal PPV detection (74 bp fragment): 80.08–81.52 °C

D strains (114 bp fragment): 84.3–84.43 °C

M strains (380 bp fragment): 85.34–86.11 °C

Internal control (181 bp fragment): 82.45–82.63 °C.

[4] The use of the brand Invitrogen for the first strand buffer, RNaseOUT™, Superscript™ II and Platinum® Taq DNA high fidelity polymerase in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

[5] See footnote 4.

[6] See footnote 4.

[7] The use of the brand Sigma for SYBR Green I in this diagnostic protocol implies no approval of it to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

[8] See footnote 4.

The method of Varga and James (2005) was evaluated using isolates of PPV-C, PPV-D, PPV-EA, PPV-M and an uncharacterized strain in *Nicotiana* and *Prunus* species.

4. Identification of Strains

This section describes additional methods (using DASI-ELISA, RT-PCR, Co-RT-PCR and real-time RT-PCR) for identification of PPV strains (see Figure 1). Strain identification is not an essential component of PPV identification but an NPPO may wish to determine the identity of the strain to assist in predicting its epidemiological behaviour.

Given the variability of PPV, techniques other than sequencing or some PCR-based assays (see below) may provide erroneous results with a small percentage of isolates. However, it is generally possible to discriminate the D and M types of PPV using the serological or molecular techniques described (Cambra *et al.*, 2006a; Candresse and Cambra, 2006; Capote *et al.*, 2006).

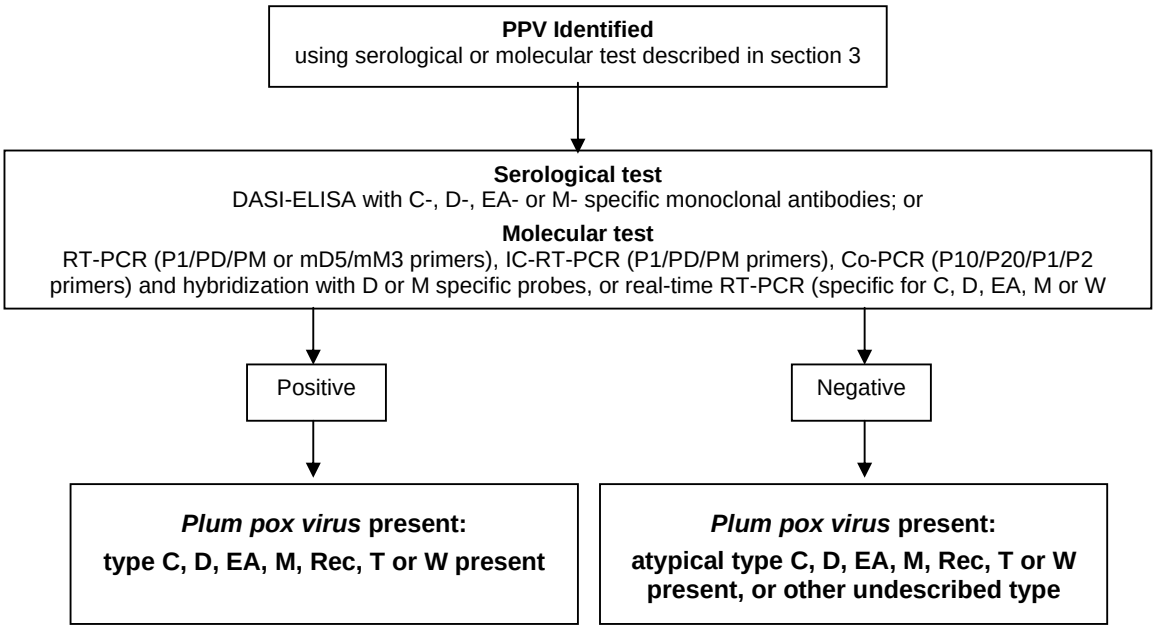

Figure 1: Methods for the identification of strains of *Plum pox virus*.

Further tests may be done in instances where the NPPO requires additional confidence in the identification of PPV type. Sequencing of the complete PPV genome, or complete or partial coat protein, P3-6K1 and cytoplasmic inclusion protein genes should also be done where atypical or undescribed types are present.

4.1 Serological identification of strains

DASI-ELISA for differentiation between the two main PPV types (D and M) should be performed according to Cambra *et al.* (1994), using D- and M-specific monoclonal antibodies (Cambra *et al.*, 1994; Boscia *et al.*, 1997), according to the manufacturer's instructions.

This method has been validated in the DIAGPRO ring-test showing an accuracy of 84% for PPV-D detection and 89% for PPV-M detection (Cambra *et al.*, 2006c; Olmos *et al.*, 2007). The 4D monoclonal antibody is PPV-D specific but does not react with all PPV-D isolates. In addition, the AL monoclonal antibody used for PPV-M detection reacts with isolates belonging to strains M, Rec and T since these groups share the same coat protein sequence. Therefore a molecular test is required to differentiate between M, Rec and T types detected using an M-specific monoclonal antibody.

Serological identification of PPV isolates from EA and C groups may be done by DASI-ELISA using the EA- and/or the C-specific monoclonal antibodies described by Myrta *et al.* (1998, 2000). However, these tests need to be validated.

4.2 Molecular identification of strains

4.2.1 Reverse transcription-polymerase chain reaction

PPV-D and PPV-M are identified using the primers described by Olmos *et al.* (1997):

P1 (5′-ACC GAG ACC ACT ACA CTC CC-3′)

PD (5′-CTT CAA CGA CAC CCG TAC GG-3′) or PM (5′-CTT CAA CAA CGC CTG TGC GT -3′).

The 25 µl reaction mixture is composed as follows: 1 µM of P1 primer, 1 µM of either PD or PM primer, 250 µM dNTPs, 1 unit AMV reverse transcriptase (10 units μl^{-1}), 0.5 units Taq DNA polymerase (5 units μl^{-1}), 2.5 µl 10 × Taq polymerase buffer, 1.5 mM $MgCl_2$, 0.3% Triton X-100, 2% formamide and 5 µl RNA template. The RT-PCR is performed under the following thermocycling conditions: 45 min at 42 °C, 2 min at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 60 °C, and 1 min at 72 °C, followed by a final extension for 10 min at 72 °C. The PCR products are analysed by gel electrophoresis. The P1/PD and P1/PM primers produce a 198 bp amplicon. The method was evaluated using six isolates of PPV-D and four PPV-M isolates.

PPV-Rec is identified using the mD5/mM3 Rec-specific primers described by Šubr, Pittnerova and Glasa (2004):

mD5 (5′-TAT GTC ACA TAA AGG CGT TCT C-3′)

mM3 (5′-CAT TTC CAT AAA CTC CAA AAG AC-3′).

The 25 µl reaction mixture is composed as follows (modified from Šubr, Pittnerova and Glasa, 2004): 1 µM of each primer, 250 µM dNTPs, 1 unit AMV reverse transcriptase (10 units μl^{-1}), 0.5 units Taq DNA polymerase (5 units μl^{-1}), 2.5 µl 10 × Taq polymerase buffer, 2.5 mM $MgCl_2$, 0.3% Triton X-100 and 5 µl of extracted RNA (see section 3.3). The PCR product of 605 bp is analysed by gel electrophoresis.

4.2.2 Immunocapture reverse transcription-polymerase chain reaction

The immunocapture phase should be performed as described in section 3.3.2. The PCR reaction mixture is added directly to the coated PCR tubes. Identification of PPV-D and PPV-M detection is done as described in section 4.2.1.

4.2.3 Co-operational reverse transcription-polymerase chain reaction

Identification of PPV-D or PPV-M should be done as described in section 3.3.3 using 3′DIG-labelled probes specific for D and M strains (Olmos, Bertolini and Cambra, 2002):

PPV-D Specific Probe: 5′-CTT CAA CGA CAC CCG TAC GGG CA-DIG-3′

PPV-M Specific Probe: 5′-AAC GCC TGT GCG TGC ACG T-DIG-3′.

The prehybridization and hybridization steps are performed at 50 °C with standard prehybridization and hybridization buffers + 30% formamide (for PPV-D identification) and + 50% formamide (for PPV-M identification). The blocking solution is used at 2% (w/v).

4.2.4 Real-time reverse transcription-polymerase chain reaction

PPV-D and PPV-M are specifically identified using either SYBR Green I chemistry according to the method of Varga and James (2005) (see section 3.3.4) or the TaqMan method described by Capote *et al.* (2006).

The primers and TaqMan probes used in the method of Capote *et al*. (2006) are:

PPV-MGB-F primer (5′-CAG ACT ACA GCC TCG CCA GA-3′)

PPV-MGB-R primer (5′-CTC AAT GCT GCT GCC TTC AT-3′)

MGB-D probe (5′- FAM-TTC AAC GAC ACC CGT A-MGB-3′)

MGB-M probe (5′-FAM-TTC AAC AAC GCC TGT G-MGB-3′).

The 25 µl reaction mixture is composed as follows: 1 µM of each primer, 150 nM MGB-D or MGB-M FAM probe, 1 × TaqMan Universal PCR Master Mix (Applied Biosystems)[9], 1 × MultiScribe and RNase Inhibitor Mix (Applied Biosystems)[10] and 5 µl of RNA template (see section 3.3). The RT-PCR is performed under the following thermocycling conditions: 30 min at 48 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C, and 60 s at 60 °C. The PCR products are analysed in real time according to the manufacturer's instructions. The method has been evaluated using 12 isolates each of PPV-D and PPV-M, and 14 samples co-infected with both types.

PPV-C, PPV-EA and PPV-W are specifically identified using SYBR Green I chemistry according to the method of Varga and James (2006). The primers used in this method are:

P1 (5′-ACC GAG ACC ACT ACA CTC CC-3′)

PPV-U (5′-TGA AGG CAG CAG CAT TGA GA-3′)

PPV-RR (5′-CTC TTC TTG TGT TCC GAC GTT TC-3′).

The following internal control primers may be included to ensure the correct performance of the assay:

Nad5-F (5′-GAT GCT TCT TGG GGC TTC TTG TT-3′)

Nad5-R (5′-CTC CAG TCA CCA ACA TTG GCA TAA-3′).

The 25 µl RT-PCR reaction is composed as follows: 2.5 µl of a 1:10 (v/v) water dilution of extracted RNA (see section 3.3) and 22.5 µl of master mix. The master mix has the following composition: 2.5 µl of Karsai Buffer (Karsai *et al.*, 2002); 0.5 µl each of 5 µM primers PPV-U, PPV-RR or P1, Nad5R and Nad5F; 0.5 µl of 10 mM dNTPs; 1 µl of 50 mM $MgCl_2$; 0.2 µl of RNaseOUT™ (40 units μl^{-1}; Invitrogen)[11] ; 0.1 µl of Superscript™ III (200 units μl^{-1}; Invitrogen)[12]; 0.1 µl of Platinum® Taq DNA high fidelity polymerase (5 units μl^{-1}, Invitrogen)[13]; and 1 µl of 1:5 000 (in TE, pH 7.5) SYBR Green I (Sigma)[14] in 16.1 µl water. The reaction is performed under the following thermocycling

[9] The use of the brand Applied Biosystems for the TaqMan Universal PCR Master Mix and the MultiScribe and RNase Inhibitor Mix in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

[10] See footnote 9.

[11] The use of the brand Invitrogen for RNaseOUT™ , Superscript™ II and Platinum® Taq DNA high fidelity polymerase in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

[12] See footnote11.

[13] See footnote 11.

conditions: 10 min at 50 °C, 2 min at 95 °C, 29 cycles of 15 s at 95 °C, and 60 s at 60 °C. Melting curve analysis is performed by incubation at 60 °C to 95 °C at 0.1 °C s^{-1} melt rates with a smooth curve setting averaging 1 point. Following the conditions of Varga and James (2006), the melting temperatures for each product are:

C strain (74 bp fragment): 79.84 °C

EA strain (74 bp fragment): 81.27 °C

W strain (74 bp fragment): 80.68 °C.

This method was evaluated using one isolate each of PPV-C, PPV-D, PPV-EA and PPV-W.

5. Records

The records required to be kept are listed in section 2.5 of ISPM 27:2006.

In instances where other contracting parties may be affected by the results of the diagnosis, in particular in cases of non-compliance and where the virus is found in an area for the first time, the following additional material should be kept:

- The original sample (labelled appropriately for traceability) should be kept frozen at −80 °C or freeze-dried and kept at room temperature.
- If relevant, RNA extractions should be kept at −80 °C and/or spotted plant extracts or printed tissue sections paper on paper or nylon membranes should be kept at room temperature.
- If relevant, RT-PCR amplification products should be kept at −20 °C.

6. Contact Points for Further Information

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[14] The use of the brand Sigma for SYBR Green I in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

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8. References

Barba, M., Hadidi. A., Candresse. T. & Cambra, M. 2011. *Plum pox virus*. *In*: A. Hadidi, M. Barba, T. Candresse & W. Jelkmann, eds. *Virus and virus-like diseases of pome and stone fruits*, Chapter 36. St. Paul, MN, APS Press. 428 pp.

Boscia, D., Zeramdini, H., Cambra, M., Potere, O., Gorris, M.T., Myrta, A., DiTerlizzi, B. & Savino, V. 1997. Production and characterization of a monoclonal antibody specific to the M serotype of plum pox potyvirus. *European Journal of Plant Pathology*, 103: 477–480.

CABI. 2011. Crop Protection Compendium. http://www.cabi.org/cpc/, accessed 26 October 2011.

Cambra, M., Asensio, M., Gorris, M.T., Pérez, E., Camarasa, E., García, J.A., Moya, J.J., López-Abella, D., Vela, C. & Sanz, A. 1994. Detection of plum pox potyvirus using monoclonal antibodies to structural and non-structural proteins. *Bulletin OEPP/EPPO Bulletin*, 24: 569–577.

Cambra, M., Boscia, D., Myrta, A., Palkovics, L., Navrátil, M., Barba, M., Gorris, M.T. & Capote, N. 2006a. Detection and characterization of *Plum pox virus*: serological methods. *Bulletin OEPP/EPPO Bulletin*, 36: 254–261.

Cambra, M., Capote, N., Myrta, A. & Llácer, G. 2006b. *Plum pox virus* and the estimated costs associated with sharka disease. *Bulletin OEPP/EPPO Bulletin*, 36: 202–204.

