# National Diagnostic Protocol

## Khapra Beetle

## *Trogoderma granarium* Everts


*NDP 45 V1*

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**Purpose**

National Diagnostic Protocols (NDPs) are diagnostic protocols for the unambiguous taxonomic identification of plant pests. NDPs:

- are a verified information resource for plant health diagnosticians
- are consistent with ISPM No. 27 – Diagnostic Protocols for Regulated Pests
- provide a nationally consistent approach to the identification of plant pests enabling transparency when comparing diagnostic results between laboratories; and,
- are endorsed by regulatory jurisdictions for use (either within their own facilities or when commissioning from others) in a pest incursion.

Where an International Plant Protection Convention (IPPC) diagnostic protocol exists it should be used in preference to NDPs although NDPs may contain additional information to aid diagnosis. IPPC protocols are available on the IPPC website:

https://www.ippc.int/core-activities/standards-setting/ispms

**Process**

NDPs are facilitated and endorsed by the Subcommittee on Plant Health Diagnostics (SPHD). SPHD reports to Plant Health Committee and is Australia's peak technical and policy forum for plant health diagnostics.

NDPs are developed and endorsed according to Reference Standards developed and maintained by SPHD. Current Reference Standards are available at https://www.plantbiosecuritydiagnostics.net.au/initiatives/national-diagnostic-protocols/

NDPs are living documents. They are updated every 5 years or before this time if required (i.e. when new techniques become available).

**Document status**

This version of the National Diagnostic Protocol (NDP) for Khapra beetle – *Trogoderma granarium* Everts is current as at the date contained in the version control box below.

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**Further information**

Inquiries regarding technical matters relating to this project should be sent to: sphds@agriculture.gov.au

# Contents

# 1 INTRODUCTION

*Trogoderma granarium*, the Khapra beetle, is a serious exotic pest of grains and stored dry foodstuffs. It has been categorised as one of the top five exotic threats to the Australian grains industry and is high on the list of target organisms for exclusion by quarantine. Identification of adults and larvae requires specialist knowledge. There are other exotic pest *Trogoderma* species, such as the economically significant Warehouse beetle, *T. variabile,* that is already established and widespread in Australia and causes significant damage to stored products in the Eastern States. A few minor exotic pest *Trogoderma* species exist. Their introduction to Australia will unlikely cause any major damage but their presence in export shipments will increase the chances of misidentification and should they become established, would make a Khapra beetle incursion more difficult to detect.

Australia has a large, poorly studied native *Trogoderma* fauna. Many of them are still undescribed, their estimated number is likely to be over 100 species (Szito pers. comm.). Occasionally native Australian species can occur in stored commodities (Szito 1997). Their identification and at least separation from the pestiferous exotic *Trogoderma* presents a serious problem.

This protocol has been prepared to distinguish *Trogoderma granarium* from the other exotic *Trogoderma* species and from the already introduced *Trogoderma variabile* as well as the Australian native *Trogoderma* and related Dermestid species by both morphological and molecular methods. Companion protocols include ISPM 27 DP 03: *Trogoderma granarium* Everts (2012) (morphological), and Olson *et al.* (2014) (molecular).

## 1.1 Host range

Unlike most Dermestidae the Khapra beetle feeds by preference on grain and cereal products. Primary hosts are cereals (rice, wheat, oats, barley, sorghum, maize etc.), cereal products (rolled oats and barley, ground corn, corn meal, wheat flour, bran products, pasta, etc.), pulses (chickpea, soybean, and lentil), various vegetable seeds (cucurbit, capsicum, tomato, etc.), herbs, spices and various nuts (peanut, pecan, walnut, almond etc.) and can be a serious pest of such commodities in store. It can successfully complete its lifecycle in copra, dried fruits, various gums and many different dried products of wholly or partial animal origin such as skins, dried dog food, dried blood, dead insects and dried animal carcasses. The damage occurs in the larval stage, as the adults do not normally feed. They can feed on products with as little as 2% moisture content and can develop on animal matter such as dead mice, dried blood, and dried insects (Hinton 1945, Lindgren *et al*. 1955).

A list of primary host plants and hosts of plant origin on which *T. granarium* Everts has been intercepted at US entry points (Pasek 1998) are presented in Appendix 8.1.

Contaminated shipping containers, pallets, and packaging, especially hessian bags used for packaging and shipping non-host materials present a very significant risk.

Further information on the life history and dispersal of the Khapra beetle is presented in Appendix 8.2.

# 2 TAXONOMIC INFORMATION

**Taxonomic Position**

*Trogoderma granarium* Everts, 1898

| | |
|---|---|
| Phylum: | Arthropoda |
| Class: | Insecta |
| Order: | Coleoptera |
| Superfamily: | Bostrichoidea |
| Family: | Dermestidae |
| Subfamily: | Megatominae |
| Tribe: | Megatomini |
| Genus: | *Trogoderma* Dejean, 1821 |
| Species: | *Trogoderma granarium* Everts, 1898 |

**Synonyms**

*Trogoderma quinquefasciata* Leesberg, 1906 nec Jacquelin du Val, 1859 - Leesberg 1906: in Mroczkowski, 1968

*Trogoderma khapra* Arrow, 1917: Everts, 1923

*Trogoderma koningsbergeri* Pic, 1933

*Trogoderma afrum* Priesner, 1951: Howe and Burgess, 1956

*Trogoderma granarium* ssp. *afrum*: Attia and Kamel, 1965

**Common names**

Khapra beetle (English)

Khaprakäfer (German)

Trogoderme (dermeste) du grain, dermeste des grains (French)

Trogoderma de los granos, escarabajo khapra, gorgojo khapra, dermeste de los granos (Spanish)

Dermeste dei Cereali (Italian)

Kapra (Turkish)

Kaprabille (Norway)

Chipushit hagargirim (Hebrew)

Kaprabogár (Hungarian)

Капровый жук (Russian)

# 3 DETECTION

## 3.1 Symptoms of *Trogoderma granarium* Everts damage

*Trogoderma granarium* infestations are usually recognised by (1) the presence of the pest especially larvae and exuviae (cast larval skin) and (2) symptoms of infestation. The short-lived adults are often not present either due to non-emergence or because the larvae have consumed most of the body of dead adults. Damage to the commodities can be a warning sign, but often it is a result of the feeding of other common stored product pests. Early instar larvae feed on broken or damaged kernels, whereas older larvae feed on whole kernels. 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[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.

**Table 1 Pattern of damage and activity of *Trogoderma granarium* in storage facilities. Primary hosts are all affected in a similar way, but damage to secondary hosts may vary from this.**

| Plant part | Attacked | Insect Life Stage |
|---|---|---|
| Root | No | Not reported on living plant |
| Stem | No | Not reported on living plant |
| Leaves | No | Not reported on living plant |
| Growing tips | No | Not reported on living plant |
| Buds | No | Not reported on living plant |
| Flowers | No | Not reported on living plant |
| Fruit and Seeds | No | Larvae feed on stored seeds, nuts, and dried fruits. They can also feed on dry material of animal origin such as hide, milk powder, dog biscuit etc. |

**Table 2 Life-stage activity of *Trogoderma granarium*. All life stages behave in a similar way on any produce suitable for consuming and that enables them to complete their lifecycle.**

| Life Stage | Site of activity |
|---|---|
| Eggs | Single eggs are laid in host material loosely. |

[1] See Appendix 8.4 Annex X to ISPM 27:2006 *Trogoderma granarium* Everts [figures], Figure 1.

| | |
|---|---|
| Larvae<br>(5–15 instars) | Early instar larvae feed on organic debris and damaged seeds. Early instar larvae are also capable of damaging whole soft seeds, like nuts. Late instars are able to damage whole, healthy seeds. |
| Pupa | Pupation in the last larval skin. |
| Adult | Adults do not feed. Females lay their eggs individually on the surface of the commodity. |

## 3.2 Specimen collection, handling and preservation

It is a very important requirement that the inspector should collect multiple specimens of the pest, preferably in both larval and adult stages. Primarily because there are often more than one species of Dermestid beetle present. *Attagenus, Anthrenus, Anthrenocerus, Phradonoma, Reesa* and exotic and native *Trogoderma* species may occur concurrently in stored products with Khapra beetle; though they appear to be scavengers only, feeding on dead bodies of other insects. Their presence may mask a low-level *T. granarium* infestation. There are many non-pest insect species that occur in stored commodities and in packaging material that could easily be confused with Khapra beetle under hand magnification.

Live adults, pupae, larvae or eggs MUST NOT BE TRANSPORTED, unless considered essential, and then only in approved biosecure transport containers to be opened only in PC3 or BC3 secure containment facilities.

Adults should be placed in 100% ethanol for initial preservation. With the specimen lying on its back, the hind and mid left legs can be removed at the coxae and placed into 100% ethanol in a cryo-tube for later DNA analysis.

Larvae require blanching in almost boiling water (approximately 95°C) for at most 20 seconds before transferring to 100% ethanol. A small section of the lower left ventral abdominal wall of the larvae can be excised and preserved in 100% ethanol in a cryo-tube for later DNA analysis.

At a low level of infestation or when the circumstances are unfavourable to the larvae, we may find only a few cast skins in a corner undisturbed by wind or material movement. The larvae are most likely diapausing, that is, hiding in an inactive state. Though it is least likely to yield viable DNA every attempt needs to be made to attempt DNA extraction from the cast skins. Specimens not required for DNA analysis can be transferred to 70% ethanol, before final curation by an experienced entomologist as per section 4.1.

# 4 IDENTIFICATION

Identification of adults should be reasonably reliable if carried out by a diagnostician experienced in Dermestidae, particularly *Trogoderma* identification. However, morphological characters should be treated with great caution in the case of damaged adult specimens. Most of the Dermestid larvae found in stored product will partially or wholly consume the dead adults. During their feeding activities they prefer to eat or at least severely damage the abdomen with the associated genitalia. In case of stored product movement, particularly cereals, the moving grain will damage the dry dead adults. In most cases the legs and antennae will break off and most of the setae on the elytra and pronotum will be rubbed off.

Morphological taxonomic methods based on keys and descriptions are not entirely adequate for identification of *T. granarium*. Suspected *T. granarium* adults must be pinned by an experienced entomologist.

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.

The descriptions in this NDP are sufficient for tentative morphological identification of adult and larval *T. granarium*, but for reliable species level identification molecular methods should be used, also provided in this Diagnostic Protocol. This protocol includes electron microscope photographs, line drawings and digital images of exotic *Trogoderma* pest species and Dermestids within an Australian context (Figures 1 – 36). It is recommended to be read as a companion protocol to ISPM 27 DP 03: *Trogoderma granarium Everts* (2012). All figures in ISPM 27 are cross referenced in this protocol and presented in Appendix 8.4.

To aid in determining a pathway to identification, a decision matrix is provided below.

**Table 3 Decision matrix for identification of suspect *Trogoderma granarium* based on life stage and quality of material present. Agreement of at least two separate diagnostic methods is required to identify *Trogoderma granarium*.**

| | | **Material type** | | | | | |
|---|---|---|---|---|---|---|---|
| | | **Eggs** | **Larval skin** | **Partial larva** | **Whole larva** | **Partial adult** | **Whole adult** |
| **Diagnostic method** | **Morphological screen** | No | Possibly[a] | Possibly[a] | Recommended | Possibly[a] | Recommended |
| | **Conventional PCR and sequencing** | Possibly | Possibly | Yes | Recommended | Yes | Recommended |
| | **LAMP assay** | Possibly | Possibly | Yes | Yes | Yes | Yes |
| | **Real time PCR** | Possibly | Yes | Yes | Yes | Yes | Yes |

[a] Morphological features of partial larvae and adults may confirm the identification of *T. granarium* if molecular assays have provided a detection and sufficient characters are discernible.

## 4.1 Preparation of specimens for examination

### *4.1.1 Equipment and chemicals required for dissection and mounting*

- Hotplate or preferably dry block heater
- Insect pins
- Micropins 0.015 mm diameter attached to the end of a toothpick with hot-glue, wax or nail polish
- Insect pins (No. 1) and/or micropins with hook or loop end
- 10% KOH and/or NaOH solution
- Test tubes or, if a dry block heater is being used, Eppendorf tubes
- Fine dissection forceps, preferably Dumont jeweller's forceps #4
- Eye surgery scissors
- Cavity microscope slides
- Circular coverslip #1, 19 mm diameter
- Cavity glass block (embryo glass)
- Fine brush
- Distilled water
- Permanent mounting agents such as Canada balsam/ Euparal/ CMCP10 Water based mounting agents like Hoyer's media should be avoided
- 5% Decon 90 solution

### *4.1.2 Preparation of larvae*

Before dissection, the larva should be examined under a stereo microscope. Size, body color, 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.

**Methods**

The preparation method in Hoyer's media described below is recommended for current identification of larvae in phytosanitary laboratories. However, as Hoyer's mountant is water-based, it cannot be considered permanent. Evaporation occurs over time, leaving cavities and the slide will deteriorate. Permanent slides made using CMCP10, Euparal or Canada Balsam for mounting are preferred. Mounting in Canada Balsam requires laborious dehydration technique, but the slide will be of high quality and permanent. Chloral phenol should be avoided, partially due to the lack of availability, toxicity and most importantly, if not washed out properly, in the long run, will destroy the mounted specimen. A supporting *Trogoderma* larvae dissection YouTube video produced by the Department of Primary Industries and Regional Development (DPIRD), Western Australia (WA) is available at http://youtube/lUPHdRN88ss (DPIRD, 2013[a]).

For identification, larvae should be mounted on a microscope slide using the following method:

1. Place the specimen ventral side up on a microscope slide. Cut open the whole body along the mid-line from under the head capsule to the last abdominal segment using eye surgery scissors, to ensure that the specimen can be completely opened up for mounting.

2. Put the larva into a test tube containing 10% potassium hydroxide (KOH) solution and heat either in a boiling water bath or preferably in a dry block heater until larval tissues loosen and begin to separate from the cuticle (using NaOH solution instead of KOH is more time consuming but lessens the possibility of damaging the specimen.)
3. Rinse thoroughly in warm distilled water.
4. Remove all the internal tissues using a very fine, short hair brush or the convex surface of a hooked tip of a No. 1 insect pin, or preferably a loop formed of a micropin (0.015 mm diameter). All setae should be removed from one side of the 7th and 8th abdominal segments. Staining is not necessary but if the specimen is teneral (recently moulted) it may help to make diagnostic features easier to see.
5. Remove head capsule and put it back in the hot KOH solution for 5 minutes.
6. Rinse the head capsule in warm distilled water.
7. Depending on personal preference, the dissection of the head can be performed in a few drops of glycerol on a microscope slide or in water in an excavated glass block (embryo glass). The former gives easier access to the specimen then the latter.
8. Turn the head ventral side up and fix it to the glass with a blunt No. 1 insect pin. Remove the mandibles, maxillae and labial palpi using jeweller's forceps #4, and micropins attached to toothpicks. Remove the epipharynx and antennae,
9. Mount the head capsule and the mandibles in the cavity of the microscope slide
10. 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 coverslip. (If they are mounted in the cavity the parts will rotate rendering the examination difficult or in case of the epipharynx impossible.) All body parts should be mounted on the same microscope slide.
11. In the case of larval exuviae before proceeding with the dissection, the specimen should be soaked in 5% Decon 90 solution 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 media without clearing.
12. Slides should be labelled immediately after mounting the specimen to prevent possible mix-ups. Labels should be written using pencil, pigment pen, Indian ink or printed using laser printer. Fountain pens or Biro should not be used.
13. The slides should be placed in an oven for a few days at 40°C to improve their quality (the best slides are obtained after 2–4 weeks).
14. After drying slides should be ringed using Glyptol, Brunseal, or at least two layers of nail polish in order to prevent the Hoyer's media from drying and possibly damaging the specimen. However, microscope slides may be examined immediately after preparing.

The identification should be performed using a high powered (at least 400x) compound microscope. Depending on the quality of the microscope oil, immersion oil may need to be used to achieve satisfactory resolution.

Note: Banks (1994) recommends placing the larva ventral side up and cutting the head vertically just posterior to the mandibles using a razor blade. Remove the mandibles and the palpi in order to prevent them covering the epipharynx after mounting. Although it is a good way of preparing the larva, if the

blade used is not in perfect condition and the person carrying out the dissection is not experienced enough it is very easy to damage or even destroy the epipharynx.

### *4.1.3 Preparation of adults*

Adult *Trogoderma* specimens may need to be cleaned before identification. If the specimen was caught in a sticky trap the glue can be dissolved using a number of solvents (e.g. Limolene, Citricide, Dissolv-It, baby oil or kerosene). Considering that strong solvents can damage the specimen DNA, at least one (1) insect leg (preferably a middle leg) should be removed and submitted for molecular analysis before the solvent(s) is applied. The solvent and any dirt can be removed by any laboratory detergent, like Decon 90. Dirty specimens can be cleaned using an ultrasonic cleaner.

There are different ways of processing adults. Banks (1994) recommends complete dissection and mounting of the whole adult on a cavity microscope slide. We believe it is not necessary because mounting will obscure important characters e.g. colour of setae. Also placing a cover slip on the microscope slide will inevitably lead to movement, turning and sometimes congregation of the body parts rendering examination of important features very difficult or impossible. A supporting *Trogoderma* adult dissection YouTube video produced by DPIRD is also available at http://youtube/4luzHxTv4PY (DPIRD, 2013[b]).

The procedure for preparation of adults is as follows:

**Methods**

1. Soak adults in warm distilled water for about an hour.
2. Remove abdomen while the specimen is still in the water using fine forceps.
3. Dry the specimen (minus abdomen) and mount it either on the tip of a cardboard triangle or preferably laterally on a cardboard rectangle. On a rectangle the specimen is less exposed therefore less likely to get damaged. Gluing them on the side make the specimens accessible for both dorsal and ventral examination.
4. Cut the abdomen laterally open leaving the last abdominal segment untouched. Place it in 10% KOH or NaOH solution in a hot water bath or preferably in an Eppendorf tube in the dry block heater for about 10 minutes.

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. Usually, the genitalia need to be macerated further in the caustic solution. Experienced entomologists should be able to separate the aedeagus from the periphallic tergite and the 9th abdominal segment using micropins. Staining is not recommended considering how difficult it is to locate small body parts in the stain and the discriminatory features are well visible without staining. In case of teneral specimens, staining appears a viable option, but the DNA identification will be decisive anyway.

Though a permanent mounting agent is an option, genitalia can be mounted on a microscope slide using a water miscible mountant. The preferred method is examining the genitalia 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. Then the microvial can be attached to the pinned body by driving the pin through the stopper of the vial. 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 labeled immediately after mounting the specimen to prevent possible mix-ups. Labels should be written using pencil, pigment pen, Indian ink or printed using a laser printer. Fountain pens or Biro should not be used. The slides should be placed in an oven for a few days at $40^0$ C. After drying all slides mounted using water miscible mountant should be ringed using Glyptol, Brunseal, or at least two layers of nail polish in order to prevent the Hoyer's from drying and possibly damaging the specimen.

## 4.2 Genera of the family Dermestidae frequently occurring in stored commodities and domestic environments

Besides *Trogoderma,* other Dermestid genera may also be found in stored products such as *Anthrenus*, *Anthrenocerus*, *Attagenus*, *Dermestes*, *Phradonoma* and *Reesa*. During the last decade, *Attagenus* and *Phradonoma* species also have been picked up in domestic situations and in the native bush, respectively. The first step of diagnosis of collected specimens is identification to genus. Adults and in some cases larvae of these beetles can be identified using one of the keys of Mound (1989), Kingsolver (1991), Haines (1991), Banks (1994), Rees (2004) and/or Kingsolver (2002). Unfortunately, Háva's (2004) 'Keys to the Genera and subgenera of Dermestidae' contains quite a few factual and observational errors, therefore it needs to be avoided.

The simple keys below should quickly enable *Trogoderma* to be distinguished from four other Dermestid genera commonly found in stored commodities[2]. It should also be noted that other genera of Dermestid beetles may also be present and are known to occur in stores. These genera include *Evorinea, Thorictodes, Neoanthrenus, Thaumaglossa*, *Orphinus* and *Phradonoma* (Delobel and Tran, 1993; Szito Pers Comm). However, stores are not typical habitats for them, so most of them are not included in the above-mentioned keys. Dermestid beetles that commonly occur in Australian stores and households are shown (Figures 25–30). *Trogoderma variabile* has also been found in grain stores throughout Australia (Figures 17–19, 20, 21).

Even experienced diagnosticians should use identification keys including other Dermestidae genera relevant to the given area and the country of origin. If the diagnostic key being used was not specifically written to include area of origin (and interception) of the specimens, the key should be used with caution as there are many genera and species of Dermestidae of limited distribution. Due to their nutritional requirements, they often feed on dead stored product pests. Unfortunately, often the origin of the goods or insects is not known.

### *4.2.1 Differentiation of Dermestidae larvae*

Dermestid larvae may be differentiated using a simple key (Dermestid Larval Key). 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 4.3.

**Dermestid Larval Key**

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

[2] Distinguishing characters are illustrated in Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* [figures], Figures 2–23.

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

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

   Dorsal surface with hastisetae[3], 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 sclerotised 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 3 times as long as wide at the widest point, Anticostal sutures darkly coloured and well-defined across entire tergite ..... ***Anthrenocerus* spp.**

   Second antennal segment not twice as long as last segment, head of hastisetae about 4 times as long as wide at the widest point, Anticostal sutures prominent and tergites strongly sclerotised ..... ***Reesa vespulae*.**

   Second and last antennal segments subequal, head of hastisetae less than three times as long as wide at widest point, Anticostal sutures not always well defined and tergites yellowish to pale brown..... ***Trogoderma* spp.**

### *4.2.2 Differentiation of Dermestidae adults*

Adult Dermestids may be differentiated using a simple key (Dermestid Adult Key). Adult insect specimens identified to the *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 4.3.

**Dermestid Adult Characters**

- 1-13 mm long, oblong to broadly ovate body shape
- Cuticle never metallic, setose, setae often more than one kind, sometimes modified to scales
- Head hypognathous, labrum visible, mandibles short and palpi simple
- Antennae short, 5–11 segmented, usually clubbed, club formed by 1–8 segments, shape often exhibits sexual dimorphism, ultimate segment often enlarged
- Antennae often received into more or less well-defined grooves on the underside of prothorax
- Pronotal hypomera usually concave and often distinctly and sharply defined excavated for the reception of antennae
- Elytra usually entire, covering abdomen, epipleura usually well-defined at base
- Abdomen with five visible sternites
- Fore coxae usually projecting, conical, sometimes globose and their cavities are open behind
- Legs short and more or less closely retracted; posteroventral surface of femora concave for the reception of tibiae concavities often deep and well defined

[3] Appendix 8.3.2 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figure 18A

- Aedeagus modified trilobed type

**Dermestid Adult Key**

1. Median ocellus absent; fore coxae touching at apices .....***Dermestes* spp.**[4]

   Median ocellus present; fore coxae not touching, separated by prosternal process ..... **2**

**2.** Body covered with scale-like setae, antennal cavity filled by antennae, fully visible from anterior view[5] .....***Anthrenus* spp.**[6]

   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, antennae 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.**[7]

**5.** Antennal cavity poorly defined, depression in hypomeron, body elongate relative to width..... ***Reesa vespula***

**6**. Antennal cavity carinate posteriorly, carina does not reach lateral margin of pronotum, posterior margin of hind coxa straight, arcuate or sinuate, first segment of posterior tarsus longer than second segment..... ***Trogoderma* spp.**[8]

## 4.3 Differentiation of various native and exotic pest *Trogoderma* species from *Trogoderma granarium*

### *4.3.1 Identification of Trogoderma larvae*

There is no published key that covers all *Trogoderma* species. Partially because all existing keys encompass the fauna of a geographical unit or includes only the economically important species. In addition, there are still many undescribed species. Several keys have been published for the economically important species. Kingsolver (1991) and Barak (1995) published keys to adult and larvae of some Dermestid beetles, including a few *Trogoderma* species. Banks (1994) published a key to adults and larvae of the genus *Trogoderma* associated with stored products, as well as keys to larvae of 14 species of *Trogoderma* from different parts of the world, including stored product pests. He has also placed his work in an Australian context; hence it is a very valuable tool. Mitsui (1967) published illustrated keys for identification of larvae and adults of some Japanese *Trogoderma* species. Zhang *et al.* (2007) published a key for identification of eight economically important species in the genus

[4] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figure 15
[5] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figure 14A
[6] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figure 17
[7] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figure 16
[8] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figures 2A, 4A, 14B

*Trogoderma*. Some native Australian *Trogoderma* species collected in and around Australian grain storage facilities are presented in Figures 31–36.

**Quick screening identifiers of *Trogoderma* larvae**

1. elongate, cylindrical, hairy larvae
2. hastisetae present on sclerotized part of tergites
3. pretarsal setae on the ventral side of claws unequal
4. antennal segments are subequal
5. antecostal sutures not well defined and acrotergites with numerous small spicisetae

If all of the listed features are present for the larva or larval skin it is very likely that the specimen is a *Trogoderma* species therefore it is warranted to check the detailed list of features below.

### *4.3.2 Discriminating features of Trogoderma larvae*

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

1) body elongated, cylindrical, somewhat flattened, roughly six times as long as wide, nearly parallel-sided but gradually tapering toward rear
2) head well developed, sclerotised, 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 type of setae spicisetae, hastisetae and/or fiscisetae[9]
6) head of hastisetae not more than three times longer than wide[10]
7) numerous hastisetae on all nota and tergites 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 sclerotised part of tergites 5, 6 and 7)
8) urogomphi absent.

Larval identification based on morphological features only should be considered unreliable. This is because in some species the intraspecific variation is such that it is overlapping to other species. In addition, large numbers of non-pest Dermestidae and more specifically *Trogoderma* species may occur in stored commodities and many of their characteristics are not well studied or not known at all.

### *4.3.3 Discriminating features of* Trogoderma granarium *larvae*

1) antennal segments subequal
2) setae of basal antennal segments almost completely encircling the segment, reaching or surpassing apex of second segment, at least three-fourth as long as second antennal segment
3) second antennal segment of last instar usually with one seta or sometimes no setae
4) last antennal segment with at least one sensory pore in basal quarter

[9] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figures 18 and 20
[10] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figure 20

5) epipharynx[11] with four papillae in distal sensory cup, usually in a single unit[12]
6) fiscisetae absent
7) mesally directed tergal setae absent
8) at least six small spicisetae on first abdominal tergite, posterior to antecostal suture, anterior to large spicisetae
9) anterior-median small spicisetae anterior to antecostal suture not extending over the suture
10) large median spicisetae on first abdominal segment smooth or covered with inconspicuous scales, tips smooth for at least four times the diameter of seta
11) 7th abdominal tergite with suture faint or interrupted
12) antecostal suture of 8th abdominal tergite almost always absent, but if present, faint and interrupted
13) no greyish pigmentation (pigmentation may be very vague) on sides of thoracic and other segments, not even at the base of large lateral spicisetae.

### *4.3.4 Identification key for* Trogoderma granarium *larvae*

Larvae of *T. granarium*[13] may be separated from other *Trogoderma* species occurring in stores using the following short key. This key does not allow for identification of all *Trogoderma* species known to occur in stores. 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).

1. Epipharynx with 4 distal papillae, usually in a single sensory cup[12] ..... **2**

   Epipharynx with 6 distal papillae in a distal sensory cup; sometimes one or two papillae outside or in a separate sensory cup[14] ..... **3**

2. Tergites uniformly yellowish-brown, without greyish pigmentation at base of large spicisetae; acrotergites weakly sclerotised; antecostal sutura on 8th abdominal segment almost always absent if present then faint and usually interrupted; apical antennal segment with sensory pores(s) in basal quarter, antennae usually with single setae on second antennal segments, setae on basal antennal segment almost completely encircling segment; hastisetae morphology as in[15] ..... ***Trogoderma granarium*** **Everts**

   Tergites usually dark greyish-brown, at least at base of major spicisetae; acrotergites brownish, sclerotised; antecostal sutura on 8th abdominal segment distinct; second antennal segment without setae; hastisetae morphology as in[16] ..... ***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[17]; compare

[11] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figure 22
[12] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figure 23A
[13] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figures 2C, 2D, 21
[14] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figure 23B, C
[15] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figure 20A, B
[16] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figure 20C, D
[17] Appendix 8.4 Annex X to ISPM 27:2006 *Trogoderma granarium* Everts [figures], Figure 19C

with[18]; hastisetae[19] very sparse on thoracic and anterior abdominal tergites[20]; tergites with single row of large spicisetae[21].....***Trogoderma variabile* Ballion**

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

### *4.3.5 Identification of Trogoderma adults*

**Quick screening identifiers of *Trogoderma* adults**

1. median ocellus present;
2. antennal cavity well defined by a posterior carina and open laterally;
3. antennal outline smooth, antennal club at least three segmented;
4. body setose, covered in two or often three different type of hairs (long, coarse, semierect; short coarse, semierect or decumbent; long or short but soft; often yellowish-white, flattened (ensiform) setae also present particularly in the exotic pests (Figure 3). If there are lighter coloured patterns including bands present in the elytral integument, then the arrangement of different setae usually follow them, particularly the ensiform setae. In *T. granarium*, the banding is very vague giving a mottled appearance. In exotic *Trogoderma* the elytra usually have three transverse bands of pale (ensiform) setae. Please, note; setae of dead adults are often rubbed off.

If all of the above listed features can be observed on the adult beetle it is very likely that the specimen is a *Trogoderma* species therefore it is warranted to check the detailed list of features listed below.

### *4.3.6 Discriminating features of Trogoderma adults*

Adapted from Banks (1994), Beal (1954, 1960), Haines (1991), Hinton (1945), Kingsolver (1991), Lawrence & Britton (1991, 1994), Lawrence *et al*. (1999b), Okumura and Blanc (1955) and Peacock (1993).

1) body ovate, densely setose, setae simple, usually 2–3 different types, recumbent, yellowish-white slightly flattened, (ensiform) setae often present, lighter pattern in elytral integument often present
2) median ocellus present
3) pronotum without lateral carina
4) antennal cavity of antero-ventral surface not, or slightly visible in anterior view[22]
5) antennal cavity carinate posteriorly at least to half but usually at least to three quarters of length and open laterally
6) prosternum well developed. forming a "collar" anteriorly that covers or abuts against mouth parts when head is retracted
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

[18] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figure 19D
[19] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figure 20E, F
[20] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figure 19A
[21] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figure 19B
[22] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figure 14B

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.

### *4.3.7 Discriminating features of* Trogoderma granarium *adults*

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 sometimes appears uncoloured, light brown or reddish brown, almost always vaguely mottled without a clearly defined pattern (Figure 11)

2) elytral setae predominantly brown; yellowish or white (Figures 11, 12, 14), ensiform setae forming no clearly defined banded pattern may also be present; these hairs are gradually rubbed off as the beetle moves around and the adult develops a shiny appearance

3) antennae with 9–11 segments; male antennal club with 4–5 segments (Figure 4); female antennal club with 3–4 segments (Figure 3)

4) inner eye margin straight or sinuate

5) metacoxa narrowing gradually

6) post-coxal lines absent

7) posterior margin of terminal abdominal ventrite smooth

8) anteromedial metasternal process rounded (rarely rudimentary nipple present)

9) 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

10) serrate sclerites of bursa copulatrix (Figure 8, 9A) of female small, not longer than corrugated part of spermatheca, with 10–15 teeth

11) male genitalia with bridge straight, and evenly wide, broader at connections to the parameres (Figures 8, 10A, 16).