Cambra, M., Capote, N., Olmos, A., Bertolini, E., Gorris, M.T., Africander, N.L., Levy, L., Lenardon, S.L., Clover, G. & Wright, D. 2006c. Proposal for a new international protocol for detection and identification of *Plum pox virus*. Validation of the techniques. *Acta Horticulturae*, 781: 181–191.

Candresse, T. & Cambra, M. 2006. Causal agent of sharka disease: historical perspective and current status of *Plum pox virus* strains. *Bulletin OEPP/EPPO Bulletin*, 36: 239–246.

Capote, N., Bertolini, E., Olmos, A., Vidal, E., Martínez, M.C. & Cambra, M. 2009. Direct sample preparation methods for detection of *Plum pox virus* by real-time RT-PCR. *International Microbiology*, 12: 1–6.

Capote, N., Gorris, M.T., Martínez, M.C., Asensio, M., Olmos, A. & Cambra, M. 2006. Interference between D and M types of *Plum pox virus* in Japanese plum assessed by specific monoclonal antibodies and quantitative real-time reverse transcription-polymerase chain reaction. *Phytopathology*, 96: 320–325.

Damsteegt, V.D., Scorza, R., Stone, A.L., Schneider, W.L., Webb, K., Demuth, M. & Gildow, F.E. 2007. *Prunus* host range of *Plum pox virus* (PPV) in the United States by aphid and graft inoculation. *Plant Disease*, 91: 18–23.

Damsteegt, V.D., Waterworth, H.E., Mink, G.I., Howell, W.E. & Levy, L. 1997. *Prunus tomentosa* as a diagnostic host for detection of *Plum pox virus* and other *Prunus* viruses. *Plant Disease*, 81: 329–332.

Desvignes, J.C. 1999. *Virus diseases of fruit trees*. Paris, CTIFL, Centr'imprint. 202 pp.

EPPO. 2004. Diagnostic protocol for regulated pests: *Plum pox potyvirus*. *Bulletin OEPP/EPPO Bulletin*, 34: 247–256.

EPPO. 2006. Current status of *Plum pox virus* and sharka disease worldwide. *Bulletin OEPP/EPPO Bulletin,* 36: 205–218.

García, J.A. & Cambra, M. 2007. *Plum pox virus* and sharka disease. *Plant Viruses*, 1: 69–79.

Gentit, P. 2006. Detection of *Plum pox virus*: biological methods*. Bulletin OEPP/EPPO Bulletin*, 36: 251–253.

ISPM 27. 2006. *Diagnostic protocols for regulated pests*. Rome, IPPC, FAO.

ISPM 31. 2008. *Methodologies for sampling of consignments*. Rome, IPPC, FAO.

James, D. & Glasa, M. 2006. Causal agent of sharka disease: New and emerging events associated with *Plum pox virus* characterization*. Bulletin OEPP/EPPO Bulletin*, 36: 247–250.

Karsai, A., Müller, S., Platz, S. & Hauser, M.T. 2002. Evaluation of a homemade SYBR Green I reaction mixture for real-time PCR quantification of gene expression. *Biotechniques,* 32: 790–796.

Levy, L. & Hadidi, A. 1994. A simple and rapid method for processing tissue infected with plum pox potyvirus for use with specific 3′ non-coding region RT-PCR assays. *Bulletin OEPP/EPPO Bulletin,* 24: 595–604.

Myrta, A., Potere, O., Boscia, D., Candresse, T., Cambra, M. & Savino, V. 1998. Production of a monoclonal antibody specific to the El Amar strain of plum pox virus. *Acta Virologica,* 42: 248–250.

Myrta, A., Potere, O., Crescenzi, A., Nuzzaci, M. & Boscia, D. 2000. Production of two monoclonal antibodies specific to cherry strain of plum pox virus (PPV-C). *Journal of Plant Pathology,* 82 (suppl. 2): 95–103.

Olmos, A., Bertolini, E. & Cambra, M. 2002. Simultaneous and co-operational amplification (Co-PCR): a new concept for detection of plant viruses. *Journal of Virological Methods*, 106: 51–59.

Olmos, A., Bertolini, E., Gil, M. & Cambra, M. 2005. Real-time assay for quantitative detection of non-persistently transmitted *Plum pox virus* RNA targets in single aphids. *Journal of Virological Methods,* 128: 151–155.

Olmos, A., Cambra, M., Dasi, M.A., Candresse, T., Esteban, O., Gorris, M.T. & Asensio, M. 1997. Simultaneous detection and typing of plum pox potyvirus (PPV) isolates by heminested-PCR and PCR-ELISA. *Journal of Virological Methods,* 68: 127–137.

Olmos, A., Capote, N., Bertolini, E. & Cambra, M. 2007. Molecular diagnostic methods for plant viruses. *In*: Z.K. Punja, S. DeBoer and H. Sanfacon, eds. *Biotechnology and plant disease management*, pp. 227–249. Wallingford, UK and Cambridge, USA, CAB International. 574 pp.

Osman, F. & Rowhani, A. 2006. Application of a spotting sample preparation technique for the detection of pathogens in woody plants by RT-PCR and real-time PCR (TaqMan). *Journal of Virological Methods,* 133: 130–136.

PaDIL. 2011. http://old.padil.gov.au/pbt/, accessed 26 October 2011.

Schneider, W.L., Sherman, D.J., Stone, A.L., Damsteegt, V.D. & Frederick, R.D. 2004. Specific detection and quantification of *Plum pox virus* by real-time fluorescent reverse transcription-PCR. *Journal of Virological Methods*, 120: 97–105.

Šubr, Z., Pittnerova, S. & Glasa, M. 2004. A simplified RT-PCR-based detection of recombinant *Plum pox virus* isolates. *Acta Virologica,* 48: 173–176.

Ulubaş Serçe, Ç., Candresse, T., Svanella-Dumas, L., Krizbai, L., Gazel, M. & Çağlayan, K. 2009. Further characterization of a new recombinant group of *Plum pox virus* isolates, PPV-T, found in the Ankara province of Turkey. *Virus Research*, 142: 121–126.

Varga, A. & James, D. 2005. Detection and differentiation of *Plum pox virus* using real-time multiplex PCR with SYBR Green and melting curve analysis: a rapid method for strain typing. *Journal of Virological Methods,* 123: 213–220.

Varga, A. & James, D. 2006. Real-time RT-PCR and SYBR Green I melting curve analysis for the identification of *Plum pox virus* strains C, EA, and W: Effect of amplicon size, melt rate, and dye translocation. *Journal of Virological Methods*, 132: 146–153.

Wetzel, T., Candresse, T., Macquaire, G., Ravelonandro, M. & Dunez, J. 1992. A highly sensitive immunocapture polymerase chain reaction method for plum pox potyvirus detection. *Journal of Virological Methods*, 39: 27–37.

Wetzel, T., Candresse, T., Ravelonandro, M. & Dunez, J. 1991. A polymerase chain reaction assay adapted to plum pox potyvirus detection. *Journal of Virological Methods,* 33: 355–365.

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INTERNATIONAL STANDARDS FOR PHYTOSANITARY MEASURES

ISPM 27 DIAGNOSTIC PROTOCOLS

DP 3: *Trogoderma granarium* Everts

(2012)

CONTENTS

1. Pest Information

Trogoderma granarium Everts (Coleoptera: Dermestidae) is a stored product pest of great importance. Its economic importance lies not only in the serious damage it can cause to stored dry commodities but also in the export restrictions faced by countries when they have established populations of this pest. Live populations can stay in uncleaned containers, packaging material and cargo holds for extended periods of time, infesting non-host material. *Trogoderma granarium* may also increase the likelihood of contamination by *Aspergillus flavus* (Sinha and Sinha, 1990).

Trogoderma granarium may have originated from the Indian subcontinent and it is now present in some areas of Asia, the Middle East, Africa and a few countries in Europe. It is one of the very few stored products pests with a limited distribution. It is found from 35° north latitude to 35° south latitude, but occurs mainly in regions near the equator in dry and hot environments. However, viable populations should be able to survive in almost any country in a closed storage environment. *T. granarium* has very limited ability to spread without human aid because it is unable to fly, so international movement of host commodities appears to be the only means of spreading the pest. It is very important to distinguish between records that relate to interceptions of the pest in imported commodities (i.e. its finding in the commodity during the border phytosanitary control without further spread) and those of established infestations (EPPO, 2011).

T. granarium usually occurs in various dry stored products of primarily plant origin. Primary hosts are cereals, buckwheat, cereal products, pulses, alfalfa, various vegetable seeds, herbs, spices and various nuts. It can also successfully complete its life cycle in copra, dried fruits and various gums, as well as many different dried products wholly or partially of animal origin, such as milk powder, skins, dried dog food, dried blood, dead insects and dried animal carcasses. As a pest it is most prevalent under hot dry conditions, where very heavy infestations can develop. In cooler and also in hot and humid conditions it tends to be out-competed as a pest by other species such as *Sitophilus* spp. and *Rhyzopertha dominica* (Fabricius). Commodities stored in bags in traditional warehouses are more at risk from this pest than commodities that are stored at bulk.

There are important features of *T. granarium* biology that enable the pest to survive in harsh conditions.

T. granarium may have from one to more than ten generations per year depending on food availability and quality, temperature and humidity. A complete life cycle may be as short as 26 days (temperature 32–35° C) or as long as 220 days or more in a suboptimal environment. In temperate climates larvae become inactive at temperatures below 5° C, so the pest is able to survive and breed only in protected environments. There are two genetic variations of larvae: those that are able to undergo facultative diapause and those that are unable to do so. Larvae of the first type are stimulated into diapause by adverse conditions such as low or high temperatures and/or lack of food. During diapause their respiration drops to an extremely low level leading to tolerance to fumigation. Diapausing larvae are also cold-hardy and may survive temperatures below −10° C. When favourable conditions return, the pest is able to multiply rapidly and cause serious damage to the commodity (EPPO/CABI, 1997).

Trogoderma species other than *T. granarium* may also be found in stored products, but only some of these feed on such products. Among these species the biggest economic losses are caused by *T. variabile* Ballion, which may cause significant economic damage and is recognized as a quarantine pest in some countries. However, most *Trogoderma* species occurring in stored products appear to be scavengers, feeding on dead bodies of other insects. During a 12-year survey conducted in California, eight species of *Trogoderma* were found in stored seeds, animal feed and grocery commodities (Strong and Okumura, 1966). Mordkovich and Sokolov (1999) mention other *Trogoderma* species that may be found in stored products. Among them, *T. longisetosum* Chao and Lee has been noted as a stored product pest in China. It is very similar to *T. glabrum* (Herbst). Some tropical *Trogoderma* species may also be present in stored products (Delobel and Tran, 1993). One of such species is *T. cavum* Beal, which was described by Beal (1982) after examination of specimens infesting stored rice in Bolivia. Some species occurring in stored products closely resemble *T. granarium*.

For more general information on *T. granarium*, see the EPPO PQR database (EPPO, 2011) as well as Hinton (1945), Lindgren *et al*. (1955), Varshalovich (1963), Bousquet (1990) Kingsolver (1991), EPPO/CABI (1997), Pasek (1998), OIRSA (1999a), PaDIL (2011) and CABI (2011).

Diagnostic protocols for *T. granarium* have been published by two regional plant protection organizations – OIRSA (1999a) and EPPO (2002). The initial point for preparation of this protocol was the document issued by EPPO (2002).

2. Taxonomic Information

Name:	*Trogoderma granarium* Everts, 1898
Synonyms:	*Trogoderma khapra* Arrow, 1917
	Trogoderma koningsbergeri Pic, 1933
	Trogoderma afrum Priesner, 1951
	Trogoderma granarium ssp. *afrum* Attia and Kamel, 1965
Common names:	khapra beetle (English)
	Trogoderme (dermeste) du grain, dermeste des grains (French)
	Trogoderma de los granos, escarabajo khapra, gorgojo khapra (Spanish)
	الشـــعرية الحبـــوب خنفســـاء (Arabic)
Taxonomic position:	Insecta: Coleoptera: Dermestidae.

3. Detection

Trogoderma granarium has the following life developmental stages: eggs on the surface of grain and other stored products; larvae (5–11 instars) in stored products (larvae may be found in packing material or within storage structures); pupae in stored products, in the last larval exuviae (cast skins); adults in stored products.

Methods to detect *T. granarium* infestations include inspection, physical search, use of food baits and pheromone traps. Often the infested material contains only larvae because (1) adult longevity is usually between 12 and 25 days (it can be as long as 147 days in unfavourable conditions), whereas larval longevity is usually 19–190 days (and can be up to six years in diapausing larvae); (2) most of the dermestid larvae occurring in stored products will partially or wholly consume dead adults; and (3) adults are most prevalent when conditions are favourable for population growth. Larval exuviae are usually not consumed so their presence is a clear indication of a possible active infestation. Larvae are extremely cryptic by nature, particularly diapausing larvae that may stay inactive for long periods in cracks and crevices where they are very difficult or nearly impossible to locate.

Many other dermestid species belonging to genera other than *Trogoderma* may occur in stored products. Members of *Dermestes* and *Attagenus* genera are frequently found feeding on materials of animal origin, such as dog biscuits, dried meat and dried blood. They also feed on rat, mice and bird carcasses. *Anthrenus* and *Anthrenocerus* species can be serious pests of wool and woollen products. In stored products heavily infested with other stored products pests, non-pest *Trogoderma, Anthrenus* and *Anthrenocerus* are usually found feeding on carcasses of these pests.

T. granarium infestations are usually recognized by (1) the presence of the pest (especially feeding larvae and exuviae) and (2) symptoms of infestation. The short-lived adults are sometimes not seen. Damage to the commodities can be a warning sign, but often it is a result of the feeding of other common stored product pests. Larvae usually feed first on the germ portion of cereal seeds and then on the endosperm. The seed coat is eaten in an irregular manner. In bulk commodities infestations usually concentrate in the surface layers, where numerous larval exuviae, broken setae and frass (excrement) are present (Figure 1). However, larvae can occasionally be found as deep as 3–6 m in bulk grain. It is therefore important to consider biased sampling when inspecting for these types of pests.