### *4.3.8 Identification key for* Trogoderma granarium *adults*

The following short key should be used to distinguish adult *T. granarium* from some other *Trogoderma* species frequently occurring in stored commodities. If necessary, other species can be identified with the keys of Beal (1954, 1956), Banks (1994), Kingsolver (1991) and Mordkovich & Sokolov (1999). These keys include species occurring in stored products and therefore should be used for identification of *Trogoderma* adults detected in imported consignments.

In these circumstances, Mordkovich & Sokolov (1999) mention other *Trogoderma* species that may be found in stored products. Among them *T. longisetosum* Chao & Lee (1966) has been noted as a stored product pest in China. It is very similar to *T. glabrum.* Some tropical *Trogoderma* species may be present in stored products, such as *Trogoderma cavum* Beal, described by Beal (1982) after examination of specimens infesting stored rice in Bolivia. Delobel & Tran (1993) provide an account of tropical species. Diagnostic images of adult *Trogoderma* are illustrated as follows: *T. granarium* (Figures 1, 11–15), *T. variabile* (Figures 17–19) and *T. glabrum* (Figures 2, 22 –24).

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

   Dorsal pubescence of whitish, ensiform setae in addition to yellowish and reddish-brown setae their arrangement usually follows the elytral patterns ..... **2**

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

   Elytral integument with well-defined lighter and darker areas[23]..... **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; setae on 5th sternite of males uniform, recumbent setae..... ***Trogoderma glabrum* (Herbst)[24]**

   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; Metacoxa narrowing gradually; Post-coxal lines absent; Posterior margin of terminal abdominal ventrite smooth ..... ***Trogoderma granarium* Everts**

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

   Elytral integument without a loop, distinct bands and/or spots present..... **7**

5. Anterior margin of eyes distinctly emarginate..... ***Trogoderma inclusum* LeConte[25]**

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

6. Basal loop, if present, never connected to the antemedian band ..... ***Trogoderma variabile* Ballion[26]**

   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)[27], *T. simplex* Jayne[28], *T. sternale* Jayne[29], *T. versicolor* (Creutzer)[30]**

7. Elytral integument with three well-defined (basal, submedian and apical) fasciae, setae on fasciae largely white, ensiform with very little yellowish recumbent setae ..... ***Trogoderma angustum* (Solier)[31]**

   Elytral integument with well-defined basal band and median spot..... ***Trogoderma variabile* Ballion (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

[23] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figure 3
[24] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figure 6B
[25] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figure 6D
[26] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figures 4A–4C, 5, 6H
[27] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figure 6E
[28] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figure 6F
[29] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figure 6G
[30] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figure 6I
[31] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figure 6A

the basal loop is indicated by curved anterior band, antemedian and/or median bands by small spots, and apical spots are usually missing.

In the case of abraded or damaged specimens genital dissections should be carried out. This is recommended even on undamaged specimens. The reason is that there is a large number of undescribed *Trogoderma* species and by examining the genitals, the chances of misidentifications can be significantly reduced.

Matveeva (2001) provides additional discriminating features for separation of adults of *Trogoderma granarium* from *T. variabile* and *T. glabrum.* These species are frequently found in stored products imported to Russia. These supplementary characters concern size and morphology of hide wings and can be useful for the identification of damaged specimens and help to increase the certainty of identification based on other features[32]. During dissection hind wings should be removed and mounted in glycerol or Hoyer's.

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 (unfortunately this character might be useful only in case of mixed infestation); number of setae S1 on costal vein (mean = 10) is half the number of setae on *T. variabile* and *T. glabrum* (mean = 20–23); number of small setae S2 between costal vein and pterostigma less (mean = 2, sometimes absent) is less than that for *T. variabile* and *T. glabrum* (mean = 8)[31].

For positive identification all (in case of damaged specimens most) of the features should be observed. Often the material that the discriminating features are based on have come from a limited source, therefore they may not reflect the whole range of intraspecific variation. Features evaluated against *Trogoderma* species from one restricted geographic area may not be proven to be useful on species from other areas. Examination of the genitalia should make identification more reliable without using additional, less understood features.

## 4.4 Detailed description of *Trogoderma granarium*

### *4.4.1 Immature stages*

**Eggs**

Initially milky-white, later pale-yellowish; typically cylindrical, 0.7 mm long and 0.25 mm broad; one end rounded, the other more pointed and bearing a number of spine-like projections, broader at the base and tapering distally. Eggs laid loosely and singly in the host material.

**Larvae**

Total length of the first-instar larva[33] is 1.6–1.8 mm, a little more than half of its length constituted by a long tail, made up of a number of hairs borne on the last abdominal segment. Body width is 0.25–0.3 mm, and colour uniformly yellowish-white, head and body hairs are reddish-brown. The head is as long as the combined lengths of four of the preceding segments. Simple hairs (spicisetae) are scattered over the dorsal surface of the head and body segments. The tail consists of two groups of long simple hairs, borne on the 9th abdominal segment. Barbed hairs (hastisetae) are found in pairs of tufts, borne on abdominal tergites. As the larva increases in size, the colour changes progressively from the pale

[32] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figures 9, 10
[33] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figure 2C

yellowish-white of the first-instar larva to a golden or reddish-brown. The density of the body hairs increases but the tail become much shorter in proportion to the length and width of the larval body, and in the 4th instar the hairs give the appearance of four dark transverse bands. The mature larva[34] is approximately 6–8 mm in length and 1.5 mm in width

For identification purposes final instar larvae should be used where possible because the important features are most expressed in this stage. Also, in case of early instars, the extent of intra and inter specific variation of external features is not known. To make the necessary features clearly visible, the specimen needs to be dissected and mounted on a microscope slide using Hoyer's, Euparal, or Canada balsam. Hoyer's has the great advantage that the specimen can be remounted should reorientation become necessary. The disadvantage is that the slides need to be sealed otherwise air gets under the cover slip making remounting necessary. The other mounting agents are permanent rendering remounting very difficult.

Morphologically, the mature larva of *T. granarium* can be separated with reasonable confidence from *Anthrenus* spp., *Anthrenocerus* spp. and other pestiferous exotic *Trogoderma* species though for conclusive identification adult genital dissection is necessary.

**Useful Larval Characters**

Antennae: Antennal segments are approximately the same length; setae of basal segment almost completely encircling the segment and extending beyond of the distal end of second antennal segment when antenna fully stretched; second antennal segment often with a single seta, last antennal segment with two sensory pores which usually located together in the basal quarter, or often one in the basal quarter and the other is in the basal third-half.

Epipharynx: Middle setal series of epipharyngeal margin consisting of two broad inner setae and two narrow outer setae, about half as wide as inner setae, inner setae often with longitudinal grooves on apical half; epipharyngeal sensory cup with 4 papillae. *T. granarium* shares this important feature with *Trogoderma glabrum* and possibly numerous other non-pestiferous *Trogoderma* species which larval characters are still not studied.

Legs: Three paired legs; coxae without dark pigmentation; pretarsal segment (claw) with two setae on inner surface, the longer is about twice as long as the shorter.

Setae: There are three different kinds of setae arising from tergites: hastisetae, small spicisetae and large spicisetae. Hastisetae bear many stiff, upwardly directed processes on the shaft, ending in a spearhead[35]. Head of hastiseta no more than 3 times longer than wide at the widest point. Spicisetae are simple setae with a smooth or reticulated surface[36]. Arrangement of setae on body: small spicisetae are present in front of antecostal suture; at least 6 small spicisetae present on first abdominal segment posterior to antecostal sutura, anterior to band of large spicisetae; anteriormost median small spicisetae in front of antecostal sutura, not long enough to extend over the sutura. Tufts of hastisetae arising on the sclerotised surface of caudal segments; hastisetae not convergent over the cauda.

Tergites: Yellowish-brown to medium-brown. Abdominal tergites each bearing a transverse line (antecostal sutura) near the anterior margin of the sclerotised area. Antecostal sutura is faint, or

[34] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figure 2D
[35] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figure 18A
[36] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figure 18B

interrupted on 7th abdominal tergite; very faint, incomplete, or absent on 8th abdominal segment. No urogomphi on 9th abdominal segment.

**Pupa**

Last instar larva pupate in the last larval exuvia. No key to pupae exists though the outline of sclerotised edges of the gin trap and the arrangement and number of setae of the central gin trap on the abdominal segments ought to be species specific (Beal 1954; Banks 1994).

### *4.4.2 Description of Trogoderma adults*

The adult stage of *T. granarium* is illustrated in Figures 1, 11–15 and also in Appendix 8.4[37]

**Adult male**

Body: Length 1.4–2.3 mm width 0.75–1.1 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 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 more dense on ventrites, setae fine, short, decumbent, yellowish-brown (Figures 11, 14, 15).

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 (Figure 5). 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 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, more dense laterally, on disc separated by 2–4 diameter, laterally by 1–2 diameter.

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 detail see Appendix 8.4[38]).

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.

[37] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figures 2A, B
[38] Appendix 8.4 Annex X to ISPM 27:2006, *Trogoderma granarium* Everts [figures], Figure 9

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, median bridge between parameres straight distally and proximally; width at point of crossing of aedeagus is as wide, or wider than the aedeagus; 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.

Head: antenna sometimes less than 11-segmented, antennal club 3-4 segmented (Figure 4).

Abdomen: 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.

## 4.5 Likelihood of morphological misidentification

The *Trogoderma* genus has a chequered history worldwide. Most of the Western Palearctic species are described although many immature stages are still unknown. Similarly, the North American species are well studied though still quite a few questions remain unanswered. The genus worldwide has received little attention in spite of the great economic significance of *T. granarium* Everts and *T. variabile* Ballion though the other Dermestid genera has been studied and published on primarily by Hava J., Herrmann, A., Kadej M. and others. Unfortunately, most of these publications resort to species descriptions without placement in faunistic or more importantly taxonomic context. Also, they usually appear scattered in various obscure journals often extremely difficult to get. In spite of their efforts, there are still numerous undescribed Dermestidae and particularly *Trogoderma* species worldwide. Potentially any of them could turn up in stores and import shipments and some of them may even become a stored product pest.

In Australia, there are 52 described *Trogoderma* species, not taking into account the likely synonyms. There are an estimated at least 50 more undescribed species. Rarely, native Australian *Trogoderma* species can get into stored products and lay their eggs. The emerging larvae most likely feed on dead insects but it is very unlikely that they can develop multi-generational population.

*Trogoderma* larvae are difficult to identify. There are many undescribed species worldwide and very few of the immature stages of described species are known. Also, the intraspecific variation of well-known pest species is poorly studied.

There are a few identification keys to identify the pestiferous *Trogoderma* from the harmless species, but these keys are either not comprehensive or do not take into account the poorly studied native species or both, for example, Banks 1994, Beale 1954, 1960 and Peacock, 1993. The regional keys are often outdated, for example the last (not comprehensive) key to Australian *Trogoderma* was produced by Armstrong 1945.

Unfortunately, the more easily identifiable adults rarely occur in stores because they are short lived and the larvae have the tendency to partially devour the dead adults. Movement of the infested commodity will seriously damage the dead adults by breaking off limbs and rubbing off setae. Even the colour of the integument can change depending on temperature and humidity changes and due to the time, the specimen has spent in the commodity before being found.

## 4.6 Molecular detection and identification of *Trogoderma granarium* by DNA sequencing, LAMP, and real-time PCR

DNA-based methods for detecting and identifying *Trogoderma granarium* are sensitive and specific. 'Universal' conventional (end-point) PCR assays are described which allow for the confirmation of identification via Sanger sequencing. PCR using primers designed for improved specificity to *T. granarium* are also described, to improve PCR amplifications from Khapra beetle and minimise amplifications from other species and/or DNA contaminants. Loop-mediated isothermal amplification (LAMP) (Rako *et al.* 2021) is a sensitive and specific test that can be deployed as a field diagnostic. Real-time PCR (qPCR) is a sensitive molecular tool that allows the detection and quantification of specific gene sequences and is routinely performed in diagnostic molecular laboratories. The primary real-time PCR methods described below are for the species-specific detection of *T. granarium*. Finally, a multiplex real-time PCR assay is included for the detection and differentiation of *T. granarium* and *Trogoderma variabile* (warehouse beetle).

The conventional PCR and sequencing approach is on par with morphology for confirming identification and can most easily be implemented straight after a morphological identification to provide confirmation. LAMP and real-time PCR assays provide alternative options that may be more useful if the quantity or quality of material available is inadequate or if a faster identification is required. Indeterminate results from an assay are often resolved via repeat testing. If definitive results are still not obtained, attempt to resolve using a different method from within this protocol as all lines of evidence will support or clarify each other. This can include morphology, conventional PCR and Sanger sequencing, LAMP or real-time PCR assays.

A list of equipment and reagents used for diagnostics is presented in Appendix 8.5.

**Conventional PCR**

1. Conventional **Simon PCRs** (Simon *et al*. 1994) for the universal amplification and sequencing of the mitochondrial COI -3' gene region.
2. Conventional PCRs using specific primers (Gopurenko, *in prep.*) for targeted amplification and sequencing of *T. granarium* mitochondrial $tRNA^{Tyr}$ – COI -5' gene regions.
3. Conventional – **16SAr PCR** (Simon 1994, Cognato & Volger 2001, Olson *et al.* 2014) for the amplification and sequencing of arthropod mitochondrial 16S rRNA gene.

**Loop-mediated isothermal amplification (LAMP)**

4. Loop-mediated isothermal amplification (LAMP) assay (Rako *et al*. 2021) for the detection of *T. granarium* specific mitochondrial 16S ribosomal RNA (16S rRNA) gene.

**Real-time PCR**

5. Modified Multiplex real-time PCR based on the **Furui qPCR protocol** (Furui *et al*. 2019) for the detection of *T. granarium* specific mitochondrial DNA based on various NADH dehydrogenase subunit genes (ND2 and ND6).
6. A **16S real-time PCR** protocol developed by SARDI based on the Olson qPCR protocol (Olson *et al*. 2014) for the detection of *T. granarium* specific mitochondrial 16S ribosomal RNA (16S rRNA) gene.
7. Multiplex **TgTv real-time PCR** (DDLS WA in-house assay) for the simultaneous identification and differentiation of *T. granarium* and *T. variabile* via the mitochondrial COI gene.

Note: All real-time PCR methods should include a method to quality control the DNA extraction process.

### *4.6.1 DNA extraction*

DNA extraction may be conducted destructively (i.e., the specimen is destroyed to obtain the DNA) or non-destructively (the specimen is morphologically preserved). Non-destructive DNA extraction is often preferred, especially when dealing with limited specimens, as this preserves morphological characters for post-extraction examination.

Destructive DNA extraction protocols may, however, be preferred, such as when establishing reference *T. granarium* DNA from specimens that can be readily sacrificed. Destructive DNA extraction may be preferred in these instances, as it likely yields greater quantities of genomic DNA. The primary difference between non-destructive and destructive protocols is a prior step in the latter protocol that requires physical destruction of the specimen prior to undertaking DNA extraction.

Non-destructive protocols are mostly described below but are equally applicable to destructive protocols.

**Non-destructive DNA extraction protocols**

Listed below are a suite of applicable DNA extraction protocols that retain specimens as morphologically intact. Each method is discussed in terms of sensitivity, nature of target specimen (fresh whole specimen, fragments of aged specimen, degraded larval skin), suitability for downstream diagnostic protocols, and practicality (ease of use, speed, cost and long- term storage).

**HotShot4 extraction method - Adapted from Zieritz *et al*. (2018)**

1. **Prepare HS4 Lysis Buffer** (25 mM NaOH and 0.2 mM EDTA); to make 10 mL, in a 15 mL Falcon tube, add:
   250 µL 1M NaOH,
   4 µL 0.5M EDTA
   9.746 mL sterile $H_2O$
2. **Prepare Neutralising Solution** (40 mM Tris-HCl); to make 10 mL: in a 15 mL Falcon tube add:
   400 µL 1M Tris-HCl
   9.6 mL sterile $H_2O$.
3. Use a "single-use" disposable toothpick or sterile pipette tip (e.g., 200 µL tip) to transfer the specimen to a PCR strip well, taking care to avoid cross contamination between each specimen by using a new toothpick or tip for each specimen. Add one specimen per well.

4. If a specimen has been preserved in ethanol, care must be taken to remove excess ethanol. Residual ethanol may be removed by placing the specimen on a tissue paper before transferring to PCR strip. Complete dryness sometimes leads specimens to 'fly' out of the wells because of static electricity; take additional care.
5. Pipette 25 µL of HotShot (HS4) lysis solution into PCR strip wells having the specimen.
6. Incubate samples in a PCR machine at 95°C for 10 min, then hold at 4°C for 5 min.
7. Remove sample from the thermocycler and incubate the PCR strip on ice for approximately 1 min then neutralise with 25 µL of HS4 neutralisation solution.
8. Store DNA on ice or at -20°C until required.

**Notes:**

- Highly suitable for any kind of insect tissue ranging from whole fresh specimen to degraded larval skin. Less time consuming yet yielding amplifiable DNA suitable for all downstream diagnostic process and long-term storage (at least one year).
- This extraction protocol can be undertaken in any standard PCR machine or Genie.
- The volume of lysis buffer and neutralisation solution may be altered depending on the amount of tissue used in DNA extraction. Use these solutions in 1:1 ratio. The incubation time may also be extended up to 30 min in case of larger chitinous specimens from which DNA extraction may take more time, such as whole beetles; specimen morphology has been found to remain intact following up to 12 h of lysis extraction (Porco *et al*. 2010).
- In case of trap-catch beetles, the presence of oily substances in the tissue and subsequently in the extracted DNA interferes with PCR. This can be minimised by using DNA diluted 1:10.

**Quick Extract (QE) extraction method**

1. This involves use of commercial **QuickExtract** DNA extraction 50 mL solution 1.0 (Epicentre, USA), distributed by Astral Scientific; this product is to be stored at -20°C.
2. To avoid contamination, it is recommended to pre-prepare batches of working solutions, by pipetting 1 mL of QE solution into 1.5 mL Eppendorf tubes and freezing them at -20°C until required.
3. Use a "single-use" disposable toothpick or sterile pipette tip (e.g., 200 µL tip) to transfer the specimen to a PCR/Genie strip tube, taking care to avoid cross contamination between each specimen by using a new toothpick or tip for each specimen. Add one specimen per well.
4. If a specimen has been preserved in ethanol, care must be taken to remove excess ethanol. Residual ethanol may be removed by placing the specimen on a tissue paper before transferring to PCR strip. Complete dryness sometimes leads specimens to 'fly' out of the wells because of static electricity; take additional care.
5. Pipette 25 µL of the QuickExtract solution into each well containing a specimen.
6. Incubate samples at 65°C for 6 min, followed by 98°C for 2 min, using either a standard PCR machine or similar (e.g., Genie machine).
7. After removing the samples from Thermocycler, keep on ice for approximately 1 min and then store DNA at -20°C until required.

**Notes:**

- Suitable for qPCR, but DNA yields from degraded larval skin may not be sufficient for LAMP assay.
- DNA quality may significantly degrade after a week of storage, even at -20°C.
- This extraction protocol may be especially well-suited to high throughput processing of surveillance samples at a collection site.

**Alkaline lysis KOH extraction method**

1. This protocol lyses specimens using a solution of 0.3M KOH.
   Use a "single-use" disposable toothpick or sterile pipette tip (e.g., 200 µL tip) to transfer the specimen to the PCR/Genie strip, taking care to avoid cross contamination between each specimen by using a new toothpick or tip for each specimen. Add one specimen per well.
2. If a specimen has been preserved in ethanol, care must be taken to remove excess ethanol. Residual ethanol may be removed by placing the specimen on a tissue paper before transferring to PCR strip. Complete dryness sometimes leads specimens to 'fly' out of the wells because of static electricity; take additional care.
3. Pipette 25 µL of 0.3M KOH solution into each well each well containing a specimen.
4. Incubate samples at 95°C for 5 min using either a standard PCR machine or similar (e.g., Genie machine).
5. After removing the samples from Thermocycler, keep on ice for approximately 1 min and then store DNA at -20°C until required.

**Notes:**

- Stock solution of 1M KOH (5.6 gm KOH pellets dissolved in 100 mL of $H_2O$) can be stored long term; however, it is recommended to prepare fresh 0.3M KOH for each use (to prepare 1mL, add 700 µL of $H_2O$ to 300 µL of 1M KOH).
- Alkaline lysis KOH extraction is suitable for a LAMP assay, but not as well suited for other PCR-based diagnostics. The alkalinity of extracted DNA solution may interfere with PCR.
- This extraction protocol may be especially well-suited to high throughput processing of surveillance samples at a collection site.

**Spin column extraction method (specified here for Qiagen kits. If using other kits from other spin column manufacturers refer to their manual for DNA extraction process)**

1. For specimens preserved in ethanol discard the supernatant and with an open cap allow the residual ethanol to air dry for 10–15 min.
2. Place the entire larvae, adult or skin cast in a 1.5mL mct containing 180 µL ATL buffer and 20 µL Proteinase K (larvae may be 'punctured' with a fine gauge syringe to aid extraction) and incubate at 56°C with gentle agitation for at least 1 hr (can be left overnight). For non-destructive extraction, following incubation remove the specimen and rinse with Phosphate Buffer Saline and preserve with 100% ethanol in a 1.5 mL mct (Proceed to step 3). The lysate without the specimen is subsequently processed, thus preventing the damage to the specimen.

3. A) If using the automated Qiacube platform to complete the extraction process transfer the sample lysates to the deck and run the DNeasy Tissue Protocol selecting the 200 µL elution option, or;

   B) Follow the manufacturers protocol starting from the first step post 56°C incubation. The final elution step can vary with 30-200µL of elution buffer depending on amount and nature of tissue used in the extraction.

**Destructive DNA extraction protocols**

1. Destructive protocols are primarily applied when using column-based DNA extraction methods; these result in the complete maceration of the sample that can no longer be used for morphological diagnostics. These protocols yield high-volume 'clean' DNA suitable for all downstream protocols. The primary drawback of using column-based protocols is that they are more expensive and time consuming. Follow the manufacturers protocol for any of the commercially available DNA extraction kit listed below.

   - Qiagen DNeasy® Tissue Kit
   - Sigma GenElute™ Mammalian Genomic DNA Extraction Kit
   - Bioline ISOLATE II Genomic DNA Kit

2. Use column-based DNA extraction protocols when establishing lab specific positive controls for *T. granarium* from specimens that are not required to be kept intact.

### *4.6.2 Universal conventional PCR and Sanger sequencing*

The following PCRs include 'universal' insect primer sets (Simon *et al*. 1994 and Cognato & Volger 2001) and a new primer set (Gopurenko, *in prep.*) specific to *T. granarium* and some Dermestids, for anneal to sites flanking polymorphic nucleotide regions which in combination with Sanger sequencing can identify a specimen to species level (or at a minimum determine the level of similarity to *T. granarium*). Commonly used universal primers for barcoding (e.g. mtCOI-5';LCO1490/HCO2198) are not recommended due to lack of sensitivity (mismatches in primer binding sites); however, if they produce a viable sequence product they may be used.

The Gopurenko and Simon PCRs amplify adjunct regions of the mitochondrial COI gene whereas the modified Cognato & Volger (2001) 16SAr PCR amplifies a region of the 16S mitochondrial gene. For this reason, it is useful for resolving indeterminate qPCR results and confirming positives. Sequencing of this region also allows *T. granarium* specimens to be haplotyped which could provide useful information in the event of an incursion and subsequent trace-back investigation. Olson (2014) identified three discreet *T. granarium* haplotypes and this has been corroborated during the validation/verification of this protocol (reference sequences for haplotypes are in GenBank as KJ930433, KJ930431 and KJ930432).

Sequencing of the COI gene provides additional confidence especially when dealing with a potentially undescribed species, and the greater diversity of *T. granarium* COI haplotypes available at public sequence repositories may also be informative for trace-back analyses. Two COI PCR options are provided as both primer sets may not amplify all specimens and sequence information for some species may be incomplete or absent on GenBank (http://www.ncbi.nlm.nih.gov) and or BOLD (http://www.boldsystems.org). The Gopurenko (*in prep.*) primer set targeted to $tRNA^{Tyr}$ – COI 5' gene region, is designed for improved amplification from *T. granarium* (and some Dermestids) while minimising amplification from non-target species and contaminants. The universal Simon *et al*. (1994)

primer set targeted to the COI 3' gene region, encompasses the (section 4.6.7) Multiplex *T. granarium/T. variabile* qPCR primers, and will prove useful for sequence verification of qPCR results of that target region.

**Primers**

| Primer | Sequence | Gene | Annealing temp. (°C)[a] | PCR Size (bp) | Reference |
|---|---|---|---|---|---|
| Trog-tRNA-TyrF (fwd) | 5'- CAA TCT AGC GCC TAA ACT CAG CC -3' | tRNA$^{Tyr}$ – COI 5' | 49 °C | 742 | Gopurenko (in prep.) |
| Trog-JerR (rev) | 5'- TGT CCA AAR AAY CAR AAY ARR TGT TG -3' | | | | |
| C1-J-2183 (fwd) | 5'- CAA CAT TTA TTT TGA TTT GG -3' | COI 3' | 48 °C | ~883 | Simon *et al.* 1994 |
| TL2-N-3014 (rev) | 5'- TCC AAT GCA CTA ATC TGC CAT ATT A -3' | | | | |
| LR-J-12961 (fwd) | 5'- TTT AAT CCA ACA TCG AGG -3' | 16S | 42 °C | ~480 | Simon *et al.* 1994 and Cognato & Volger 2001 |
| LR-N-13398 (rev) | 5'- CGC CTG TTT AAC AAA AAC AT -3' | | | | |

[a] Recommended annealing temperature. In-house optimisation may be required.

**PCR controls**

1. Positive control – *T. granarium* DNA for the Gopurenko primer set, any arthropod DNA extract for other primer sets.
2. No template control – An aliquot of the PCR master mix without template DNA.
3. Process extraction control – Extraction reagents containing nil sample that have gone through the extraction process.

**Preparation of master mix and template addition**

| Reagent | 1 x 25 µL reaction | Final concentration |
|---|---|---|
| 2× commercial master mix | 12.5 µL | x1 |
| Forward primer (50 µM) | 0.25 µL | 0.5 µM |

| | | |
|---|---|---|
| Reverse primer (50 µM) | 0.25 µL | 0.5 µM |
| Nuclease free water | 7 µL | n/a |
| DNA template | 5 µL | n/a |

The same master mix primer concentrations can be used for all three conventional assays. Add 5 µL of extracted DNA to freshly prepared master mix.

**PCR cycling conditions**

| **Step** | **Temperature** | **Duration** | **Cycles** |
|---|---|---|---|
| Initial denaturation | 95 °C | 15 min | 1 |
| Denaturation | 94 °C | 30 sec | 32 |
| Annealing | See primer table above | 30 sec | |
| Extension | 72 °C | 60 sec | |

The same cycling conditions can be used for all three conventional assays. Annealing temperatures, cycling times and the number of cycles can be optimised in-house for different specimen types and PCR platforms.

**Gel electrophoresis**

Conventional PCR products can be visualised on an agarose gel using standard laboratory gel electrophoresis techniques. Briefly;

1. Load 5 µL of PCR product mixed with 5 µL of 2x loading buffer on a 2% agarose gel (containing a suitable fluorescent dye e.g. SYBR Safe) in 0.5x TBE buffer.
2. Load a suitable molecular weight marker (e.g. GeneWorks DMW-100L) on to the gel.
3. Run the gel at 90 volts for 60 min.
4. Visualise gel on a UV transilluminator.

**Results/Interpretation**

**Successful amplification** – Amplification of a PCR product (single band) of the expected size (molecular weight) – see Primers section. Move on to sequencing of PCR product section below.

**Unsuccessful amplification** – No amplification visualised.

**Invalid** – Failure of one or more controls OR multiple bands – Repeat PCR.

**Sequencing of PCR products**

Conventional PCR products require sequencing to confirm the identity of suspect specimens. It is recommended the products are sequenced in both directions using the forward and reverse PCR primers.

Sequences can be quality trimmed and aligned/assembled using sequence analysis software such as Geneious (Biomatters). GenBank accession NC_053875.1 (*Trogoderma granarium* complete mitochondrial genome) allows an alignment reference to each of the target regions reported in this section. Consensus sequences can also be compared against additional reference sequences in GenBank using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and or using the BOLD systems Identification Engine (http://www.boldsystems.org/index.php/IDS_OpenIdEngine) for comparisons against a greater diversity of vouchered COI sequences. Maximum intraspecific sequence difference among public accessions of *T. granarium* on BOLD and/or GenBank is less than 1.45% at COI and 2.33% at 16S (last searched 19 Nov. 2021). The nearest genetically matched sibling species to *T. granarium* is *T. glabrum*: these species differ by at least 11.04% at COI and 5.57% at 16S. As there is no overlap between the maximum sequence difference observed in *T. granarium*, and the minimum difference to its nearest sibling species (*T. glabrum*), sequences of either of these loci from test specimens that match (or are most similar to) reported *T. granarium* accessions can be confidently used to identify this species. Caution is recommended where matched public sequence records are unpublished or not associated with vouchered specimens. Sequence statistics reported here may change following future releases of novel accessions of *T. granarium* and other species at BOLD and or GenBank.

### *4.6.3 Loop-mediated isothermal amplification (LAMP) assay*

This DNA based loop-mediated isothermal amplification (LAMP) assay is simple and species-specific, enabling rapid detection of *T. granarium*. This assay requires enough amplifiable DNA, with the minimum threshold of˜1.0 x$10^{-6}$ ng/µL (Rako *et al*., 2021). The 16S locus is the diagnostic region targeted in this assay. The inclusion of general 18S (ribosomal) insect primers as a DNA quality-control check along with *T. granarium* specific primers mitigates the risk of false negatives.