Samples of suspect products have to be visually inspected in a well-lit area, using a 10× magnification hand lens. If appropriate, samples should be passed over sieves with aperture sizes relevant to the particle size of the products. Usually sets of sieves of aperture sizes 1, 2 and 3 mm are used. The sifted material collected on particular sieves should be placed in Petri dishes and examined under at least 10× to 25× magnification through a stereoscopic microscope to detect the pest. This screening technique allows the detection of various developmental stages of the pest. However, some larvae feeding within grains may remain undetected. Therefore, it may become necessary to heat samples to 40° C to drive pests out of the grains with an extractor tool such as a Berlese funnel, especially in case of heavy infestation. Visual inspection is preferable to sieving because the latter can easily destroy or seriously damage dead adults and larval exuviae rendering the morphological identification very difficult or impossible.

Inspections for this pest are particularly difficult in cases of low-level infestations. The larvae of *Trogoderma* species are most active at dawn and dusk. Populations can persist in small quantities of residues that may occur within a structure or mode of transport. Larvae in diapause can survive long periods without food. For diapausing larvae it is important to search under piles of dirt, flaking paint and rust and also in empty packaging materials such as hessian bags, tarpaulins and corrugated cardboard. Larvae are often hiding behind wall panelling, under internal lining, between floorboards, under insulation, on dry ledges, electrical cable trays and conduits, switch boxes etc. Because larval exuviae become airborne very easily, window sills, grilles of venting holes and spider webs must be checked. Rodent traps containing baits should be always inspected.

Additionally to initial inspections, it is possible to monitor the presence of *T. granarium* using various traps. Food-baited traps (containing oil seeds, peanuts, wheat germ etc.) or attractant traps (containing wheat germ oil) can be used to attract larvae. Simple traps offering hiding places for the larvae, such as pieces of corrugated cardboard or hessian bag, can be placed on the floor. After monitoring, all the traps should be destroyed. Adults may be detected with the use of pheromone traps where the pheromone capsule is combined with a non-drying sticky trap. However, the *Trogoderma* pheromone traps are not species-specific and attract many species of dermestid beetles (Saplina, 1984; Barak, 1989; Barak *et al.*, 1990; Mordkovich and Sokolov, 2000). Traps baited both with pheromone and food bait are commercially available.

Insects found should be picked up carefully with small forceps or collected using an aspirator. It is important to collect multiple specimens of the pest. Identification of larvae is difficult; if the dissection of a single specimen is not successful and serious damage occurs to the mouthparts, exact identification is impossible. Specimens should be placed in 70% ethyl alcohol for preservation and safe shipping if the identification is not done immediately at the same locality.

4. Identification

The genus *Trogoderma* in recent years has been reported to include 117 species (Mroczkowski, 1968), 115 species (Beal, 1982), 130 species (Háva, 2003) and 134 species (Háva, 2011). There are many other species of *Trogoderma* yet to be described. Great caution needs to be exercised with the synonymies established because few of them are based on detailed comparison of the type specimens.

Identification of *Trogoderma* eggs and pupae based on external features is currently not possible. Insect eggs and pupae possess very few external features and therefore are poorly studied. Larval identification is difficult. It requires experience in identification and also good skills in dissection of small insects. Pupation takes place in the last larval cast. The larval exuviae can be used for identification, but one needs to be more cautious because the material is brittle. Adults are the easiest to identify, though misidentification is still common, so training in preparation, mounting and determination of *Trogoderma* specimens is required.

Adults in good condition can be identified by experienced staff using a stereomicroscope at 10× to 100× magnification. However, for reliable identification it is recommended that the genitalia are always examined. Movement of the stored product, particularly cereals, will damage the dead adults.

In most cases the legs and antennae will break off and also the setae on the elytra and pronotum will be rubbed off. In the case of a damaged specimen with missing body parts or morphological features not visible, identification should always be based on examination of the genitalia. Genitalia should be removed (section 4.2) and mounted temporarily on a cavity microscope slide using glycerol, Hoyer's medium (50 ml water, 30 g gum arabic, 200 g chloral hydrate, 20 ml glycerine[1]) or similar mounting media.

For larval identifications the mouthparts should be dissected out (section 4.1). The larval exuviae and dissected mouthparts should be mounted on a cavity microscope slide using Hoyer's medium (Beal, 1960) or other mounting media, such as polyvinyl alcohol (PVA). Details of mounting procedures are included in section 4.1.

Adult and larval dissection can be performed under 10× to 40× magnification using a stereomicroscope. For the examination of genitalia and larval mouthparts, particularly the papillae of the epipharynx, a good-quality compound microscope is necessary and must be capable of 400× to 800× magnification in bright field and phase contrast. Use of higher magnifications (1000×) may be necessary to achieve a more satisfactory resolution.

Methods have been developed for the identification of a limited number of pest *Trogoderma* species, using both immunological (ELISA test) and molecular techniques for specific purposes. As these methods still do not allow for a reliable and unequivocal distinction between *T. granarium* and other *Trogoderma* species that are likely to occur in stored products, they still cannot be used as quarantine diagnostic techniques for the determination of insect specimens found during inspection of stores and consignments of plant material in trade. Currently, research is being carried out this area in the USA and Australia.

4.1 Procedure for preparation of larvae and larval exuviae

Before dissection the larva should be examined under a stereomicroscope. Size, body colour, arrangement and colour of setae should be recorded. Use of microscope photography provides a record of material prior to disturbance via manipulation and handling and so allows for its independent interpretation.

For identification the larvae should be mounted in Hoyer's medium or other mounting media such as PVA on a microscope slide using the following method:

(1) First, place the specimen on a microscope slide; it is best done ventral side up in order to preserve the diagnostic characters.

(2) Cut open the whole body along the mid-line from under the head capsule to the last abdominal segment using eye surgery scissors.

(3) Next put the larva into a test-tube containing 10% potassium hydroxide (KOH) solution and heat in a boiling water bath until larval tissues loosen and begin to separate from the cuticle.

(4) Rinse thoroughly in warm distilled water.

(5) Remove all internal tissues using a very fine, short hair brush or the convex surface of a hooked tip of a no. 1 insect pin, or a loop formed from a micropin. All setae should be removed from one side of the 7th and 8th abdominal segment; stains such as acid fuchsin or chlorazol black may be used to make the analysed structures more visible.

(6) Remove the head capsule and put it back in the hot KOH solution for 5 minutes. Rinse the head capsule in warm distilled water. Dissection of the head can be performed in a few drops of Hoyer's mounting medium or glycerol on a microscope slide or in water in an excavated glass block. Turn the head ventral side up and hold it to the glass with a blunt no. 1 insect pin.

(7) Remove the mandibles, maxillae and labial palpi using jeweller's forceps and micropins. Remove the epipharynx and antennae, which may be additionally stained with a stain such as

[1] Some experts prefer Hoyer's mounting medium containing 16 ml of glycerine.

acid fuchsin or chlorazol black. Mount the head capsule and the mandibles in the cavity of the slide using Hoyer's medium or another mounting media. Mount the cleared skin, fully opened on the flat part of the microscope slide, next to the cavity. It is usually best done ventral side up. Epipharynx, antennae, maxillae and labial palpi should be mounted with the skin under the same cover slip. Mount all body parts on the same microscope slide.

(8) In the case of larval exuviae, before proceeding with the dissection soak the specimen in a 5% solution of any laboratory detergent for about two hours and rinse thoroughly in distilled water. Cut the specimen open anteriorly and dissect out the mouthparts. They can be mounted directly in Hoyer's medium without clearing.

(9) Label slides immediately after mounting specimens and place them in an oven for at least three days at 40 °C to improve their quality (the best slides are obtained after 2–4 weeks). After drying, ring the slides using any lacquer recommended for sealing of microscopic slides (e.g. Glyptal, Brunseal), or at least two layers of nail polish in order to prevent the Hoyer's medium from drying and possibly damaging the specimen. However, microscopic slides may be examined immediately after preparing.

Permanent slides can be made using Euparal or Canada balsam for mounting, but these require a laborious dehydration process.

4.2 Procedure for preparation of adults

Adult *Trogoderma* specimens may need to be cleaned before identification, with any laboratory detergent or using an ultrasonic cleaner. If the specimen was caught in a sticky trap the glue can be dissolved using a number of solvents (e.g. kerosene). These solvents can be removed from the specimen with any laboratory detergent.

Before beginning the preparation, soak the adult in warm distilled water for about an hour. Perform the preparation in the following way:

(1) First remove abdomen while the specimen is still in the water using fine forceps. Dry the specimen (minus abdomen) and mount it on a cardboard rectangle, preferably laterally. The specimen will be less exposed to damage and accessible for both dorsal and ventral examination if it is glued on the side.

(2) Next cut the abdomen laterally open, leaving the last abdominal segment untouched. Place it in a 10% KOH or sodium hydroxide (NaOH) solution in a hot water bath for about 10 minutes.

(3) Rinse the specimen in water and carefully remove the genitalia using hooked micropins. After removing the genitalia the abdomen should be glued onto the same cardboard rectangle with the insect, ventral side facing up.

(4) The genitalia need to be macerated further in the caustic solution. Separate the aedeagus from the periphallic tergum and the 9th abdominal segment using micropins. They may be stained with a stain such as acid fuchsin or chlorazol black to make them more visible.

Genitalia can be mounted on a microscope slide using Hoyer's medium or other mounting media such as PVA. The aedeagus should be mounted on a cavity microscope slide to keep its shape. Female genitalia can be mounted on a flat microscope slide.

Slides and pinned insects should be labelled immediately after mounting the specimens. The slides should be placed in an oven for at least three days at 40 ºC (the best slides are obtained after 2–4 weeks). After drying, all slides should be ringed (see section 4.1.(9)).

If there is no need for mounting the genitalia using a permanent or semi-permanent mounting agent, they can be examined in a drop of glycerol on a microscope slide. After the identification the organs can be placed in a microvial in a drop of glycerol or glued onto the cardboard rectangle next to the abdomen.

4.3 Genera of the family Dermestidae frequently occurring in stored commodities

Besides *Trogoderma*, other dermestid genera may also be found in stored products, such as *Anthrenus*, *Anthrenocerus*, *Attagenus* and *Dermestes*. The first step of diagnosis of collected specimens is identification to genus. Adults of these beetles, and in some cases larvae, can be identified using at least one of the keys of Mound (1989), Haines (1991), Kingsolver (1991), Banks (1994), Háva (2004) and Rees (2004). Genera of the North American Dermestidae can be identified using the key of Kingsolver (2002).

The simple keys below (Key 1 and Key 3) quickly enable *Trogoderma* to be distinguished from four other dermestid genera commonly occurring in stored commodities. Distinguishing characters are illustrated in section 9, Figures 2 to 23. It should be mentioned that other genera of dermestid beetles may also be found in stores. These genera include *Thaumaglossa, Orphinus* and *Phradonoma* (Delobel and Tran, 1993). However, stores are not typical habitats for them, so they are not included in above-mentioned keys.

4.3.1 Differentiation of dermestid larvae

Dermestid larvae may be differentiated using a simple key (Key 1). Larval or exuvial specimens identified to *Trogoderma* genus with this key are very likely to belong to a species from this genus and therefore it is warranted to check the detailed list of their features listed in section 4.4.1.

If the diagnostic key being used was not specifically written to include the area of origin (and interception) of the specimens, the key should be used with caution as there are many undescribed species of Dermestidae worldwide.

Key 1: Simple key for differentiation of dermestid larvae

1. Urogomphi present on 9th abdominal segment, 10th segment sclerotized, cylindrical ..... ***Dermestes* spp.**

Urogomphi absent, 10th abdominal segment not sclerotized **2**

2. Dorsal surface without hastisetae, maxillary palp 4-segmented ..... ***Attagenus* spp.**

Dorsal surface with hastisetae (Figure 18(A)), maxillary palp 3-segmented **3**

3. Posterior margins of abdominal terga sinuate, or emarginate, tufts of hastisetae placed on posterior membranous parts of terga, 8th abdominal tergum without tufts of hastisetae ..... ***Anthrenus* spp.**

Posterior margins of terga not sinuate or emarginate, tufts of hastisetae placed on sclerotized tergal plates, 8th tergum with tufts of hastisetae **4**

4. Second antennal segment about twice as long as last segment, head of hastisetae at least three times as long as wide at the widest point ..... ***Anthrenocerus* spp.**

Second and last antennal segments subequal, head of hastisetae less than three times as long as wide at widest point ..... ***Trogoderma* spp.**

4.4 Identification of *Trogoderma* larvae

There is no published key that covers all *Trogoderma* species. In part this is because there are still many undescribed species. Several keys have been published for the economically important species. Banks (1994) published a key to adults and larvae of the genus *Trogoderma* associated with stored products, as well as keys to larvae and adults of some species found in warehouses. Beal (1960) constructed an identification key to larvae of 14 species of *Trogoderma* from different parts of the world, including stored products pests. Mitsui (1967) published illustrated keys for identification of larvae and adults of some Japanese *Trogoderma* species. Kingsolver (1991) and Barak (1995) published keys to adults and larvae of some dermestid beetles, including a few *Trogoderma* species.

Zhang *et al.* (2007) published a key for identification of eight economically important species in the genus *Trogoderma.*

4.4.1 Discriminating features of *Trogoderma* larvae

Discriminating features of *Trogoderma* larvae below are adapted from Rees (1943), Hinton (1945), Beal (1954, 1960), Okumura and Blanc (1955), Haines (1991), Kingsolver (1991), Lawrence (1991), Peacock (1993), Banks (1994) and Lawrence *et al.* (1999a):

(1) body elongated, cylindrical, somewhat flattened, roughly six times as long as wide, nearly parallel-sided but gradually tapering toward rear part

(2) head well developed, sclerotized, and hypognathous

(3) three pairs of jointed legs present

(4) pretarsal setae on the ventral side of claws unequal

(5) very hairy, being covered with different types of setae: hastisetae, spicisetae and/or fiscisetae (Figures 18 and 20)

(6) head of hastisetae not more than three times longer than wide (Figure 20)

(7) numerous hastisetae on all nota and terga, with prominent tufts of erect hastisetae inserted on the posterolateral part of the tergal plates of abdominal segments 6 to 8 (in Anthrenus genus the tufts of hastisetae are inserted on the membrane behind the sclerotized part of terga 5, 6 and 7)

(8) urogomphi absent.

4.4.2 Identification of *Trogoderma* last instar larvae

Larvae of *T. granarium* (Figures 2(C), 2(D) and 21) may be separated from other *Trogoderma* species occurring in stores using the following short key (Key 2). This key does not allow for identification of all *Trogoderma* species known to occur in stores. So, if necessary, larvae of other pest and a few non-pest species can be identified, or at least separated, with reasonable confidence using the keys of Beal (1956, 1960), Banks (1994) and Peacock (1993). Features of larval specimens identified to *Trogoderma granarium* species with this key should next be compared with the detailed list of this species' features in section 4.4.3 and larval description in section 4.4.4.

Key 2: Identification key for *Trogoderma granarium* larvae

1. Epipharynx with 4 distal papillae, usually in a single sensory cup (Figure 23(A)).....**2**

Epipharynx with 6 distal papillae in a distal sensory cup; sometimes one or two papillae outside of the sensory cup (Figure 23(B), (C)).....**3**

2. Terga uniformly yellowish-brown, without greyish pigmentation at base of large spicisetae; acrotergites weakly sclerotized; antecostal suture on 8th abdominal segment almost always absent (if present, faint and usually broken); setae occupying 50 to 75% of the basal antennal segment, second segment usually with a single seta or no seta, apical segment with sensory pores in basal quarter; hastisetae morphology as in Figure 20(A), (B) .....***Trogoderma granarium* Everts**

Terga usually dark greyish-brown, at least at base of major spicisetae; acrotergites brownish, sclerotized; antecostal suture on 8th abdominal segment distinct; second antennal segment without setae; hastisetae morphology as in Figure 20(C), (D) ..... ***Trogoderma glabrum* (Herbst)**

3. Setae on basal antennal segment grouped on inner and inner-dorsal side leaving the outer and outer-ventral side glabrous; on fully extended antenna setae on basal segment not reaching apex of the second segment, sensory pore(s) on apical antennal segments not in basal quarter; median small spicisetae on acrotergites not long enough to extend over the antecostal suture (Figure 19(C); compare with Figure 19(D)); hastisetae (Figure 20(E), (F)) very sparse on thoracic and anterior abdominal terga (Figure 19(A)); terga with single row of large spicisetae (Figure 19(B)) . ***Trogoderma variabile* Ballion**

Specimen without above combination of characters .....**other *Trogoderma* spp.**

Larval identification should be considered unreliable if it is based only on one specimen, or exuviae or worn specimens. This is because in many species the intraspecific variation is such that in individual specimens features considered specific to the species may not be seen, while features specific to other species may be. In addition, large numbers of non-pest *Trogoderma* species occur in stored commodities and many of their characteristics are not well studied.

4.4.3 Discriminating features of *Trogoderma granarium* larvae

Discriminating features of *T. granarium* larvae are as follows:

(1) antennal segments subequal

(2) setae of basal antennal segment occupying 50–75% of the circumference of the segment, reaching or surpassing apex of second segment, at least three-fourths as long as the second antennal segment

(3) second antennal segment of last instar usually with one seta or sometimes no seta

(4) last antennal segment with at least one sensory pore in basal quarter

(5) epipharynx (Figure 22) with four papillae in distal sensory cup, usually in a single unit (Figure 23(A))

(6) fiscisetae absent

(7) mesally directed tergal setae absent

(8) at least six small spicisetae on first abdominal tergum, posterior to antecostal suture, anterior to large spicisetae

(9) anterior-median small spicisetae anterior to antecostal suture not long enough to reach over the suture

(10) large median spicisetae on first abdominal segment smooth or covered with inconspicuous scales with tips smooth for at least four times the diameter of seta

(11) antecostal suture of 8th abdominal tergum almost always absent, but if present, faint and interrupted

(12) antecostal suture on 7th abdominal tergum faint or interrupted

(13) no greyish pigmentation on sides of thoracic and other segments, not even at the base of large lateral spicisetae.

4.4.4 Description of *Trogoderma granarium* larvae

The first-instar larva (Figure 2(C)) is 1.6–1.8 mm long and 0.25–0.3 mm wide. Body is uniformly yellowish-white, head and hairs are reddish-brown. The mature larva (Figure 2(D)) is 4.5–6 mm long and 1.5 mm wide and body is reddish-brown. The larval body is covered with two kinds of hairs: spicisetae (Figure 18(B)), in which the shaft is covered with tiny, stiff, upwardly directed, pointed scales; and hastisetae (Figure 18(A)), in which the shaft is multi-segmented with spear-headed apex. Spicisetae are scattered over the dorsal surface of the head and body segments. Two groups of long spicisetae on the 9th abdominal segment form the tail. Hastisetae are found on all notal and abdominal segments, but on the last three or four segments they form distinctive, paired, erect tufts (Beal, 1960, 1991; EPPO/CABI, 1997).

4.5 Identification of *Trogoderma* adults

4.5.1 Differentiation of dermestid adults

Dermestid adults may be differentiated using a simple key (Key 3). Adult insect specimens identified to *Trogoderma* genus with this key are very likely to belong to a species from this genus and therefore it is warranted to check the detailed list of their features in section 4.5.2.

Key 3: Simple key for differentiation of dermestid adults

1. Median ocellus absent ..... ***Dermestes* spp.** (Figure 15)
Median ocellus present **2**

2. Body covered with scale-like setae; antennal cavity filled by antennae, fully visible from anterior view (Figure 14(A)) ..... ***Anthrenus* spp.** (Figure 17)
Body covered with simple setae, some of them whitish, flattened (ensiform) but never scale-like **3**

3. Antennal cavity completely closed behind, antennal club 3-segmented and well defined ..... ***Anthrenocerus* spp.**
Antennal cavity open behind or partially delimited by a posterior carina, antennal cavity much wider than antennae, not visible in anterior view **4**

4. Antennal cavity open behind, posterior margin of hind coxa angulate, first segment of posterior tarsus shorter than second segment ..... ***Attagenus* spp.** (Figure 16)
Antennal cavity carinate posteriorly, posterior margin of hind coxa straight, arcuate or sinuate, first segment of posterior tarsus longer than second segment..... ***Trogoderma* spp.** (Figures 2(A), 4(A), 14(B)).

4.5.2 Discriminating features of *Trogoderma* adults

The features below are adapted from Hinton (1945), Beal (1954, 1960), Okumura and Blanc (1955), Haines (1991), Kingsolver (1991), Lawrence and Britton (1991, 1994), Peacock (1993), Banks (1994), Lawrence *et al.* (1999b) and Háva (2004):

(1) body ovate, densely setose, setae simple, usually 2–3 different types, recumbent, yellowish-white slightly flattened, sword-shaped setae

(2) presence of median ocellus

(3) pronotum without lateral carina

(4) antennal cavity of anteroventral surface not, or only slightly visible in anterior view (Figure 14(B))

(5) antennal cavity carinate posteriorly at least to half of length and open laterally

(6) prosternum forming a “collar” anteriorly

(7) mesosternum deeply divided by sulcus

(8) posterior margin of hind coxal plate curved or sinuate, never angulate

(9) first segment of hind tarsus longer than second segment

(10) antennae short, 9–11-segmented, with a 3–8-segmented club, antennal outline usually smooth or rarely flabellate, terminal segment never disproportionately enlarged

(11) tarsi of all legs 5-segmented.

4.5.3 Identification of *Trogoderma* adults

The following short key (Key 4) should be used to distinguish adult *T. granarium* from some other *Trogoderma* species frequently occurring in stored commodities. This key does not allow for identification of all *Trogoderma* species known to occur in stores. So, if necessary, other species, not included in the key, can be identified with the keys of Beal (1954, 1956), Kingsolver (1991), Banks (1994), and Mordkovich and Sokolov (1999). These keys include species occurring in stored products and therefore may be used for identification of *Trogoderma* adults. It should be noted that identification of adult sex of various *Trogoderma* species is practically possible only after dissecting their genitalia (for morphology of male and female genitalia, see Figures 11 and 12). Checking of external distinguishing features as antennal club morphology should be performed on specimens surely identified to sex.

Features of adult specimens identified to *Trogoderma granarium* species with this key should be next compared with the detailed list of this species' discriminating features in section 4.5.4 and adult description in section 4.5.5.

Key 4: Identification key to *Trogoderma granarium* adults

1. Dorsal pubescence unicolorous ..... **non-pest *Trogoderma* spp.**

Dorsal pubescence not unicolorous but with pattern or pubescence completely rubbed off; (ensiform setae in addition to yellowish- and reddish-brown setae) **2**

2. Elytra without well-defined pattern, unicolorous or vaguely mottled **3**

Elytra with well-defined lighter and darker areas (Figure 3) **4**

3. Integument black, rarely with vague brownish maculation, basal loop, submedian and subapical bands formed by yellowish and whitish, ensiform setae; antennae always 11-segmented, male antennal club 5–7-segmented, female 4–5-segmented; 5th sternite of male with uniform, recumbent setae ..... ***Trogoderma glabrum* (Herbst)** (Figure 6(B))

Integument light reddish-brown, often with indistinct lighter maculation, scattered ensiform setae rarely forming 2–3 indistinct bands; antennae usually 11-, rarely 9- or 10-segmented, male antennal club 4–5-segmented, female 3–4-segmented; 5th sternite of male with apical patch of dense, coarse setae ..... ***Trogoderma granarium* Everts**

4. Elytral integument with distinct light basal loop **5**

Elytral integument with distinct bands and spots only **7**

5. Anterior margin of eyes distinctly emarginated .... ***Trogoderma inclusum* LeConte** (Figure 6(D))

Anterior margin of eyes straight or slightly sinuate **6**

6. Basal loop never connected to the antemedian band ..... ***Trogoderma variabile* Ballion** (Figures 4(A)–4(C), 5, 6(H))

Basal loop of elytral maculation connected to the antemedian band by a longitudinal band or bands (*T. inclusum* with less obvious emargination of eyes may key out here) ..... ***Trogoderma ornatum* (Say)** (Figure 6(E)), ***T. simplex* Jayne** (Figure 6(F)), ***T. sternale* Jayne** (Figure 6(G)), ***T. versicolor* (Creutzer)** (Figure 6(I))

7. Elytral integument with three well-defined (basal, submedian and apical) fasciae, setae on fasciae largely white, ensiform with very sparse yellowish recumbent setae ..... ***Trogoderma angustum* (Solier)** (Figure 6(A))

Elytral integument with well-defined basal band and median or posterior spot (Figure 5, left) ..... ***Trogoderma variabile* (reduced pattern)**

In general, elytral fasciae of *Trogoderma* species usually form a more or less complete basal loop, antemedian and median bands and apical spots. Some specimens have a reduced elytral pattern where the basal loop is indicated by curved anterior band, antemedian and/or median bands by small spots, and apical spots are usually missing.

For positive identification, all (especially in the case of damaged specimens) of the discriminating features should be observed (section 4.5.4).

Genital dissections should be carried out because there is a large number of undescribed *Trogoderma* species; by examining the genitalia, the chances of misidentifications are significantly reduced.

Maximova (2001) provides additional features for separating of adults of *Trogoderma granarium* from *T. variabile* and *T. glabrum*. Size and morphology of hind wings can be useful for identifying damaged specimens and although considering these two characteristics is not mandatory, it helps to

increase the certainty of identification based on other features (Figures 9, 10). During dissection hind wings must be removed and mounted in glycerol or Hoyer's medium.

Hind wings of *T. granarium* are smaller (mean length is 1.9 mm as compared with 2.5 mm for *T. variabile* and *T. glabrum*); they are paler in colour with less visible venation; number of setae S1 on costal vein (mean = 10) is half that on *T. variabile* and *T. glabrum* (mean = 20–23); number of small setae S2 between costal vein and pterostigma (mean = 2, sometimes absent) is less than that for *T. variabile* and *T. glabrum* (mean = 8) (Figures 9, 10).

4.5.4 Discriminating features of *Trogoderma granarium* adults

Adults of *T. granarium* are oblong-oval beetles, 1.4–3.4 mm long and 0.75–1.9 mm wide. The head is deflexed, head and pronotum darker than elytra, legs and abdomen are brownish. The elytra are brown. Females are slightly larger than males and lighter in colour.

To identify the adult stages of *T. granarium* correctly, specimens should correspond to the characters used to identify the family Dermestidae, the genus *Trogoderma* and the species *granarium*. These characters are as follows:

(1) elytral cuticle unicoloured, usually light brown or reddish-brown, or vaguely mottled without a clearly defined pattern

(2) elytral setae predominantly brown (yellowish or white setae forming no clearly defined banded pattern may also be present; these setae are gradually rubbed off as the beetle moves around and the adult thus develops a shiny appearance)

(3) antennae with 9–11 segments; male antennal club with 4–5 segments; female antennal club with 3–4 segments (Figures 7, 8).

(4) inner eye margin straight or sinuate

(5) male abdominal tergum 8 more or less evenly sclerotized, with setae along its margin sometimes tending to be grouped medially; tergum 9 with proximal margin of broader section almost U-shaped; tergum 10 with many long setae

(6) serrate sclerites of bursa copulatrix of female small, not longer than corrugated part of spermatheca, with 10–15 teeth (Figures 12, 13(A))

(7) male genitalia with bridge straight, and evenly wide, broader at connections to the parameres (Figure 11(A), (D)).

4.5.5 Description of *Trogoderma granarium* adults

The adult stage of *T. granarium* is illustrated in Figure 2(A), (B).

Adult male

Body: Length 1.4–2.3 mm (mean 1.99 mm), width 0.75–1.1 mm (mean 0.95 mm), ratio of length to width about 2.1:1. Head and pronotum dark reddish-brown; elytra reddish-brown, usually with indistinct lighter reddish-brown fasciae. Venter of thorax and abdomen reddish-brown; legs yellowish-brown.

Setae: Dorsal surface with evenly distributed, coarse, semi-erect, yellowish-brown and few, scattered, dark reddish-brown setae, with the colour of setae corresponding to the colour of the cuticle beneath; pronotum medially and laterally with indistinct patches of yellowish-white, ensiform setae, elytra with two or three indistinct bands of yellowish-white, ensiform setae. Ventral surface with dense, simple setiferous punctures, which are denser on ventrites, setae fine, short, recumbent, yellowish-brown.