**Primer details**

| **LAMP primer or amplicon** | **Sequence 5'-3'** | **Primer length (bp)** | **Predicted Tm, annealing temperature (°C)** | **Degeneracy of primer (fold)** |
|---|---|---|---|---|
| Khapra_gBlock (IDT) fragment | cccGGTAATTTAATCTTATAATCACAAGATGGcccATCATCTAATCATAAATCAATGTTTCAcccTAGTCACCCCAACCAAATTAAcccCCTAAAATTGAAAATTTCTATACTAACAAcccTTTAACAATTAAAGAAATAATAAAACTCTcccCGTCTTTTAAAAAAATTTGAGCCcccCAAAATAAAAAAGAGACAGTAATCAcccTTCGTCCAACCATTCATTCCAGTTccc | 234 | N/A | None |
| Khapra_F3 | GGTAATTTAATCTTATAATCACAAGATGG | 29 | 60.9 | None |
| Khapra_B3 | AACTGGAATGAATGGTTGGACGAA | 24 | 69.2 | None |

| | | | | |
|---|---|---|---|---|
| Khapra_FIP | TTGTTAGTATAGAAATTTTCAATTTTAGG ATCATCTAATCATAAATCAATGTTTCA | 56 | 73.8 | None |
| Khapra_BIP | TTTAACAATTAAAGAAATAATAAAACTCT TGATTACTGTCTCTTTTTTATTTTG | 54 | 70.7 | None |
| Khapra_Floop | TTAATTTGGTTGGGGTGACTA | 21 | 60.7 | None |
| Khapra_Bloop | CGTCTTTTAAAAAAATTTGAGCC | 23 | 61.6 | None |
| Insect18S-F3 | AGAGGTGAAATTCTTGGATCGTC | 23 | 62.4 | None |
| Insect18S-B3 | CCCGTGTTGAGTCAAATTAAG | 21 | 59.3 | None |
| Insect18S-FIP | GGTTAGAACTAGGGCGGTATCKAAGCGAA AGCATTTGCCA | 40 | 74.4 | 2 |
| Insect18S-BIP | GAAACCAAAGMTTTTGGRYGTGCCCTTCC GTCAATT | 36 | 73.3 | 8 |
| Insect18S-FL | GCCTTCGAACCTCTAACTTTC | 21 | 60.6 | None |
| Insect18S-BL | TCCGGGGGAAGTATGRTTG | 19 | 62.0 | 2 |

**Preparing the gBlock positive control**

1. Spin tube to ensure all dried contents are at the bottom.

2. Resuspend to a concentration of 10 ng/µL by adding 25 µL sterile water (Follow the instruction given in the tube).

3. Vortex briefly then incubate at 50°C for 20 mins.

4. Make up a 1 in 100 dilution by adding 1 µL of the 10 ng/µL solution to 99 µL sterile water. This will provide $10^8$ copies synthetic DNA/µL

5. Store the gBlock stock solution at -20°C. The 1/100 dilution can be stored as aliquots at -20°C.

**Preparation of 16S primer mix (1:4:2)**

The Khapra beetle specific primers F3 and B3 are used at 10 µM concentration, whilst FIP, BIP, Bloop and Floop are used at 100 µM concentration. Prepare the Khapra primer mix (F3/B3:FIP/BIP:Floop/Bloop) in a ratio of 1:4:2 by adding 10 µL of each F3 and B3, 4 µL of each FIP and BIP, 2 µL of each Bloop and Floop and 68 µL of water, bringing it to a total volume of 100 µL.

| **Primers** | **Stock concentration (µM)** | **Volume to add (µL)** | **Final concentration (µM)** |
|---|---|---|---|
| Khapra F3 | 10 | 10 | 0.4 |
| Khapra B3 | 10 | 10 | 0.4 |

| | | | |
|---|---|---|---|
| Khapra Fip | 100 | 4 | 1.6 |
| Khapra Bip | 100 | 4 | 1.6 |
| Khapra Floop | 100 | 2 | 0.8 |
| Khapra Bloop | 100 | 2 | 0.8 |
| Sterile water | | 68 | |
| Total | | 100 | |

**Preparation of 18S primer mix (1:6:3)**

Six novel LAMP primers targeting eight DNA regions aligned manually from diverse insect orders are used as insect DNA quality control in 18S LAMP test. Prepare 18S LAMP primer mix (F3/B3:FIP/BIP:Floop/Bloop) in a ratio of 1:6:3 by adding 10 µL of each F3 and B3, 6 µL of each FIP and BIP, 3 µL of each Bloop and Floop and 62 µL of water, bringing it to a total volume of 100 µL.

| **Primers** | **Stock concentration (µM)** | **Volume to add (µL)** | **Final concentration (µM)** |
|---|---|---|---|
| Insect18S-F3 | 10 | 10 | 0.4 |
| Insect18S-B3 | 10 | 10 | 0.4 |
| Insect18S-Fip | 100 | 6 | 2.4 |
| Insect18S-Bip | 100 | 6 | 2.4 |
| Insect18S-Floop | 100 | 3 | 1.2 |
| Insect18S-Bloop | 100 | 3 | 1.2 |
| Sterile water | | 62 | |
| Total | | 100 | |

**Preparation of LAMP mastermix (Prepare separately for 16S and 18S primers)**

The LAMP reaction mixes for Khapra specific 16S and general insect 18S assays should be prepared separately. To prepare the reaction mix for one reaction (25 µL), add 10 µL of specific primer mix and 14 µL of Isothermal Master Mix (ISO-DR001/ISO-DR004 from OptiGene). Follow the manufacturers protocol to prepare Isothermal Master Mix and, as this reagent is light sensitive, store in aluminium foil covered micro centrifuge tubes.

| **Reagent for 25 µL reaction** | **Volume for 1 reaction (µL)** |
|---|---|
| ISO-DR001 or DR004 | 14.0 |
| Primer mix | 10.0 |
| Template DNA | 1.0 |

**LAMP protocol**

1. Pipette 24 µL of LAMP mastermix into the wells of Genie strip tube
2. Add 1.0 µL of template DNA
3. Vortex for 30 seconds and spin to make sure the reaction mix and test DNA stays at the bottom of the tube
4. Place the strip tube into the Genie and run for 65°C for 35 minutes (Amplification) followed by annealing from 98°C to 73°C ramping at 0.05°C/sec.
5. Record the time to peak amplification rate and annealing temperature
6. Run only six test samples at a time in the Genie strip of eight wells, plus one known Khapra DNA as a positive control or a Khapra gBlock, and one no-template negative control.
7. In 18S LAMP test, run the same six test samples plus one known Khapra DNA as a positive control and one no-template negative control. The Synthetic Khapra gBlock (designed from mtDNA 16S locus and synthesised by IDT) cannot be used as positive control in 18S insect LAMP test.

**LAMP test results interpretation**

| **Assay** | **16S** | | **18S** | |
|---|---|---|---|---|
| | **Time (min)** | **Derivative Temperature (°C)** | **Time (min)** | **Derivative Temperature (°C)** |
| **Criteria** | 14.0–24.2<br>(>25 low template concentration) | 77.1–78.4 | 12.3–24.2<br>(>25 low template concentration) | 88.0–89.0 |
| **Positive** | √ | √ | √ | √ |
| | √ | √ | X | √ |
| | √ | √ | √ | X |
| | √ | √ | X | X |
| **Indeterminate** | √ | X | √ | √ |

| | | | | |
|---|---|---|---|---|
| | X | √ | √ | √ |
| **Negative** | X | X | √ | √ |
| **Invalid** | X | X | X | X |

**Positive** - An amplification plot of 'S' shaped sigmoid curve reflecting the fluorescence detected (see Figure below) for both 16S Khapra primers and 18S insect primers with an anneal derivative temperature and amplification time range displayed for both the primers. 16S Khapra primers and 18S insect primers show an anneal derivative temperature of 77.7°C (range: 77.1–78.4°C) and 88°C (range: 88.0–89.0°C) at an amplification time range of 14.0–24.2 min and 12.3–24.2 min respectively (Can be recorded from the Results tab displayed in the Genie II/III). Note, amplification of positive samples may occur >25 minutes in the presence of low amounts of template DNA (e.g. from poorly preserved / partial specimens), hence the recommended amplification run time of 35 minutes. NB: gBlock anneal derivative temperature is 80°C. Refer to Figure 1. If positive 16S but negative 18S, confirm correct anneal derivative for khapra beetle.

**Negative** – No fluorescence detected as evidenced from the flat line instead of 'S' shaped sigmoid amplification curve for the entire duration of LAMP run and no values displayed for time of amplification (minutes) and anneal derivative temperature (°C) for 16S Khapra primers while the 18S insect primers show sigmoidal amplification plot within optimal anneal derivative temperature and amplification time range.

**Indeterminate** – Positive sample should show both an amplification time and anneal derivative temperature. If one of these is missing, then the run has not produced positive amplification and is indeterminate (NB: If the amplification time does not show on the Genie instrument, then settings may not allow amplifications >25 minutes to be recorded. This can be adjusted in the Genie settings).

**Invalid** – Failure of 18S insect primers to amplify and failure of gBlock/positive control because of Genie instrument or reagent malfunction.

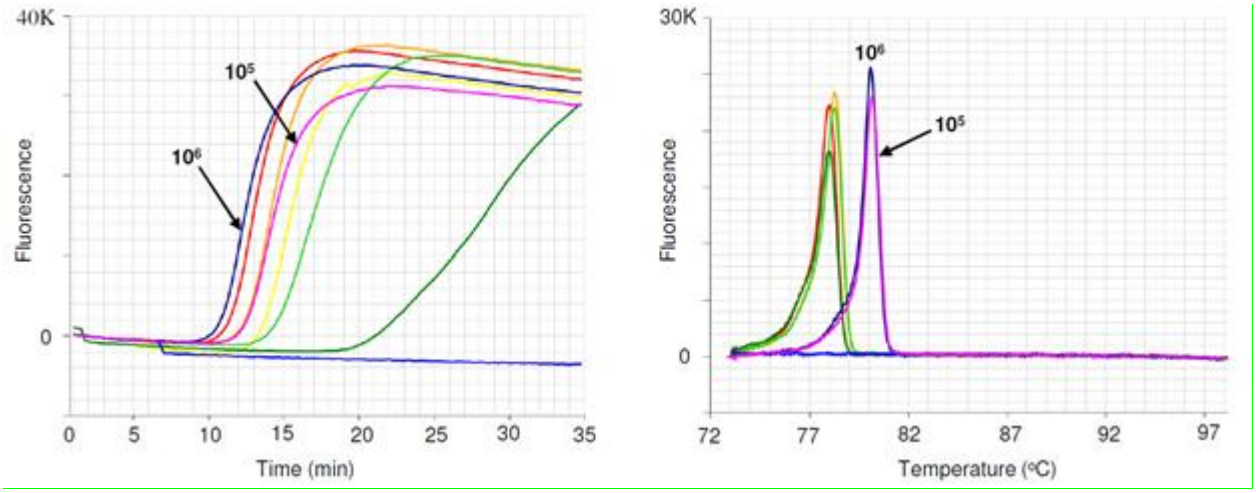


**Figure 1** Khapra 16S LAMP amplification and anneal derivative profiles. Left: Amplification of DNA and gBlock $10^5$ & $10^6$ positive controls ($10^5$, $10^6$). Right: Anneal derivatives of LAMP amplicons of Khapra 16S DNA (78°C) and gBlock $10^5$ & $10^6$ positive controls (80°C). (Source: Rako *et al.* 2021)

### 4.6.4 *Multiplex species-specific* T. granarium *real-time PCR (Furui protocol)*

This PCR is based on that published by Furui *et al.* (2018) and amplifies specific regions of the *T. granarium* mitochondrial genome. It utilises two specific primers sets, KBdetI and KBdetII, targeted at mitochondrial NADH dehydrogenase subunit II (ND2) and NADH dehydrogenase subunit VI (ND6), respectively. The published assay also includes an endogenous control primer set targeting the NADH dehydrogenase subunit I (ND1) to show the presence of dermestid DNA. The endogenous control was not included in the diagnostic protocol as it was found to provide unreliable detection of Australian dermestids. A commercially available 18S universal PCR assay has also been included to assess the presence of DNA following extraction from the starting specimen.

**Primers – Furui real-time PCR (mitochondrial NADH dehydrogenase subunits)**

| Name | Sequence | Label/Quencher* | Channel* | Length(bp) |
|---|---|---|---|---|
| KBdetI-F | CGA AAC AAT AAA ACA AAA CCA ATC A | | | 187 |
| KBdetI-R | GGA GGG GTA TCC CTA AGG C | | | |
| KBdetI-probe | CGT ACT CTC AGG ACT ATC AAT CAC CC | FAM/Tamra | Green | |
| KBdetII-F | CAG CCT TAT ATG ACT TCT CAT ACC | | | 83 |
| KBdetII-R | GAT TTC ATG TTG GGA ATG ATG | | | |
| KBdetII-probe | GCA AAT GGT GGC GAG TGT TGTC | Cy5/Tamra | Red | |
| 18SrRNA-F ** | CGGCTACCACATCCAAGGAA | | | 187 |
| 18SrRNA-R | GCTGGAATTACCGCGGCT | | | |
| 18SrRNA-probe | TGCTGGCACCAGACTTGCCCTC | VIC-Tamra | Yellow | |

* Based on the Qiagen Rotor Gene. Optimal dyes, quenchers and channels should be considered with respect to your preferred platform.

**The 18S endogenous eukaryotic DNA control is labelled with VIC and is commercially available premixed through ThermoFisher (www.thermofisher.com, Catalog number: 4319413E).

**Preparation of multiplex real-time PCR reaction mix**

Prepare reaction mixtures to achieve final concentrations specified in the table below, in a volume suitable for your preferred platform. (Face masks are recommended during preparation to avoid contamination of reaction mixtures with human DNA).

| Component | Final reaction mixture concentration |
|---|---|
| 2x qPCR master mix | 1x |

| | |
|---|---|
| KBdetI-F | 0.5µM |
| KBdetI-R | 0.5µM |
| KBdetI-probe | 0.2µM |
| KBdetII-F | 0.5µM |
| KBdetII-R | 0.5µM |
| KBdetII-probe | 0.2µM |
| 18SrRNA-F | 0.5µM |
| 18SrRNA-R | 0.5µM |
| 18SrRNA-probe | 0.2µM |

DNA template volume added to the reaction mixture can be in the range of 1µL-5µL.

Each laboratory will have its favoured method for achieving the reaction mixture concentrations listed above. An example is detailed below:

**Preparation of 20x primer-probe mix**

| **Component** | **Volume (µL)** |
|---|---|
| Forward primer (100µM) | 10 |
| Reverse primer (100µM) | 10 |
| Probe (100µM) | 4 |
| Nuclease-free water | 76 |
| TOTAL | 100 |

**Preparation of 25µL reaction mixture**

| **Reagent** | **Volume/rxn (µL)** | **Final conc.** |
|---|---|---|
| 2x qPCR master mix | 12.5 | 1x |
| 20x KBdetI primer probe mix | 1.25 | 0.5 µM fwd primer<br>0.5 µM rev primer<br>0.2 µM probe (KBI) |
| 20x KBdetII primer probe mix | 1.25 | 0.5 µM fwd primer<br>0.5 µM rev primer<br>0.2 µM probe (KBII) |

| | | |
|---|---|---|
| 20x 18SrRNA primer probe mix | 1.25 | 0.5 µM fwd primer<br>0.5 µM rev primer<br>0.2 µM probe (18S) |
| Water | 7.75 | |
| Template | 1.0 | |
| TOTAL | 25.0 | |

**PCR controls**

1. Positive amplification control – DNA extracts from known *T. granarium* specimens (Identification should be confirmed via sequencing as well as the appropriate morphological method).
2. No template control (negative amplification control) – An aliquot of the PCR master mix without template DNA.
3. Process extraction control (negative extraction control) – Extraction reagents containing nil sample that have gone through the extraction process.
4. It is recommended that samples, including controls be run in duplicate for greater confidence.

**Real-time PCR cycling conditions**

| **Step** | **Temperature** | **Duration** | **Cycles** |
|---|---|---|---|
| Initial denature | 95°C | 10 min | |
| Denature | 95°C | 30 sec | 40 cycles |
| Anneal/extend | 59°C | 60 sec | |
| Acquire in all channels (Green/Yellow/ Red) at end of extension step | | | |

**Results/Interpretation**

| **Results** | **Assays** | | | Interpretation |
|---|---|---|---|---|
| | **KBdetI** | **KBdetII** | **18SrRNA** | |
| Positive | Ct value ≤40 | Ct value ≤40 | Ct value ≤40 | DNA consistent with *T. granarium* **detected** |
| Indeterminate | Only one of these assays producing a Ct ≤40 | | Ct value ≤40 | Further investigation -Repeat |

| | | | | |
|---|---|---|---|---|
| Negative | No Ct | No Ct | Larval/beetle sample: Ct value ≤40<br><br>Larval skin/insect piece, may or may not produce a Ct | *T. granarium*<br><br>**NOT detected** |
| Invalid | Failure of controls | | | Repeat |

**Note:** If the 18S probe amplifies in the negative control, this is NOT a failure. 18S will amplify from any eukaryotic DNA in a sample including contamination from humans and in dust. It is an indicator of DNA quantity and quality.