Head: Punctures large, largest anteriorly, ocellate, separated by a distance of about the diameter of one to five punctures, surface between them shiny. Antennae yellowish-brown, 9-, 10- or 11-segmented with 4- or 5-segmented club. Antennal fossa shallow, loosely filled in by antenna. Eyes medially straight, or sometimes slightly sinuate.

Thorax: Anterior margin of pronotum with row of yellowish-brown, coarse setae pointing to middle of anterior margin, setae on anterior half of disc pointing backward, on posterior half pointing to the scutellum. Punctures slightly larger and more dense along anterior and lateral margins, and medially, otherwise small, simple on disc and separated by about 2–4 diameters.

Posterolateral end smooth, shining, otherwise very finely and densely punctured. Prosternum densely punctured, sides of posterior process straight and gradually tapering to apex.

Elytra densely punctured by setiferous punctures, punctures small, denser laterally, on disc separated by 2–4 diameters, laterally by 1–2 diameters.

Hind wings with vague venation; mean number of larger setae S1 on costal vein is 10, mean number of small setae S2 between costal vein and pterostigma is 2, but sometimes these are missing (for additional details see Figure 9).

Tibiae with small spines along outer edge. Proximal segment of hind tarsus about same length as second; distal segment about twice as long as fourth segment.

Abdomen: First ventrite with or without weak femoral lines. Ventrites covered by fine, yellowish-brown, recumbent setae, posterior half of penultimate ventrite with very dense, coarser, semi-erect, dark yellowish-brown setae.

Genitalia: Distal end of median lobe of aedeagus shorter than apices of parameres. Parameres wide, with sparse, short setae on inner and outer margins, setae extending to half the length of aedeagus. Paramere bridge is located at about one third of the total length from distal end, straight distally and proximally, bridge is as wide as or wider than aedeagus at crossing, basal process is tapered.

Adult female

Body: Length 2.1–3.4 mm (mean 2.81 mm); width 1.7–1.9 mm (mean 1.84 mm); ratio of length to width about 1.6:1.

Antenna sometimes less than 11-segmented, club 3–4-segmented.

Posterior half of penultimate ventrite without a dense fringe of semi-erect, yellowish-brown, coarse setae.

Other external morphological characters as in male above.

Genitalia: Bursa copulatrix with two small, dentate sclerites, length of sclerites equal to or shorter than the length of the corrugated part of spermatheca.

5. Records

Records and evidence should be retained as described in section 2.5 of ISPM 27.

In cases where other contracting parties may be adversely affected by results of the diagnosis, the records and evidence (in particular, preserved larvae and adults, slide-mounted specimens, photographs) should be kept for at least one year.

6. Contact Points for Further Information

Further information on this protocol can be obtained from:

Department of Agriculture and Food Western Australia, Biosecurity & Research Division, Plant Biosecurity Branch, Entomology Unit, 3 Baron-Hay Court, South Perth, WA 6151, Australia (tel: +61 8 9368 3248, +61 8 9368 3965; fax: +61 8 9368 3223, +61 8 9474 2840; e-mail: aszito@agric.wa.gov.au).

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Laboratorio de Plagas y Enfermedades de las Plantas. Servicio Nacional de Sanidad y Calidad Agroalimentaria (SENASA), Av. Ing. Huergo 1001, C1107AOK Buenos Aires, Argentina (tel:

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Disinfection Department of All-Russian Plant Quarantine Centre, 32 Pogranichnaya street, Bykovo-2, Ramensky area, Moscow region, Russian Federation (tel: +7 499 2713824, fax: +7 4952237241, e-mail: artshamilov@mail.ru).

7. Acknowledgements

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8. References

Banks, H.J. 1994. *Illustrated identification keys for* Trogoderma granarium, T. glabrum, T. inclusum *and* T. variabile *(Coleoptera: Dermestidae) and other* Trogoderma *associated with stored products*. CSIRO Division of Entomology Technical Paper, No. 32. Commonwealth Scientific and Industrial Research Organisation, Canberra. 66 pp.

Barak, A.V. 1989. Development of new trap to detect and monitor Khapra beetle (Coleoptera: Dermestidae). *Journal of Economic Entomology*, 82: 1470–1477.

—— 1995. Chapter 25: Identification of common dermestids. *In* V. Krischik, G. Cuperus & D. Galliart, eds. *Stored product management,* pp. 187–196. Oklahoma State University, Cooperative Extension Service Circular No. E-912 (revised).

Barak, A.V., Burkholder, W.E. & Faustini, D.L. 1990. Factors affecting the design of traps for stored-products insects. *Journal of the Kansas Entomological Society*, 63(4): 466–485.

Beal, R.S. Jr. 1954. Biology and taxonomy of nearctic species of *Trogoderma. University of California Publications in Entomology,* 10(2): 35–102.

—— 1956. Synopsis of the economic species of *Trogoderma* occurring in the United States with description of new species (Coleoptera: Dermestidae). *Annals of the Entomological Society of America*, 49: 559–566.

—— 1960. *Descriptions, biology and notes on the identification of some* Trogoderma *larvae (Coleoptera, Dermestidae)*. Technical Bulletin, United States Department of Agriculture, No. 1226. 26 pp.

——1982. A new stored product species of *Trogoderma* (Coleoptera: Dermestidae) from Bolivia. *The Coleopterists Bulletin*, 36(2): 211–215.

—— 1991. Dermestidae (Bostrychoidea) (including Thorictidae, Thylodriidae). *In* F.W. Stehr, ed. *Immature insects*, pp. 434–439. Duboque, Iowa, Michigan State University, Kendall/Hunt. Vol. 2, xvi+ 975 pp.

Bousquet, Y. 1990. *Beetles associated with stored products in Canada: An identification guide*. Agriculture Canada Research Branch Publication 1837. Ottawa, Supply and Services Canada. 214 pp.

CABI. 2011. *Trogoderma granarium*. *In* Crop Protection Compendium, Wallingford, UK, CAB International (available online) http://www.cabi.org.

Delobel, A. & Tran, M. 1993. Les coléoptères des denrées alimentaires entreposées dans les régions chaudes. Faune tropicale XXXII. Paris, ORSTOM. 424 pp.

EPPO/CABI. 1997. *Trogoderma granarium*. *In* I.M. Smith, D.G. McNamara, P.R. Scott, & M. Holderness, eds. *Quarantine pests for Europe*, 2nd edition. Wallingford, UK. CAB International. 1425 pp.

EPPO. 2002. Diagnostic protocols for regulated pests, *Trogoderma granarium*. *Bulletin OEPP/EPPO Bulletin*, 32: 299–310.

—— 2011. PQR - EPPO database on quarantine pests (available online). http://www.eppo.int.

Green, M. 1979. The identification of *Trogoderma variable* Ballion, *T. inclusum* and *T. granarium* Everts (Coleoptera, Dermestidae), using characters provided by their genitalia. *Entomologists Gazette*, 30: 199–204.

Haines, C.P. (ed.) 1991. Insects and arachnids of tropical stored products: their biology and identification (a training manual). Chatham Maritime, UK, Natural Resources Institute. 246 pp.

Háva, J. 2003. *World catalogue of the Dermestidae (Coleoptera)*. Studie a zprávy Okresního muzea Praha-Východ, Supplementum 1. 196 pp.

—— 2004. World keys to the genera and subgenera of Dermestidae (Coleoptera) with descriptions, nomenclature and distributional records. *Acta Musei Nationalis Pragae, Series B, Natural History*, 60 (3–4): 149–164.

—— 2011. Dermestidae of the world (Coleoptera). Catalogue of the all known taxons. Available online: http://www.dermestidae.wz.cz/catalogue_of_the_all_known_taxons.pdf, accessed January 2012.

Hinton, H.E. 1945. *A monograph of the beetles associated with stored products*, Vol. 1. London, British Museum (Natural History). 443 pp.

Kingsolver, J.M. 1991. Dermestid beetles (Dermestidae, Coleoptera). *In* J.R. Gorham, ed. *Insect and mite pests in food. An illustrated key*, pp. 113–136. Washington, DC, USDA ARS and USDHHS, PHS, Agriculture Handbook No. 655, Vol. 1: 324 pp.

—— 2002. Dermestidae. *In* R.H. Arnett Jr., M.C. Thomas, P.E. Skelley, & J.H. Frank, eds. *American beetles*, Vol. 2, pp. 228–232. Boca Raton, Florida, CRC Press. 861 pp.

Lawrence, J.F. (coordinator). 1991. Order Coleoptera. *In* F.W. Stehr, ed. *Immature insects,* pp. 144–658. Dubuque, Iowa, Kendall/Hunt, Vol. 2. xvi + 975 pp.

Lawrence, J.F. & Britton, E.B. 1991. Coleoptera (beetles). *In* CSIRO, ed. *Insects of Australia*, 2nd edition, Vol. 2, pp. 543–683. Carlton, Melbourne University Press. 2 vols, xvi + 1137 pp.

——. 1994. *Australian beetles.* Carlton, Melbourne University Press. x + 192 pp.

Lawrence, J.F., Hastings, A.M., Dallwitz, M.J., Paine, T.A. & Zurcher, E.J. 1999a. Beetle larvae of the world: Descriptions, illustrations, and information retrieval for families and subfamilies. CD-ROM, Version 1.1 for MS-Windows. Melbourne, CSIRO Publishing.

—— 1999b. Beetles of the world: A key and information system for families and subfamilies. CD-ROM, Version 1.0 for MS-Windows. Melbourne, CSIRO Publishing.

Lindgren, D.L., Vincent, L.E. & Krohne, H.E. 1955. The Khapra beetle, *Trogoderma granarium* Everts. *Hilgardia*, 24(1): 1–36.

Maximova, V.I. 2001. Идентификациа капрового жука, *Защита и карантин растиений*, 4: 31.

Mitsui, E. 1967. [On the identification of the Khapra beetle.] *Reports of the Japan Food Research Institute, Tokyo*, 22: 8–13. (in Japanese)

Mordkovich, Ya.B. & Sokolov, E.A. 1999. Определитель карантинных и других опасных вредителей сырья, продуктов запаса и посевного материала, Колос, Москва: 384.

—— 2000. Выявление капрового жука в складских помещниях, *Защита и карантин растиений*, 12: 26–27.

Mound, L. (ed.) 1989. Common insect pests of stored food products. A guide to their identification. London, British Museum (Natural History). 68 pp.

Mroczkowski, M. 1968. Distribution of the Dermestidae (Coleoptera) of the world with a catalogue of all known species. *Annales Zoologici,* 26(3): 1–191.

OIRSA. 1999a. Trogoderma granarium Everts. In OIRSA, Hojas de Datos sobre Plagas y Enfermedades de Productos Almacenados de Importancia Cuarentenaria y/o Económica para los Países Miembros del OIRSA, pp. 120–145. El Salvador, OIRSA. Vol. 6. 164 pp.

—— 1999b. Trogoderma variabile Ballion. In OIRSA, Hojas de Datos sobre Plagas y Enfermedades de Productos Almacenados de Importancia Cuarentenaria y/o Económica para los Países Miembros del OIRSA, pp. 146–161. El Salvador, OIRSA. Vol. 6. 164 pp.

Okumura, G.T. & Blanc, F.L. 1955. Key to species of *Trogoderma* and to related genera of Dermestidae commonly encountered in stored grain in California. *In* California Legislature Joint Interim Committee on Agricultural and Livestock Problems*, Special Report on the Khapra Beetle,* Trogoderma granarium, pp. 87–89. Sacramento, California.

PaDIL. 2011. Khapra beetle *(Trogoderma granarium)*. Pest and Diseases Image Library (PaDIL), available online: http://www.padil.gov.au/pests-and-diseases/Pest/Main/135594, accessed 15 November 2011.

Pasek, J.E. 1998. *Khapra beetle (*Trogoderma granarium *Everts): Pest-initiated pest risk assessment*. Raleigh, NC, USDA. 46 pp.

Peacock, E.R. 1993. Adults and larvae of hide, larder and carpet beetles and their relatives (Coleoptera: Dermestidae) and of derontid beetles (Coleoptera: Derontidae). Handbooks for the identification of British insects No. 5, Royal Entomological Society, London. 144 pp.

Rees, B.E. 1943. Classification of the Dermestidae (larder, hide, and carpet beetles) based on larval characters, with a key to the North American genera. USDA Miscellaneous Publication No. 511. 18 pp.

Rees, D.P. 2004. *Insects of stored products*. Melbourne, Australia, CSIRO Publishing; London, UK, Manson Publishing. viii +181 pp.

Saplina, G.S. 1984. Обследование складских помещений с помощью ловушек. *Защита растиений***,** 9: 38.

Sinha, A.K. & Sinha, K.K. 1990. Insect pests, *Aspergillus flavus* and aflatoxin contamination in stored wheat: A survey at North Bihar (India). *Journal of Stored Products Research,* 26(4): 223–226.

Strong, R.G. & Okumura, G.T. 1966. *Trogoderma* species found in California, distribution, relative abundance and food habits. *Bulletin, Department of Agriculture, State of California,* 55: 23–30.

Varshalovich, A.A. 1963. Капровый жук – опаснейший вредитель пищевых запасов. Сельхоиздат, Москва: 1–52.

Zhang, S.F., Liu H. & Guan, W. 2007. [Identification of larvae of 8 important species from genus *Trogoderma*], *Plant Quarantine,* 21(5): 284–287 (in Chinese).