### *4.6.5 16S real-time PCR protocol*

This PCR developed by the South Australian Research and Development Institute (SARDI) is based on specific amplification of the 16S region of the *T. granarium* mitochondrial genome. It is modified from the assay published by Olson *et al*. (2014) to be more specific for *T. granarium*. The TaqMan MGB probe is FAM labelled. Initially, it was designed to be singleplex assay for high-throughput screening of samples, but it is compatible with multiplexing and can replace one of the primer-probe sets in the Furui (2019) protocol (*see section* 4.6.5). Alternatively, it can be combined with the 18S eukaryotic DNA internal control for greater reliability and interpretation of possible false positives.

**Primers – SARDI 16S real-time PCR (mitochondrial 16S gene)** *

| **Name** | **Sequence (5'-3')** | **Label/Quencher*** | **Channel*** | **Length (bp)** | **Reference** |
|---|---|---|---|---|---|
| KB-F | GAA TGA ATG GTT GGA CGA AAT G | | | 161 | SARDI* in-house |
| KB-R | CTA AAA TTG AAA ATT TCT ATA CTA ACA ATT TAA CA | | | | |
| KB-probe | AGC CTT TTA ACT CAA AAG T | FAM/MGB | Green | | |

*South Australian Research and Development Institute (Unpublished). Based on requirements for the Qiagen Rotor Gene instrument. Optimal dyes and channels should be considered with respect to your preferred platform.

**Preparation of singleplex real-time PCR reaction mix**

Prepare reaction mixtures to achieve final concentrations specified in the table below, in a volume suitable for your preferred platform.

| **Component** | **Final reaction mixture concentration** |
|---|---|
| 2x qPCR master mix | 1x |
| KB-F | 0.4µM |

| | |
|---|---|
| KB-R | 0.4µM |
| KB-probe | 0.2µM |

DNA template volume added to the reaction mixture can be in the range of 1µL-5µL.

Each laboratory will have its favoured method for achieving the reaction mixture concentrations listed above. An example is detailed below:

**Preparation of 50x primer-probe mix**

| **Component** | **Volume (µL)** |
|---|---|
| KB-F (100µM) | 20 |
| KB-R (100µM) | 20 |
| KB-probe (100µM) | 10 |
| Nuclease-free water | 50 |
| TOTAL | 100 |

**Preparation of 10µL reaction mixture**

| **Reagent** | **Volume/rxn (µL)** | **Final conc.** |
|---|---|---|
| 2x qPCR master mix | 5.0 | 1x |
| 50x primer probe mix | 0.2 | 0.4 µM fwd primer<br>0.4 µM rev primer<br>0.2 µM probe |
| Water | 0.8 | |
| Template | 4.0 | |
| TOTAL | 10.0 | |

**PCR controls: -** as described previously in section 4.6.4

**Real-time PCR cycling conditions**

| Step | Temperature | Duration | Cycles |
|---|---|---|---|
| Initial denature | 95°C | 15 min | |
| Denature | 95°C | 15 sec | 45 cycles |
| Anneal/extend | 60°C | 60 sec | |
| Acquire in green channel at end of extension step | | | |

**Results/Interpretation**

**Positive** –Ct value ≤45. If used with a general internal control, the specific channels should have a lower Ct value than the general channels (e.g. 18S).

**Indeterminate** – Where replicates are run equivocal results where one replicate is positive and the other negative.

**Negative** – No Ct value.

**Invalid** – Failure of one or more controls – Repeat PCR.

### *4.6.6 Multiplex* T. granarium/T. variabile *real-time PCR (TgTv real-time PCR)*

This multiplex assay allows for the simultaneous identification and differentiation of *T. granarium* and *T. variabile* and targets the mitochondrial COI gene. Since its first detection in 1977 *T. variabile* has become established in Australia and is a minor pest in storage structures (Rees *et al.* 2003). *T. variabile* is of particular significance due to its taxonomic similarity to *T. granarium*. This assay offers a differential identification tool in which the exotic *T. granarium* beetle can be excluded whilst also confirming the presence/absence of the morphologically similar *T. variabile*.

**Primers – TgTv real-time PCR (mitochondrial COI rRNA gene)**

| Primer | Sequence 5'-3' | Label/ Quencher | Size (bp) | Reference |
|---|---|---|---|---|
| Tgran-F | CCC ACT CTA TGA GCA CTA GGAT | | 97 | DDLS WA* in-house |
| Tgran-R | CGT GTA GAA CGA TGT CGA TTG A | | | |
| Tgran-probe | TAT TCA CCG TAG GAG GAC TAA CAG GAG T | FAM/BHQ1 | | |
| Tvari-F | TTT AAT CCA ACA TCG AGG | | 89 | |
| Tvari-R | CGC CTG TTT AAC AAA AAC AT | | | |
| Tvari-probe | -ACG CTG TTA TCC CTA AGG TAA TT | TET/BHQ1 | | |

*DDLS WA –Department of Primary Industries and Regional Development, Diagnostics and Laboratory Services, Western Australia.

**PCR controls: -** as described previously in section 4.6.4

**Preparation of multiplex real-time PCR reaction mix**

Prepare reaction mixtures to achieve final concentrations specified in the table below, in a volume suitable for your preferred platform.

| Component | Final reaction mixture concentration |
|---|---|
| 2x qPCR master mix | 1x |
| Tgran-F | 0.5µM |
| Tgran-R | 0.5µM |
| Tgran-probe | 0.2µM |
| Tvari-F | 0.5µM |
| Tvari-R | 0.5µM |
| Tvari-probe | 0.2µM |

DNA template volume added to the reaction mixture can be in the range of 1µL-5µL.

Each laboratory will have its favoured method for achieving the reaction mixture concentrations listed above. An example is detailed below:

**Preparation of 25µL reaction mixture**

| Component | Volume (µL) |
|---|---|
| 2 x Rotor-Gene Multiplex master mix (Qiagen) | 12.5 |
| Tgran-F (50µM) | 0.25 |
| Tgran-R (50µM) | 0.25 |
| Tgran-probe (10µM) | 0.5 |
| Tvari-F (50µM) | 0.25 |
| Tvari-R (50µM) | 0.25 |
| Tvari-probe (10µM) | 0.5 |
| Nuclease-free water | 5.5 |
| DNA template | 5.0 |

**PCR cycling conditions**

| Step | Temperature | Duration | Cycles |
|---|---|---|---|
| Initial denaturation | 95°C | 5 min | 1 |
| Denaturation | 95°C | 15 sec | 45 |
| Annealing | 64°C | 30 sec | |

| | | | |
|---|---|---|---|
| Extension* | 72°C | 30 sec | |

*Acquire in green and yellow channel at the completion of the extension

**Results/Interpretation**

| **PCR Results** | **Assays** | | **Interpretation** |
|---|---|---|---|
| | **Tg** | **Tv** | |
| Positive | Larval/beetle sample: Ct value ≤36<br>Larval skin/insect piece ≤45 | No Ct | DNA consistent with *T. granarium* **detected** |
| Positive | No Ct | Larval/beetle sample: Ct value ≤36<br>Larval skin/insect piece ≤45 | DNA consistent with *T. variabile* **detected** |
| Indeterminate | Where replicates are run equivocal results where one replicate is positive and the other negative | | Further investigation -Repeat |
| Negative | No Ct | No Ct | *T. granarium/T. variabile*<br>**NOT detected** |
| Invalid | Failure of controls | | Repeat |

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# 8 APPENDICES

## 8.1 Primary Host Plants and US Border entry intercepts (Pasek, 1998) for *Trogoderma granarium*

| Scientific name | Common name | Primary Host Plants | Intercepted at US entry points (Pasek, 1998) |
|---|---|---|---|
| *Allium cepa* | Onion | | ✓ |
| *Apium graveolens* | Wild celery | | ✓ |
| *Apium graveolens* var. *dulce* | Celery | | ✓ |
| *Arachis hypogaea* | Peanut | ✓ | ✓ |
| *Avena sativa* | Oat | ✓ | |
| *Brassica carinata* | Ethiopian rape/mustard or Abyssinian mustard | | ✓ |
| *Cajanus cajan* | Pigeon Pea | | ✓ |
| *Capsicum* spp. | Capsicum/peppers | | ✓ |
| *Carya illinoensis* | Pecan | ✓ | |
| *Cicer arietinum* | Garbanzo | ✓ | ✓ |
| *Citrullus lanatus* | Watermelon | | ✓ |
| *Citrullus* spp. | Desert melons | | ✓ |
| *Coriandrum sativum* | Coriander | | ✓ |
| *Corylus* spp. | Hazel | | ✓ |
| *Cucumis melo* | Musk/honeydew melon | | ✓ |
| *Cucumis* spp. | Melon | | ✓ |
| *Cucurbita maxima* | Cultivated squash | | ✓ |
| *Cucurbita* spp. | Gourds | | ✓ |
| Cucurbitaceae | Pumpkin, Melon, Cucumber | ✓ | ✓ |
| *Cuminum* spp. | Cumin | | ✓ |
| *Cupressus* spp. | Cypress | | ✓ |
| *Cyamopsis tetragonoloba* | Guar | | ✓ |
| *Cydonia oblonga* | Quince | | ✓ |
| *Elettaria cardamomum* | True cardamom | | ✓ |
| *Fabaceae* | Legume peas | | ✓ |
| *Foeniculum vulgare* | Fennel | | ✓ |
| *Glycine max* | Soybean | ✓ | ✓ |
| *Gossypium* | Cotton | ✓ | |
| *Gossypium* spp. | Cotton | | ✓ |
| *Hordeum vulgare* | Barley | ✓ | ✓ |

| | | | |
|---|---|---|---|
| *Juglans* spp. | Walnut | ✓ | |
| *Lathyrus spp.* | Peavine | | ✓ |
| *Lens culinaris* | Lentil | ✓ | ✓ |
| *Momordica charantia* | Bitter melon | | ✓ |
| *Oryza sativa* | Rice | ✓ | ✓ |
| *Oryza* spp. | Rices | | ✓ |
| *Panicum milliaceum* | Millet | ✓ | |
| *Phaseolus lunatus* | Lima bean | | ✓ |
| *Phaseolus* spp. | Climbing beans | | ✓ |
| *Phaseolus vulgaris* | Common Bean | | ✓ |
| *Pinus* spp. | Pine | | ✓ |
| *Pistacia* spp. | Cashew | | ✓ |
| *Pistacia vera* | Pistachio | | ✓ |
| *Pisum sativum* | Garden pea | ✓ | ✓ |
| *Prunus amygdalus* | Sweet almond | | ✓ |
| *Prunus dulcis* | Almond | ✓ | ✓ |
| *Prunus* spp. | Stone fruits | | ✓ |
| *Sesamum indicum* | Sesame | ✓ | ✓ |
| *Solanaceae* | Nightshades | | ✓ |
| *Sorghum bicolor* | Grain sorghums | ✓ | |
| *Tamarindus indica* | Tamarind | | ✓ |
| *Trigonella foenum-graecum* | Fenugreek | | ✓ |
| *Triticum aestivum* | Wheat/Bread wheat | ✓ | ✓ |
| *Triticum* spp. | Wheats | | ✓ |
| *Vicia faba* | Broad bean | | ✓ |
| *Vigna radiata* | Mung bean | | ✓ |
| *Vigna* spp. | Pea/Bean | | ✓ |
| *Vigna unguiculata* | Cowpea | ✓ | ✓ |
| *Zea mays* subsp. *mays* | Corn | ✓ | |

## 8.2 Life History and dispersal of *Trogoderma granarium*

### *8.2.1 Life History*

Development rates and survival vary considerably depending upon temperature, light, moisture content, and host species (Lindgren *et al.* 1955). Khapra beetle may have one to nine or more generations per year as a result. High humidity has a depressing effect on population build-up (Ramzan and Chahal 1986; 1989) hence it will increase the population of other primary stored product pests. At favourable temperatures, eggs, pupae, and adults each last about a week, whereas the larval stage may last a month to several years (if it enters diapause) (Burges 1959). The average time to complete development from egg to adult in complete darkness was 220, 166, 37, and 26 days, respectively, at 20, 27, 32 and 35°C (Lindgren *et al.* 1955). At 27–32°C larvae had an average of 7–8 instars, whereas at 32–35°C larvae moulted only 4 times. Under adverse conditions, larvae may moult up to 15 times (Voelkel 1924). Infestations of khapra beetle generate heat (Burges 1959; Mason 1924; Rahman *et al.* 1945), which could help maintain suitable living conditions, but may increase mortality of larvae when temperatures become too high (Bains *et al.* 1974).

Development times at 32–35°C for a variety of host materials tested varied from 29 days to eight months (Lindgren *et al.* 1955). Larval survival varied significantly depending upon type of food; 89–91% of larvae completed development to the adult stage when reared on crushed wheat or whole wheat flour at 30°C compared to 56% survival when reared on rice grains (Pasek 1998). Rahman *et al.* (1945) found that larvae developed fastest on wheat, maize, and rice, somewhat slower on barley and grain, and slowest on walnut, while survival was greatest on rice. Larvae consumed an average of 3–12 mg of food during their development, with females eating about double the amount as compared to males.

More food was consumed in constant darkness; however, constant light accelerated development but reduced oviposition. Larval survival was 81% in constant darkness versus 51% in constant light for Khapra beetle reared on white rice at 28.5°C.

Larvae can prolong development time by entering a facultative diapause, whereby they are capable of surviving without food for several years. Diapause is induced by accumulation of larval faecal pellets in food, crowding, or low temperature (Burges 1959, 1962; Nair and Desai 1973). However, diapause cannot be induced after the 16th day following hatching, apparently a critical time for onset of pupation (Aggarwal *et al.* 1981). Diapausing larvae remain mobile. Where some food is available, some larvae can live in diapause for 6 years (Burges 1962). Diapause is broken by a substantial rise in temperature or provision of fresh food and reduction in crowding (Burges 1959, 1962; Nair and Desai 1973). Some larvae also break diapause spontaneously after a period of time (Nair and Desai 1973).

Sex ratio of emerging adults was 1:1 (Lindgren *et al.* 1955). Adult longevity varied from 25 days at 21°C to 12 days at 32°C. Under darkness and reared on ground dog food, fecundity increased with increasing temperature and females laid averages of 65 and 93 eggs at 32°C and 35°C, respectively. Adults derived from diapausing larvae tended to lay more eggs than those from non-diapausing larvae, although adults of diapausing larvae that had been starved produced fewer eggs than those of non-starved diapausing larvae (Pasek 1998). Eggs are usually laid singly amongst host material (Voelkel 1924). Adults feed very little during their short lives and do not fly (Voelkel 1924).

Most larvae more than a day old and adults are negatively phototropic through the majority of their lives, but adults become positively phototropic shortly before dying. Mating occurs only at night, and between temperatures of 10–42°C (Voelkel 1924 in Hinton 1945).

Little or no mortality of eggs, larvae, and pupae reared on ground dogfood occurred at temperatures above 21°C (Lindgren *et al.* 1955). Voelkel (1924) did not find Khapra beetle living in environments of higher than 44.2°C. Husain and Bhasin (1921) (in Pasek 1998) reported that all stages of Khapra beetle died within five hours at 50°C. Bains *et al.* (1974) noted a lethal effect at 41.5°C. Larvae are also extremely cold tolerant; only 52% of fourth instar larvae died when exposed for 25 hours to -10°C (Voelkel 1924).

Populations of parasites and predators were found to remain low in rural wheat stores in Punjab, India, and played only a minor role in regulating populations of khapra beetle (Bains *et al.* 1974).

### *8.2.2 Dispersal*

**Natural dispersal**

The species is almost entirely reliant on human activity for dispersal. Larvae and adults can crawl slowly and short distances. Though adults have fully developed hind wings, they have never been observed flying. It is reported that first instar larvae could be distributed by wind.

**Human-aided dispersal**

The primary means of dispersal over both short and long distances is by transport of infested materials by humans or technology (Lindgren *et al.* 1955). Adults and inactive larvae seek out cracks and crevices and may remain in trucks, rail cars, ship holds, and packing materials, such as bagging and crates, for many years feeding on remnants of the previous cargo. The spread of the Khapra beetle in the 19th century was largely due to the increase of trade and the lack of quarantine. During the 20th century it was probably associated with the military movements and shipments. The spread of khapra beetle is an indication of the capacity of this pest to be moved about through artificial means.

## 8.3 Images of Dermestidae

### *8.3.1 Scanning electron microscope photographs and line drawings -* Trogoderma granarium *Everts*

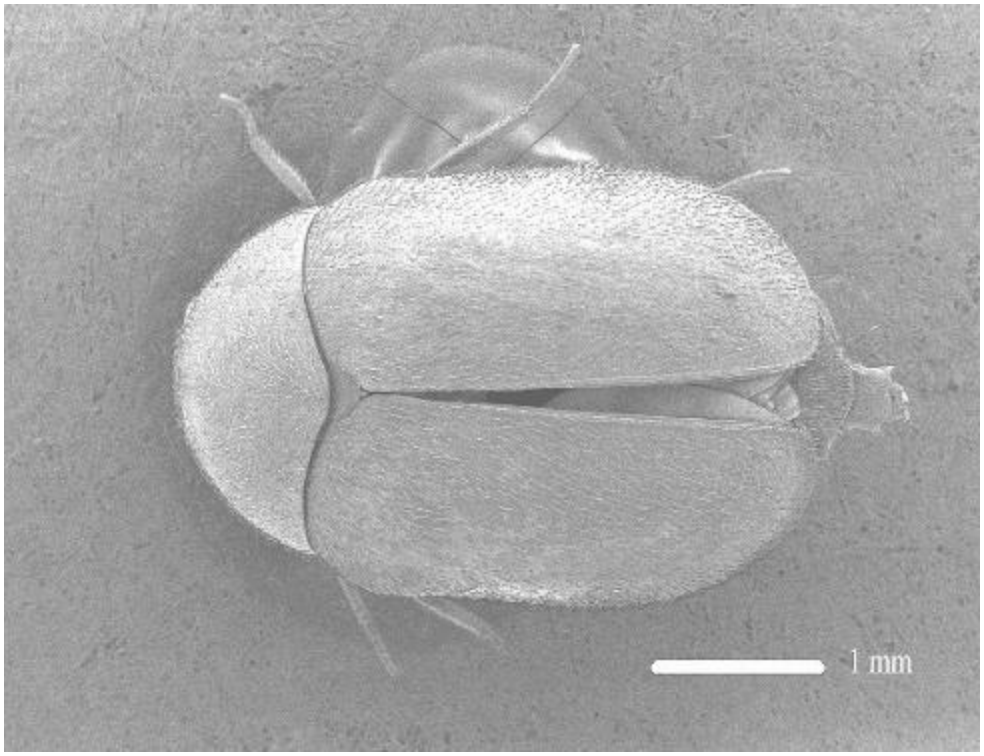


**Figure 1** Adult *Trogoderma inclusum*, dorsal view (DPIRD, A Szito).

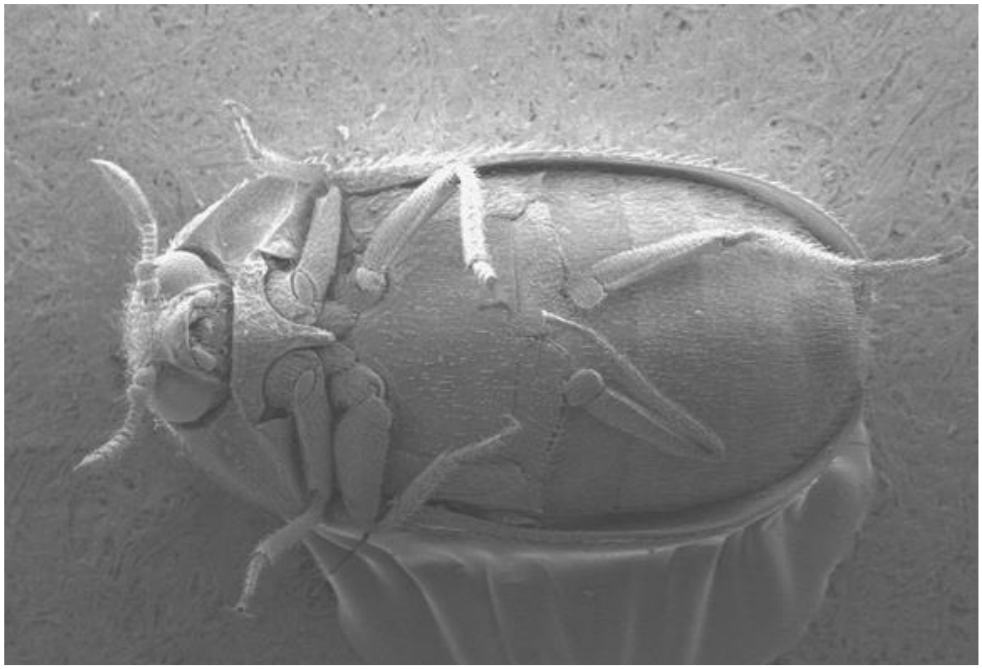