9. Figures

(A)

(B)

(C)

(D)

Figure 1: Symptoms of infestation of stored products with *Trogoderma granarium*: (A) damaged wheat grain; (B) infested rape seeds; (C) totally destroyed wheat grain (dust and remains of grains); (D) larval exuviae (cast skins) contaminating stored product (Paweł Olejarski, Instytut Ochrony Roślin - Państwowy Instytut Badawczy, Poznań, Poland)

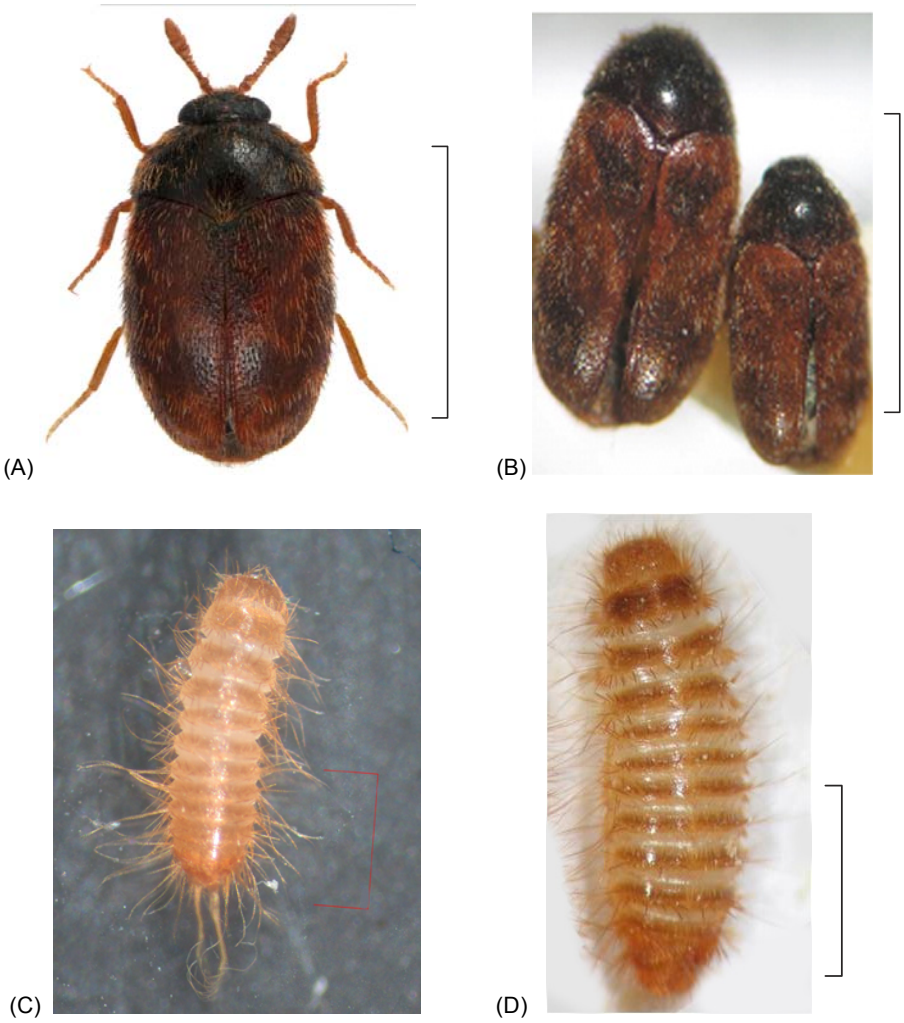


Figure 2: ***Trogoderma granarium*****:** (A) adult, female; (B) comparison of shape of female (left) and male (right); (C) young larva; (D) mature larva. Scale bar: (A), (B), (D) = 2 mm; (C) = 1 mm. ((A), Tomasz Klejdysz, Instytut Ochrony Roślin - Państwowy Instytut Badawczy, Poznań, Poland; (B), (D), Ya.B. Mordkovich and E.A. Sokolov, All-Russian Plant Quarantine Centre, Bykovo Russia); (C), Cornel Adler, Julius Kűhn-Institut; (JKI) Germany))

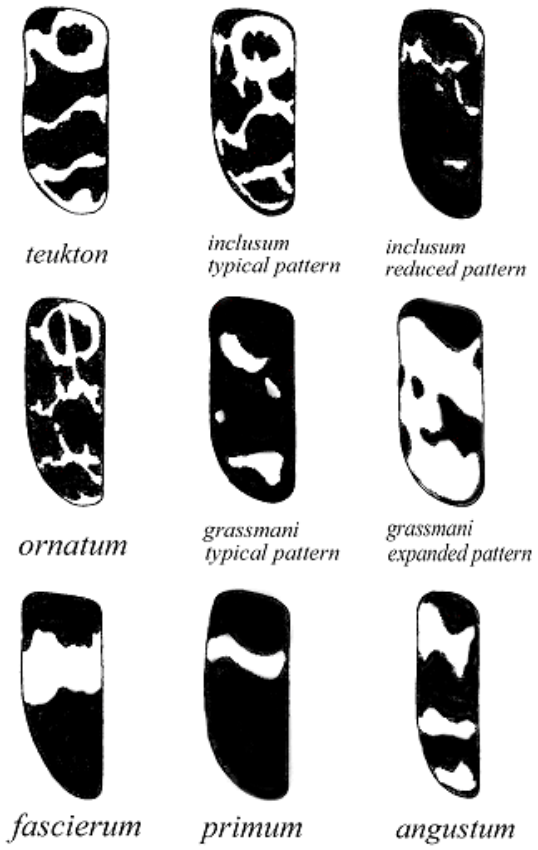


Figure 3: *Trogoderma* spp. elytral pattern (Beal, 1954)

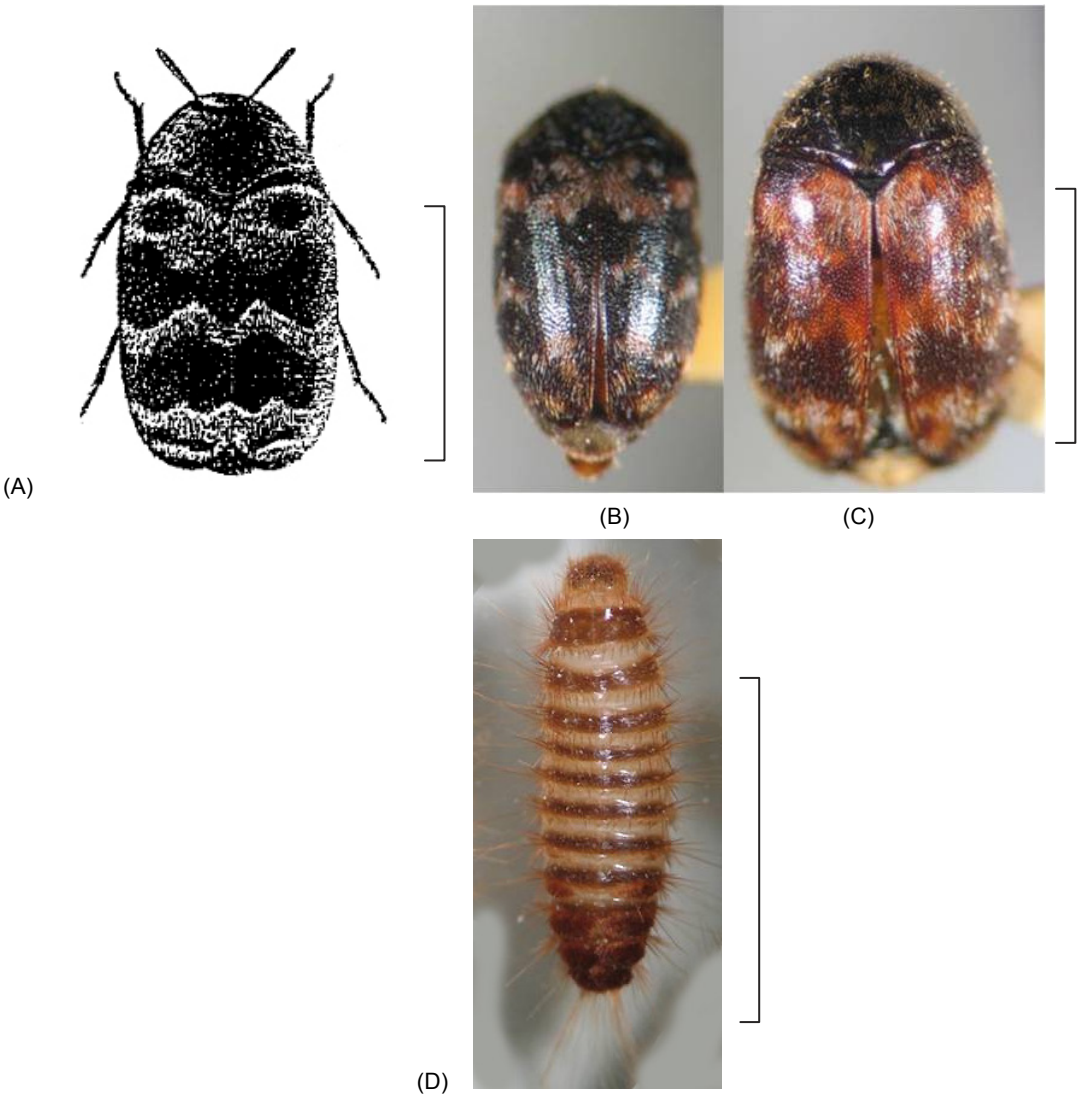


Figure 4: *Trogoderma variabile*: (A) schematic drawing of the adult; (B) male; (C) female; (D) larva. Scale bar = 2 mm. ((A), OIRSA (1999b); (B)–(D), Ya.B. Mordkovich and E.A. Sokolov, All-Russian Plant Quarantine Centre, Bykovo, Russia)

Figure 5: Elytral pattern of *Trogoderma variabile*: *left*, reduced pattern; *centre*, typical; *right*, expanded (Beal, 1954)

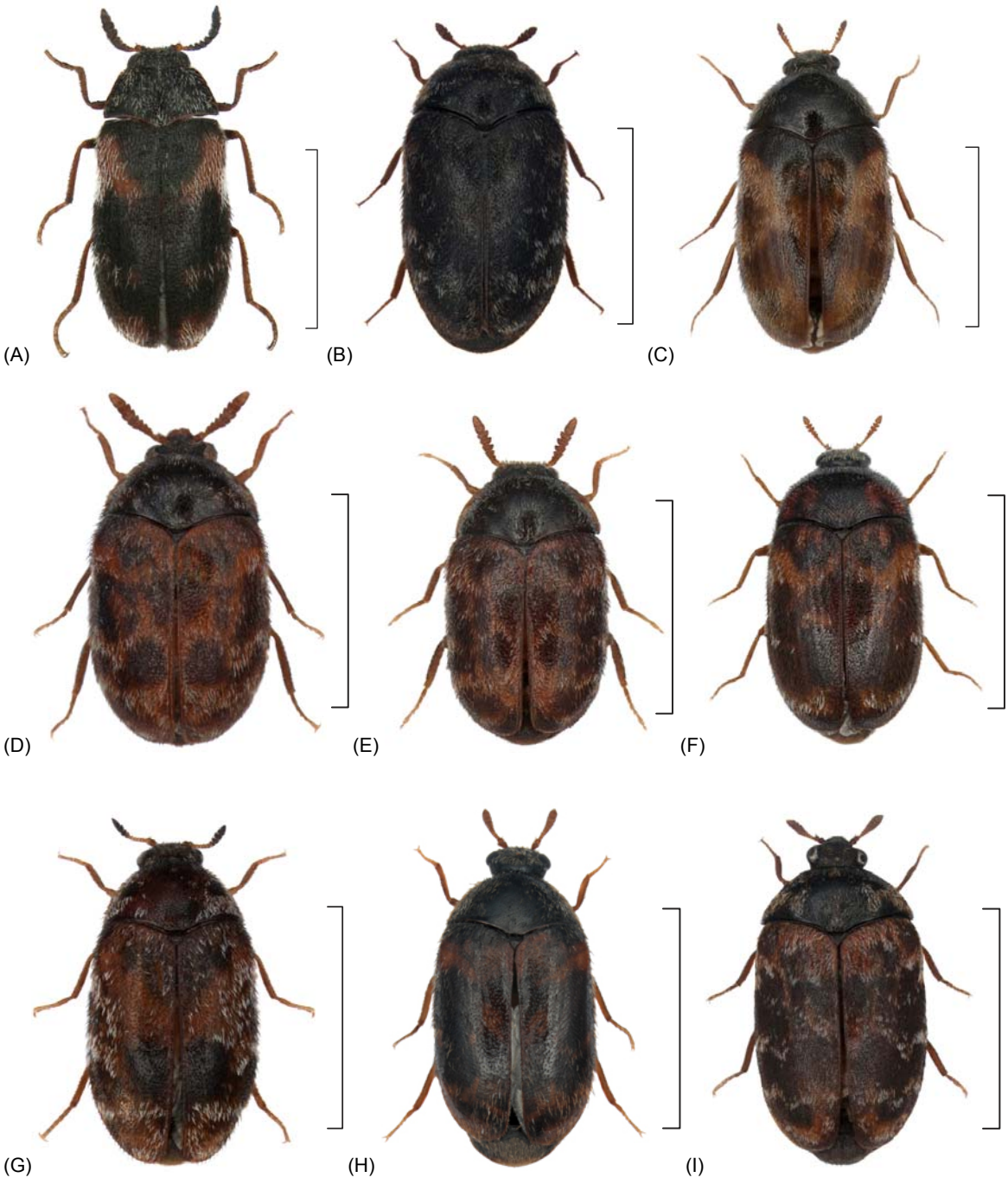


Figure 6: Comparison of females of some Trogoderma non-granarium species: (A) *T. angustum*; (B) *T. glabrum*; (C) *T. grassmani*; (D) *T. inclusum*; (E) *T. ornatum*; (F) *T. simplex*; (G) *T. sternale*; (H) *T. variabile*; (I) *T. versicolor*. Scale bar = 2 mm. (Tomasz Klejdysz, Instytut Ochrony Roślin - Państwowy Instytut Badawczy, Poznań, Poland)

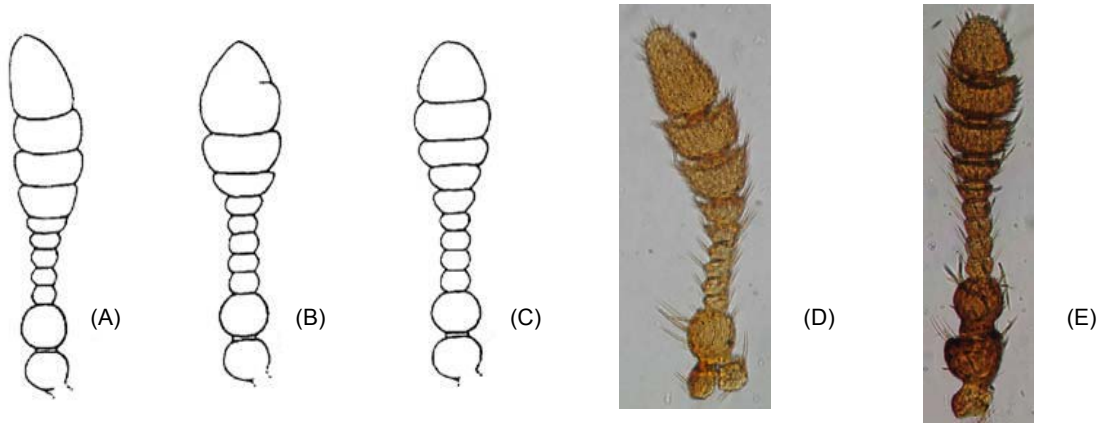


Figure 7: Antennae of *Trogoderma granarium*: (A), (D) male antenna with normal number of segments; (B) female antenna with reduced number of segments; (C), (E) female antenna with normal number of segments ((A)–(C), Beal (1956); (D), (E), Ya.B. Mordkovich and E.A. Sokolov, All-Russian Plant Quarantine Centre, Bykovo, Russia)

Figure 8: Antennae of some *Trogoderma* species: (A) *T. variabile*; (B) *T. glabrum*; (C) *T. teukton*; 1, male antenna with normal number of segments; 2, female antenna with normal number of segments (Ya.B. Mordkovich and E.A. Sokolov, All-Russian Plant Quarantine Centre, Bykovo, Russia)

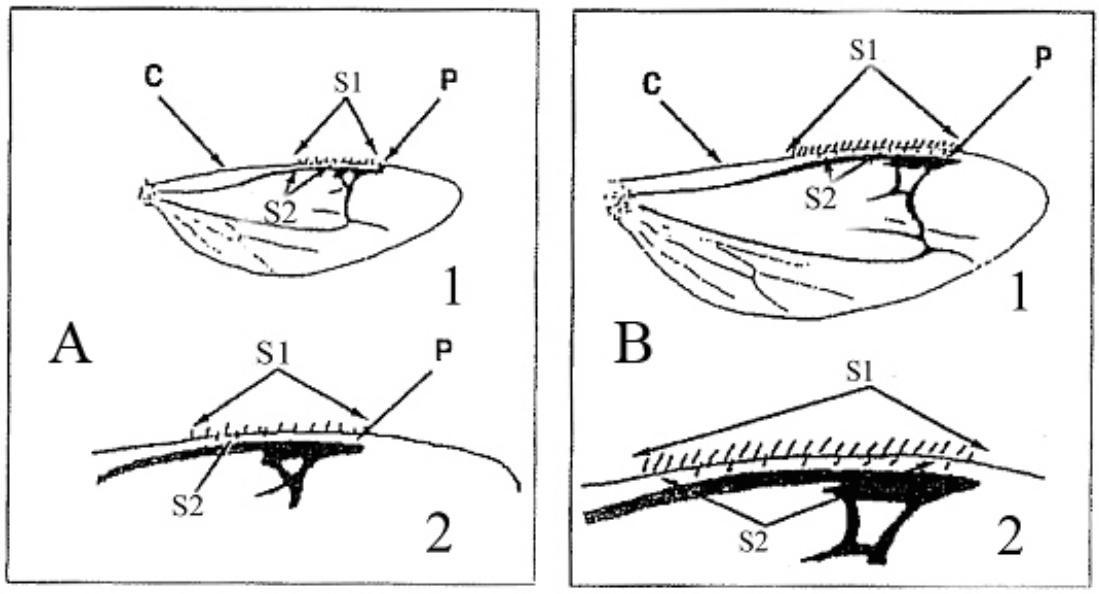
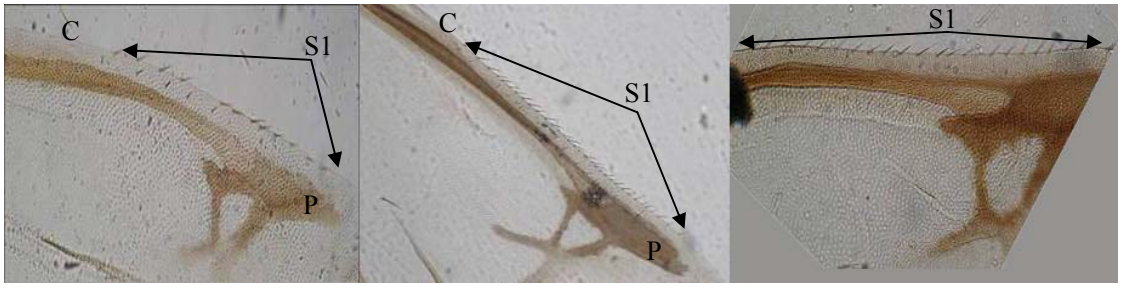


Figure 9: Schematic representation of the morphology of the hind wing: (A) *Trogoderma granarium* (Maximova, 2001), with up to 14 S1 setae on costal vein (mean = 10 S1), and 2–5 S2 setae, or with no S2 setae, between costal vein and pterostigma (mean = 2 S2); (B) *Trogoderma variabile* and *T. glabrum* with 16 or more than 16 S1 setae.
Details: 1, general morphology of the wing; 2, enlarged anterior part of the wing (C, costal vein; P, pterostigma; S1, setae on costal vein; S2, small setae between costal vein and pterostigma). The number of S2 setae is not used for the diagnosis because this character is not known for other species.



(A) (B) (C)

Figure 10: Morphology of hind wings: (A) *T. granarium*; (B) *T. glabrum*; (C) *T. variabile* (Ya.B. Mordkovich and E.A. Sokolov, All-Russian Plant Quarantine Centre, Bykovo, Russia)

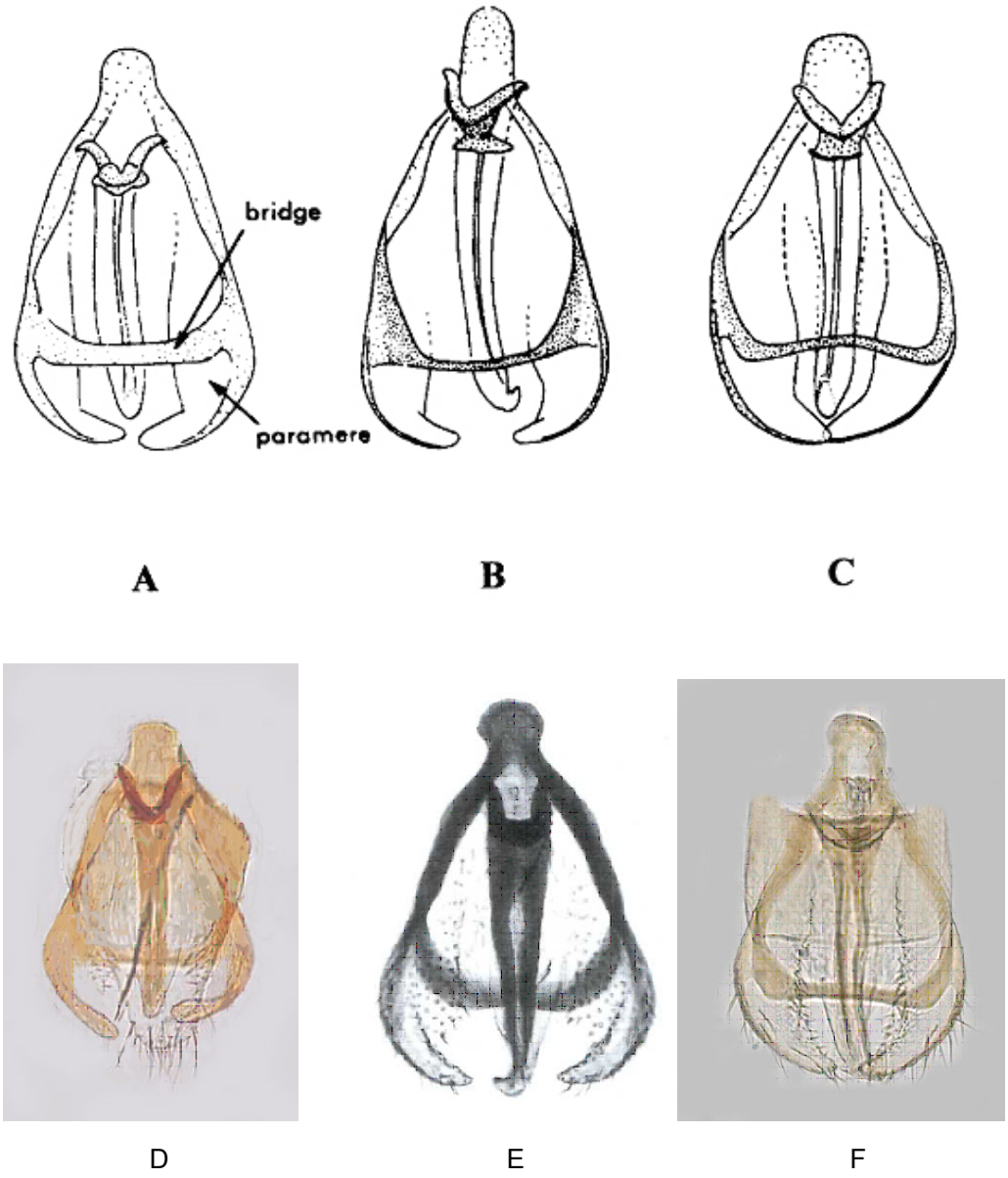


Figure 11: Male genitalia: (A), (D) *Trogoderma granarium*; (B) *T. inclusum*; (C), (F) *T. variabile*; (E) *T. glabrum* ((A)–(C), Green (1979); (D)–(F), Ya.B. Mordkovich and E.A. Sokolov, All-Russian Plant Quarantine Centre, Bykovo, Russia).

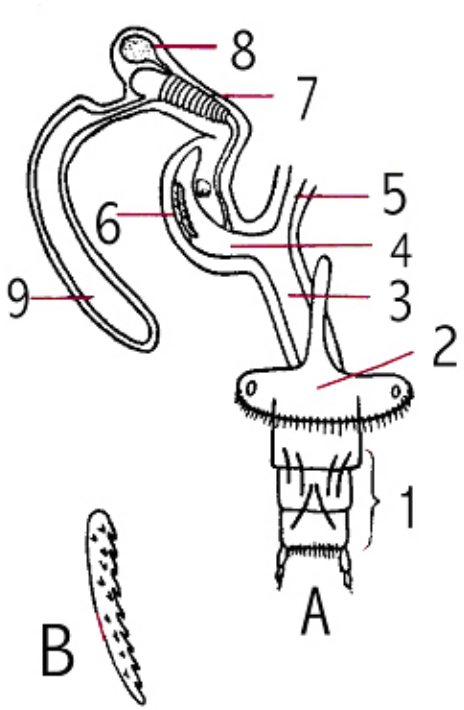
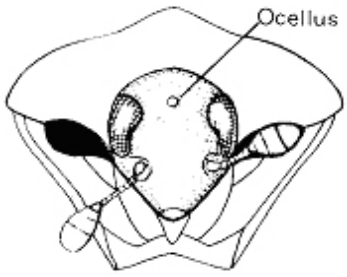
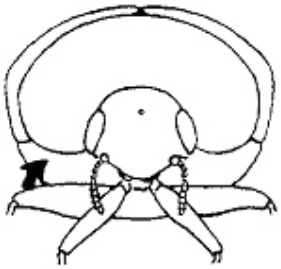


Figure 12: Female genitalia of *Trogoderma granarium:* (A) general view of genitalia; (B) one of the serrate sclerites from the bursa copulatrix (Varshalovich, 1963). Details: 1, ovipositor; 2, 7th abdominal sclerite; 3, vagina; 4, bursa copulatrix; 5, oviduct; 6, two serrate sclerites on bursa copulatrix; 7, corrugated part of spermatheca; 8, spermatheca; 9, accessory glands.

Figure 13: Serrate sclerites from the bursa copulatrix of female genitalia of various *Trogoderma* species: (A) *T. granarium*; (B) *T. variabile*; (C) *T. glabrum*; (D) *T. teukton* (Ya.B. Mordkovich and E.A. Sokolov, All-Russian Plant Quarantine Centre, Bykovo, Russia)



(A)

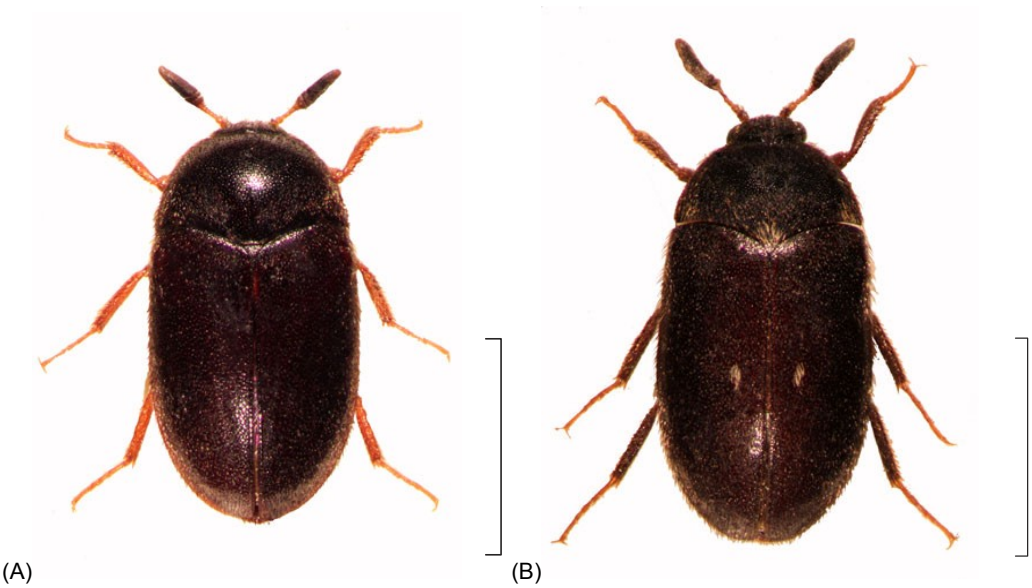


(B)

Figure 14: Antennal cavity: (A) antennal cavity clearly visible in anterior view (*Anthrenus*), antennae fully filling the cavity; (B) antennal cavity not visible in anterior view (*Trogoderma*), antennae loosely fit in the cavity ((A), Mound (1989), copyright: Natural History Museum, London, UK; (B), Kingsolver (1991))

(A) (B)

Figure 15: Adults of *Dermestes* species: (A) *D. lardarius*; (B) *D. maculates. S*cale bar = 2 mm. (Marcin Kadej, Instytut Zoologiczny, Uniwersytet Wrocławski, Wrocław, Poland)



(A) (B)

Figure 16: Adults of *Attagenus* species: (A) *A. unicolor*; (B) *A. pellio*. Scale bar = 2 mm. (Marcin Kadej, Instytut Zoologiczny, Uniwersytet Wrocławski, Wrocław, Poland)

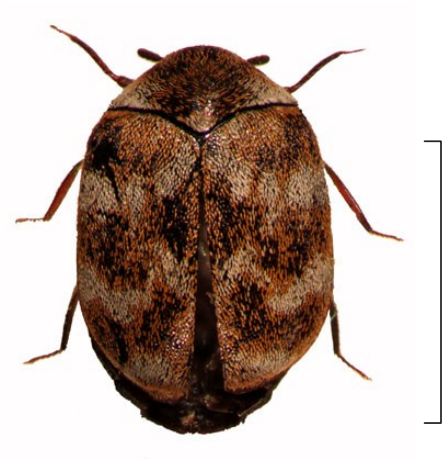


Figure 17: Adult of *Anthrenus verbasci*: Scale bar = 2 mm. (Marcin Kadej, Instytut Zoologiczny, Uniwersytet Wrocławski, Wrocław, Poland)

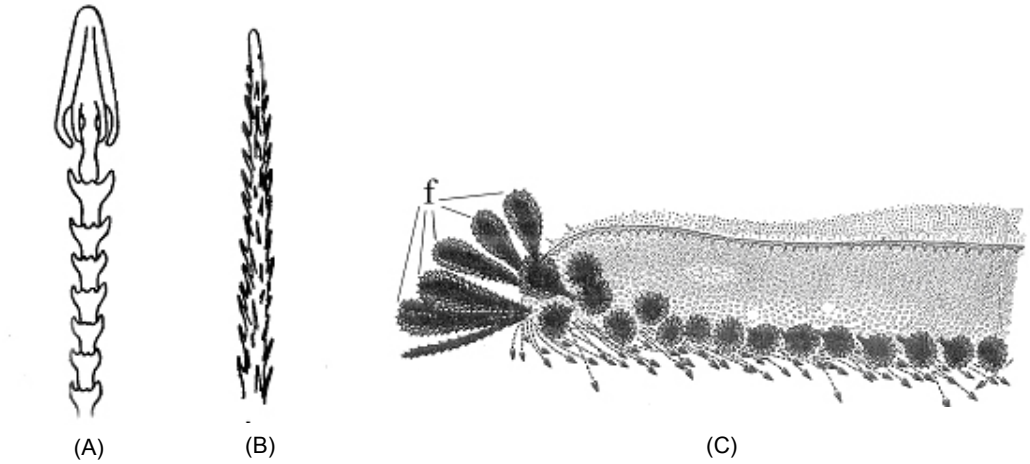


Figure 18: Larval setae: (A) hastiseta; (B) spiciseta; (C) fiscisetae (f) on first abdominal tergum of *Trogoderma carteri* larva ((A), (B), Varshalovich (1963); (C), Beal (1960))

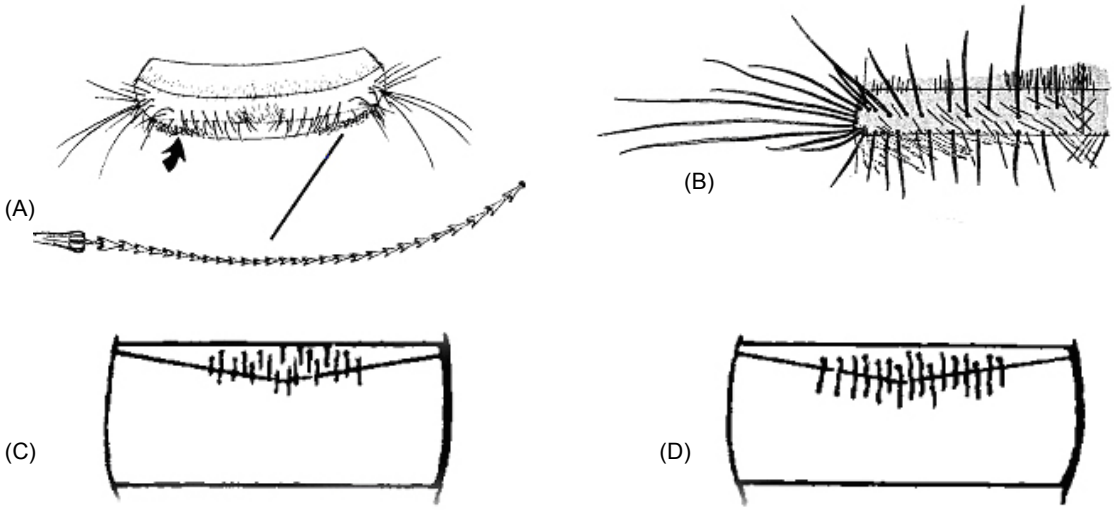


Figure 19: Abdominal tergite and setae: (A) abdominal tergite of *Trogoderma variabile* larva with enlarged hastiseta; (B) first abdominal tergite of *T. variabile* larva; (C) setae of the anterior portion of first abdominal tergite not long enough to extend caudally over the antecostal suture (*T. variabile*); (D) the same setae long enough to extend caudally through the antecostal suture (*T.* non-*variabile*) ((A), Kingsolver (1991); (B), Beal (1954); (C), (D), OIRSA (1999a))

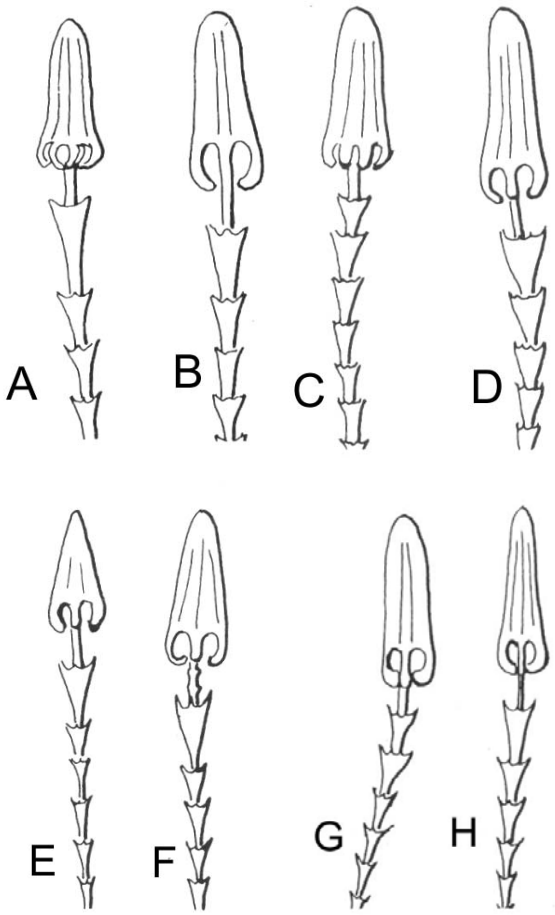


Figure 20: Comparison of hastisetae morphology of various *Trogoderma* larvae: (A), (B) *T. granarium*; (C), (D) *T. glabrum*; (E), (F) *T. variabile*; (G), (H) *T. inclusum*; copyright: Natural History Museum, London, UK (Peacock, 1993)

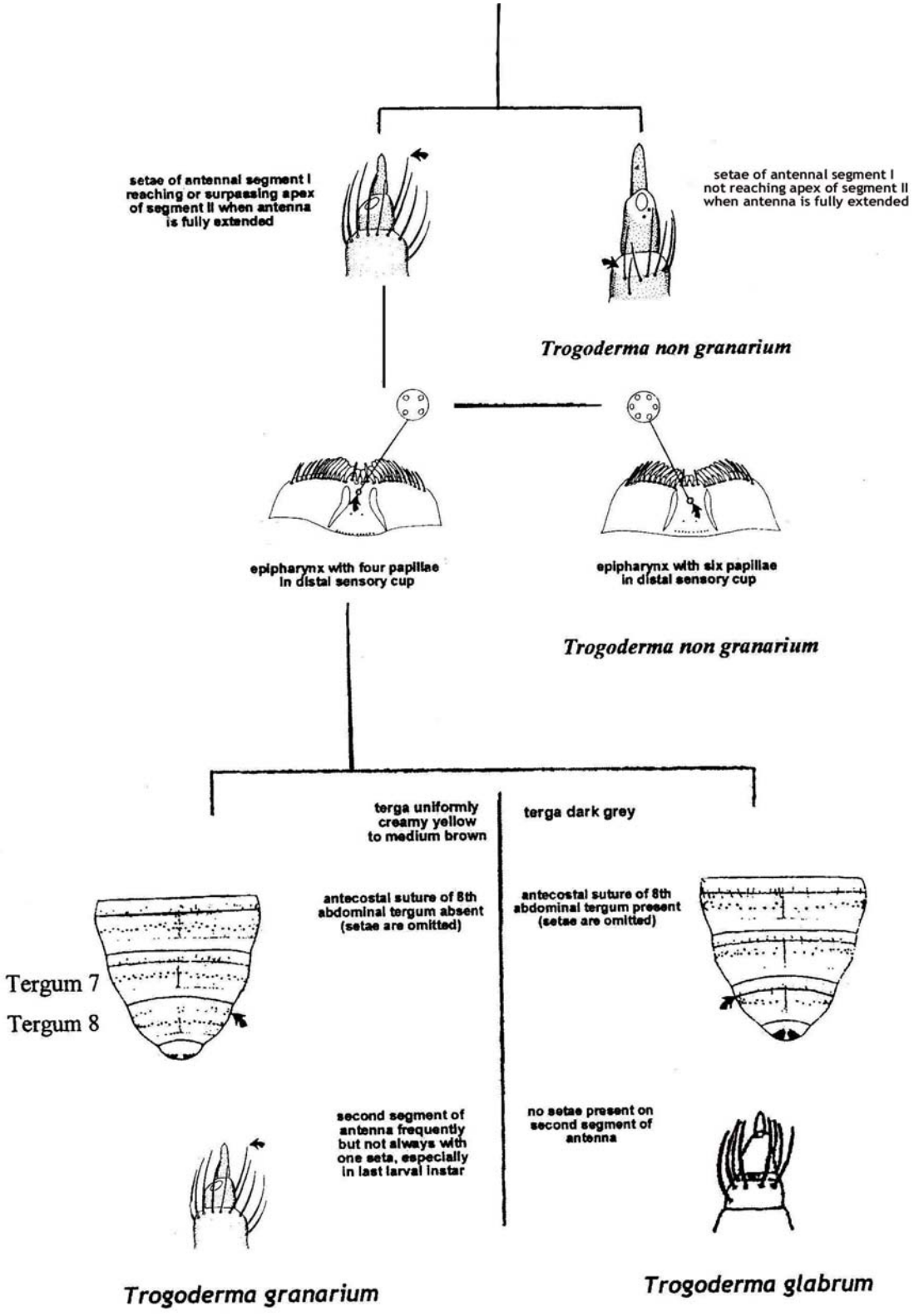


Figure 21: Pictorial key for distinguishing larvae of *Trogoderma granarium* from other species of *Trogoderma* (Kingsolver, 1991; OIRSA, 1999a)

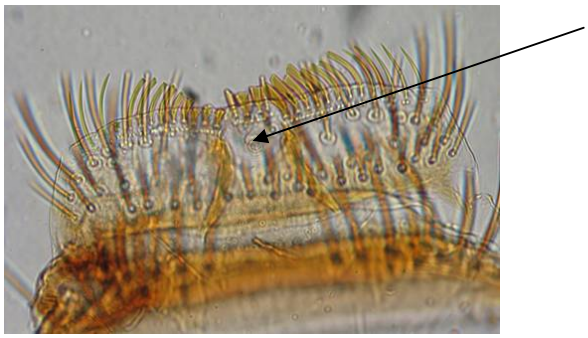
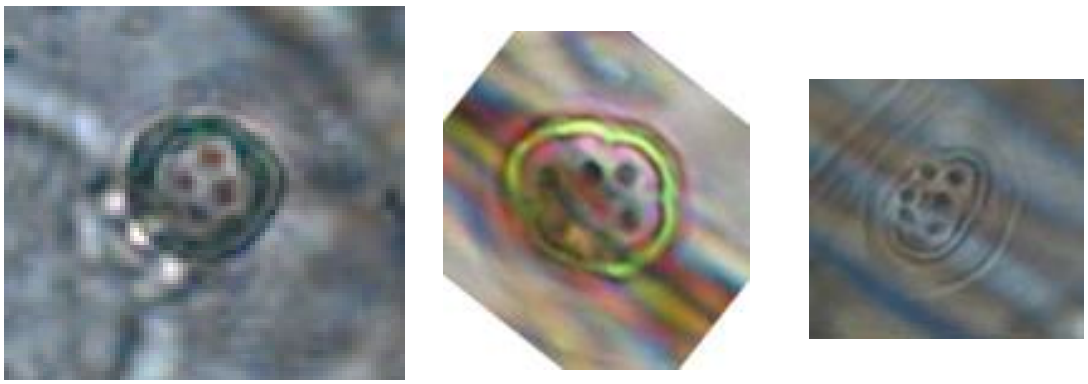


Figure 22: Epipharynx of *Trogoderma* sp. larva with a distal sensory cup marked with an arrow (Ya.B. Mordkovich and E.A. Sokolov, All-Russian Plant Quarantine Centre, Bykovo, Russia)



(A) (B) (C)

Figure 23. Distal papillae: (A) four distal papillae in sensory cup of *T. granarium* larva; (B) six distal papillae in *T. variabile*; (C) six distal papillae in *T. glabrum*. (Ya.B. Mordkovich and E.A. Sokolov, All-Russian Plant Quarantine Centre, Bykovo, Russia)

Publication history

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2006-04 CPM-1 added topic diagnostic protocol for *Trogoderma granarium* (2004-006)

2008-09 SC approved for member consultation via e-mail

2011-06 member consultation

2012-03 CPM-7 adopted Annex 3 to ISPM 27

ISPM 27. 2006: **Annex 3** *Trogoderma granarium* Everts (2012)

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