**Figure 2** Adult female *Trogoderma granarium*, ventral view (DPIRD, A Szito).

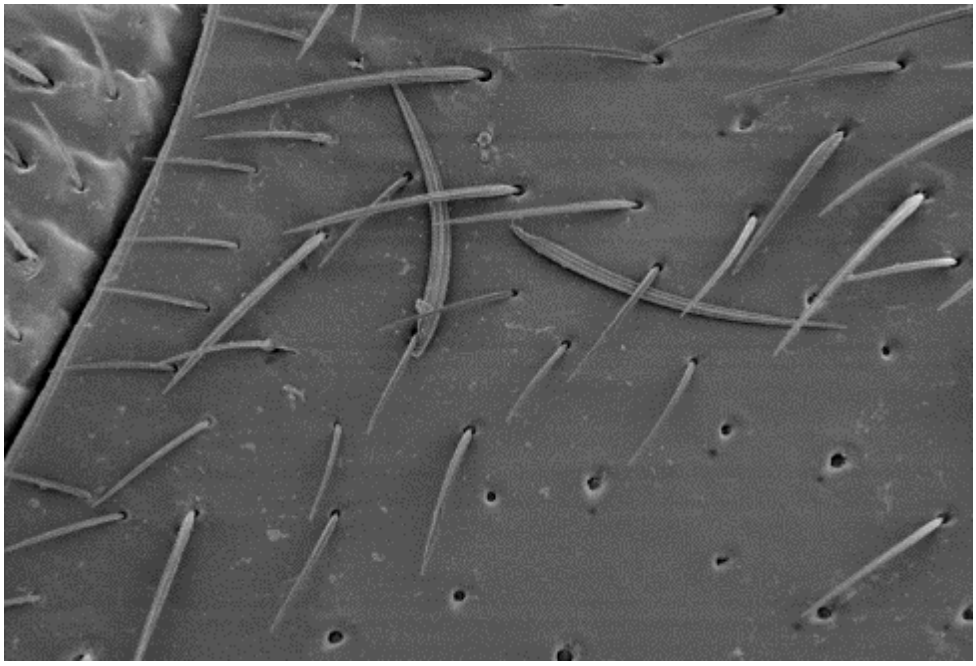

**Figure 3** Two kind of setae (normal and ensiform) on a *Trogoderma* species (DPIRD, A Szito).

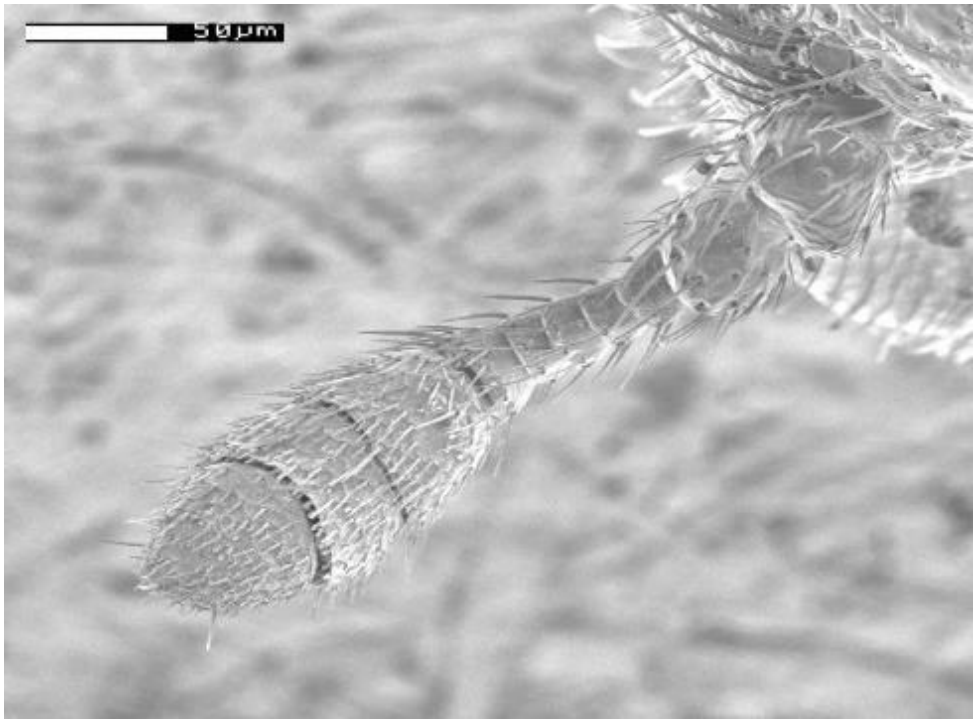


**Figure 4** Ten-segmented antenna of female *Trogoderma granarium* (DPIRD, A Szito).

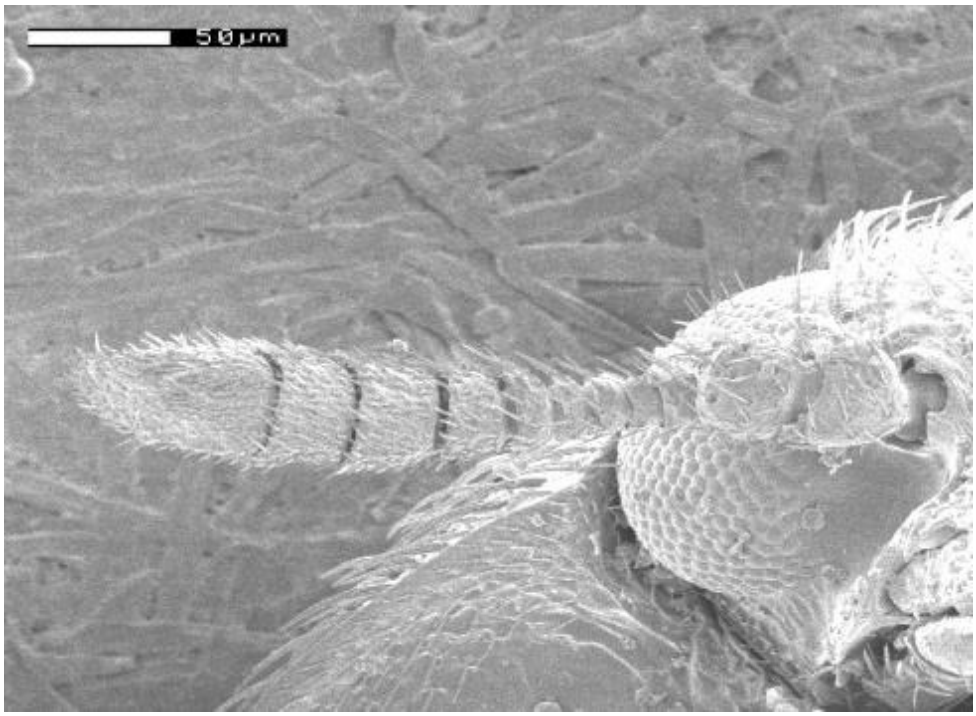


**Figure 5** Antenna of male *Trogoderma granarium* (DPIRD, A Szito).

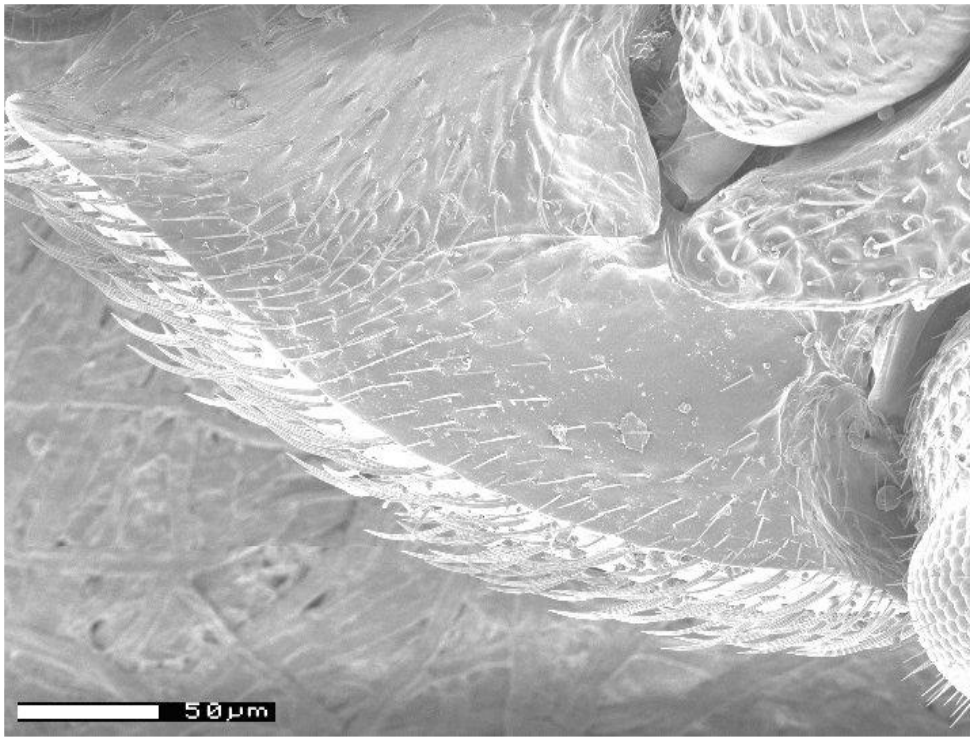


**Figure 6** Antennal cavity of female *Trogoderma granarium* (DPIRD, A Szito).

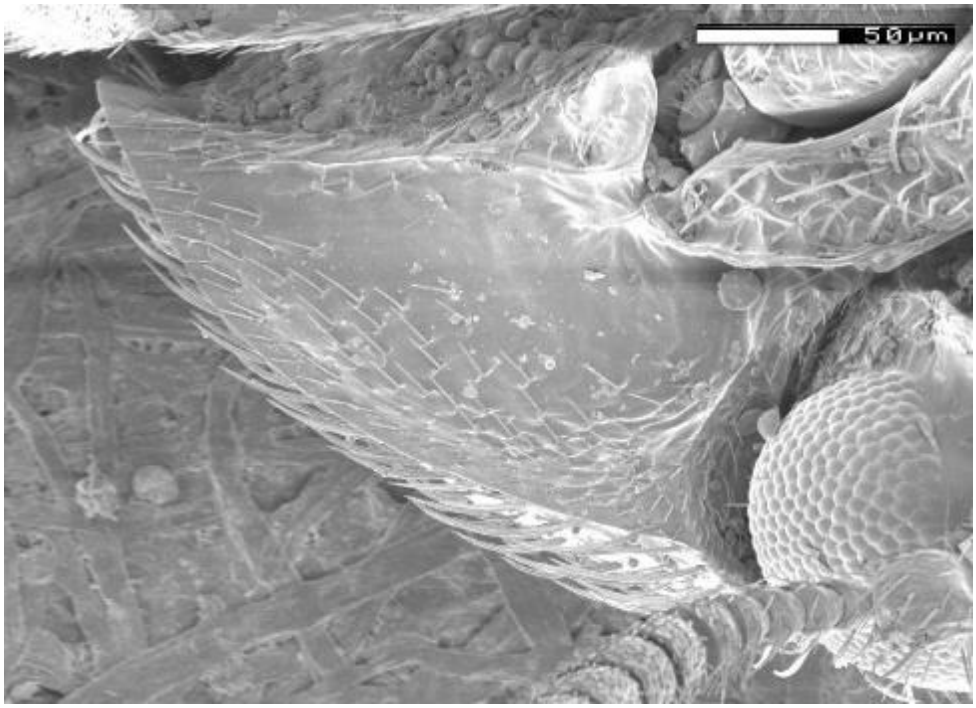


**Figure 7** Antennal cavity of male *Trogoderma granarium* (DPIRD, A Szito).

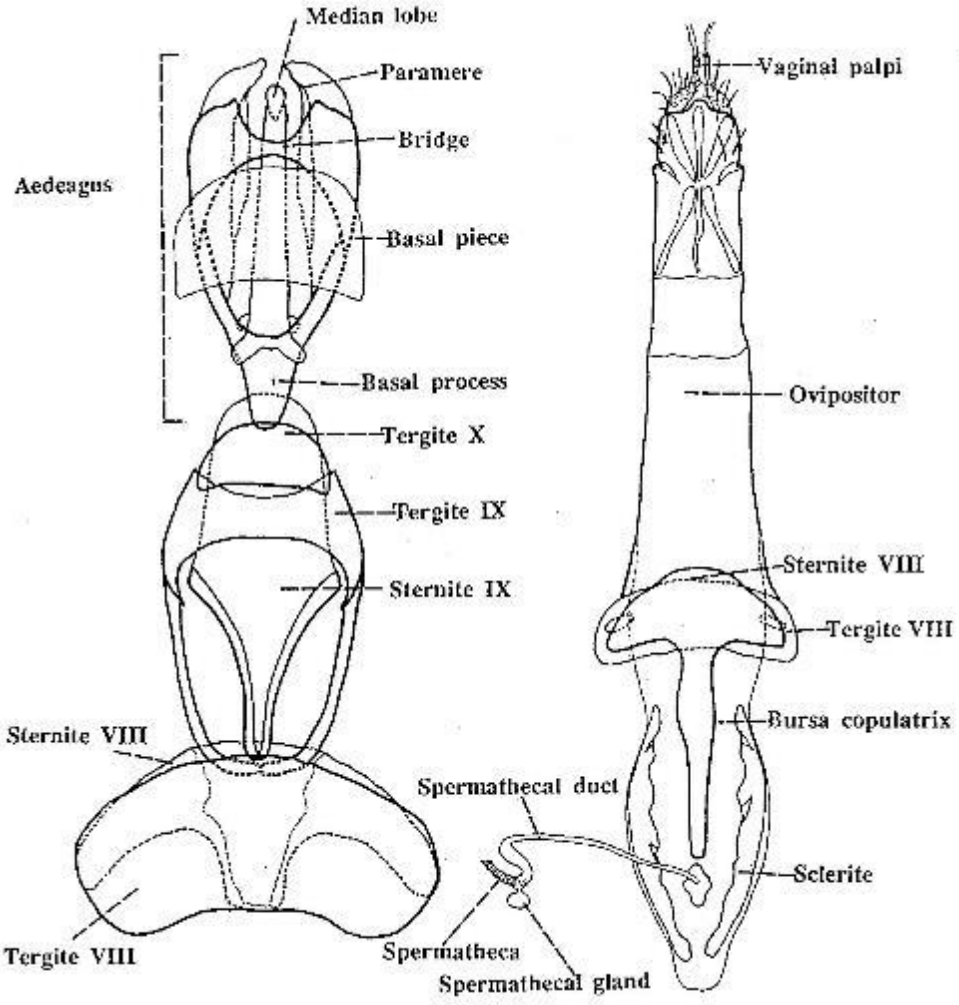


**Figure 8** Male and female genitalia of a *Trogoderma*.

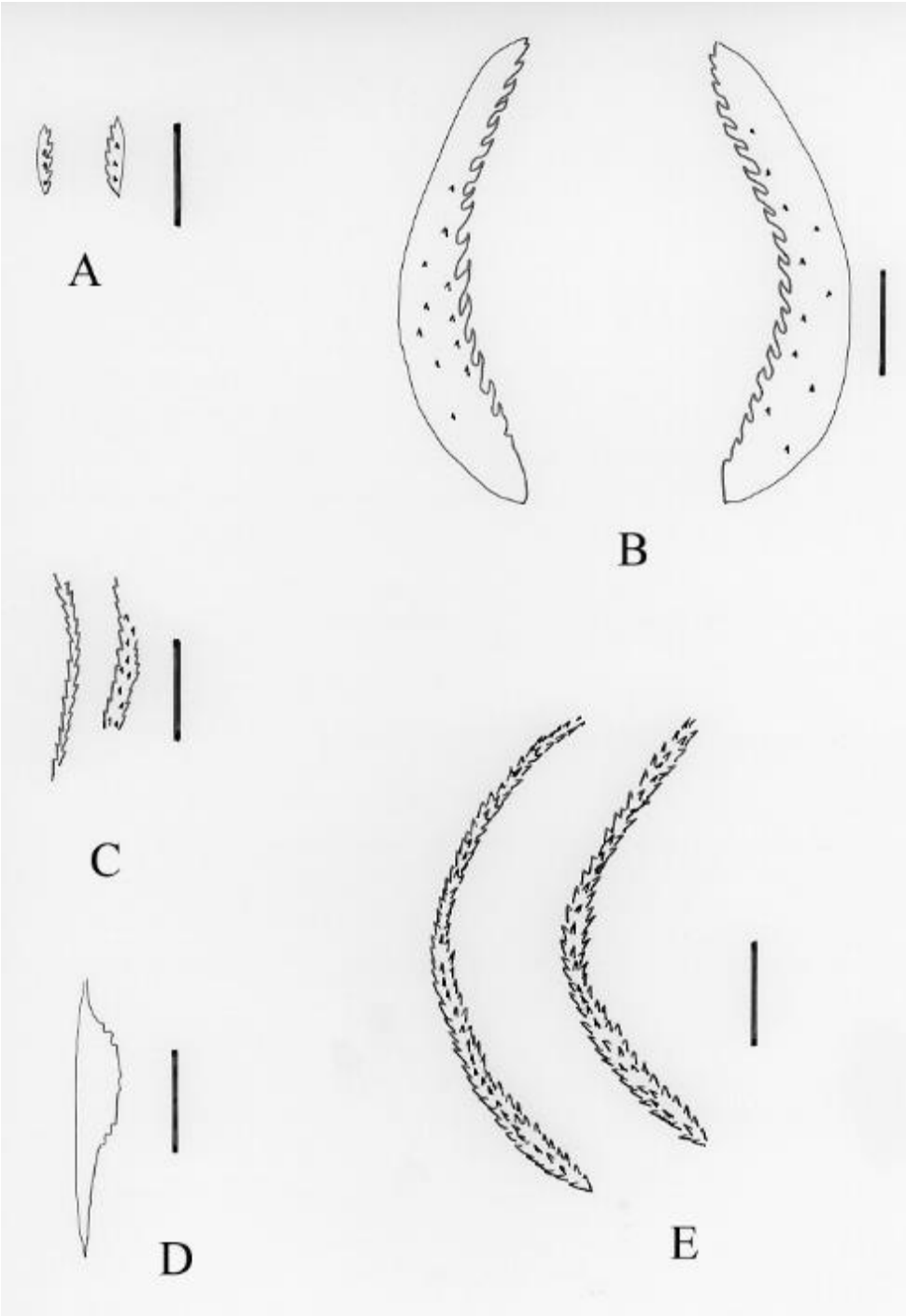


**Figure 9** Sclerites of bursa copulatrix of exotic pest *Trogoderma* species; (A) *Trogoderma granarium* Everts, (B) *T. variabile* Ballion, (C) *T. glabrum* Herbst, (D) *T. sternale* Jayne, (E) *T. inclusum* LeConte (Scale bar = 100 μm) (DPIRD, A Szito).

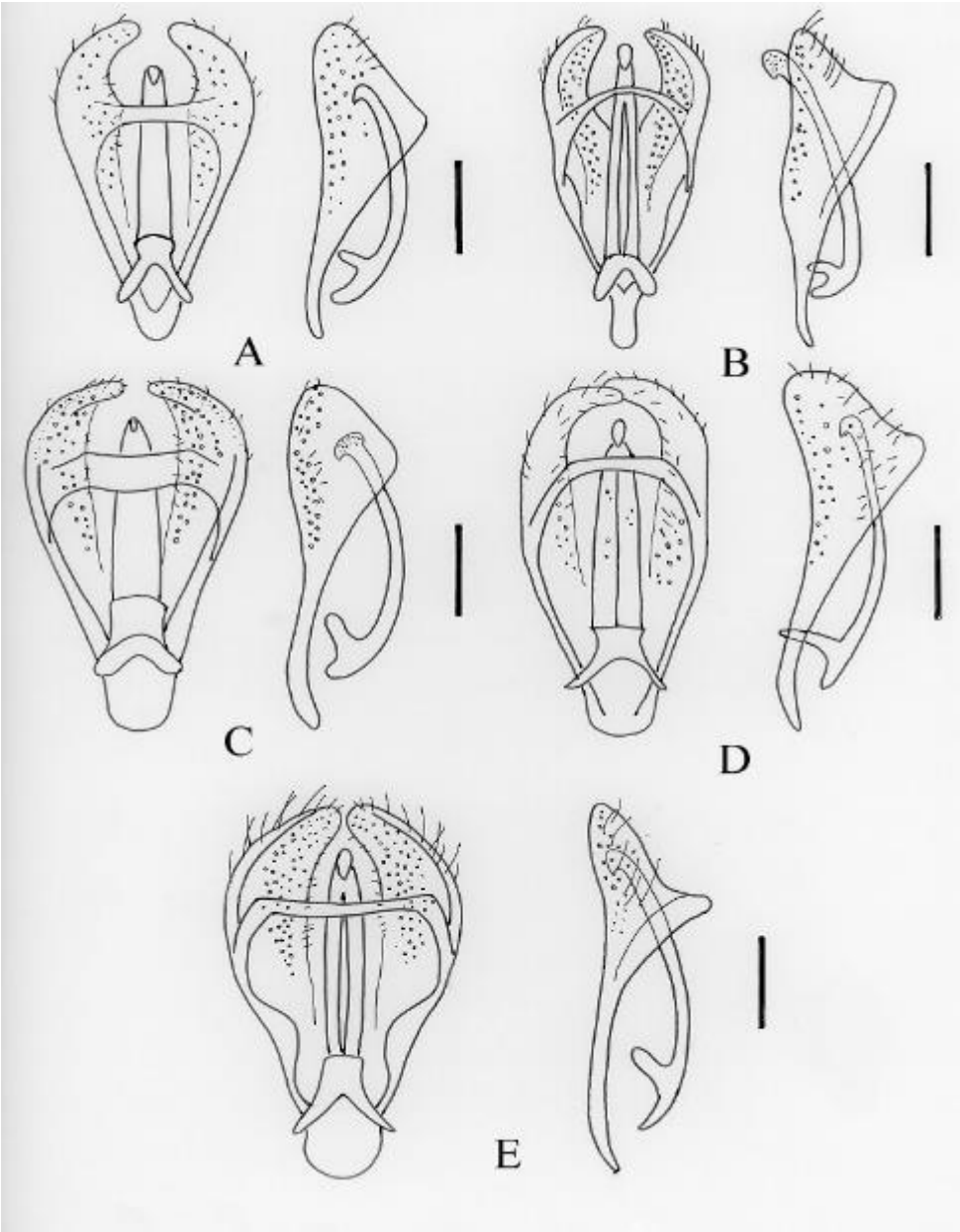


**Figure 10** Male genitalia of pest *Trogoderma* species; (A) *Trogoderma granarium* Everts, (B) *T. sternale* Jayne, (C) *T. glabrum* Herbst, (D) *T. inclusum* LeConte, *T. variabile* Ballion. (Scale bar = 100 μm) (DPIRD, A Szito).

### *8.3.2 Images of* Trogoderma granarium *Everts*

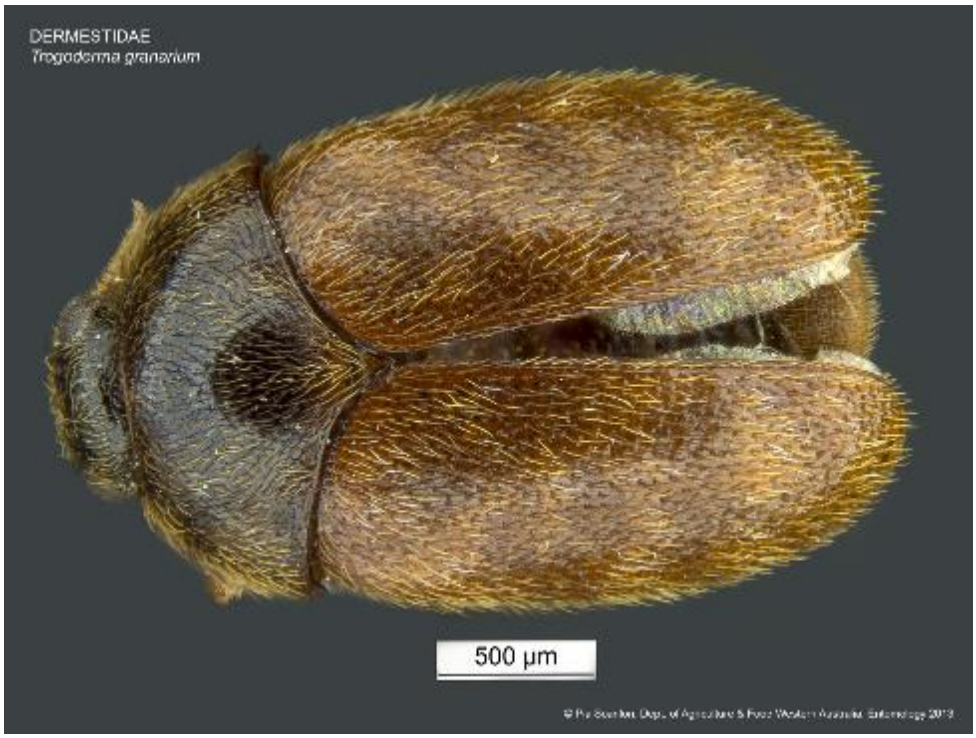


**Figure 11** Adult *Trogoderma granarium*, dorsal view. Two different coloured setae clearly visible (DPIRD, P Scanlon)

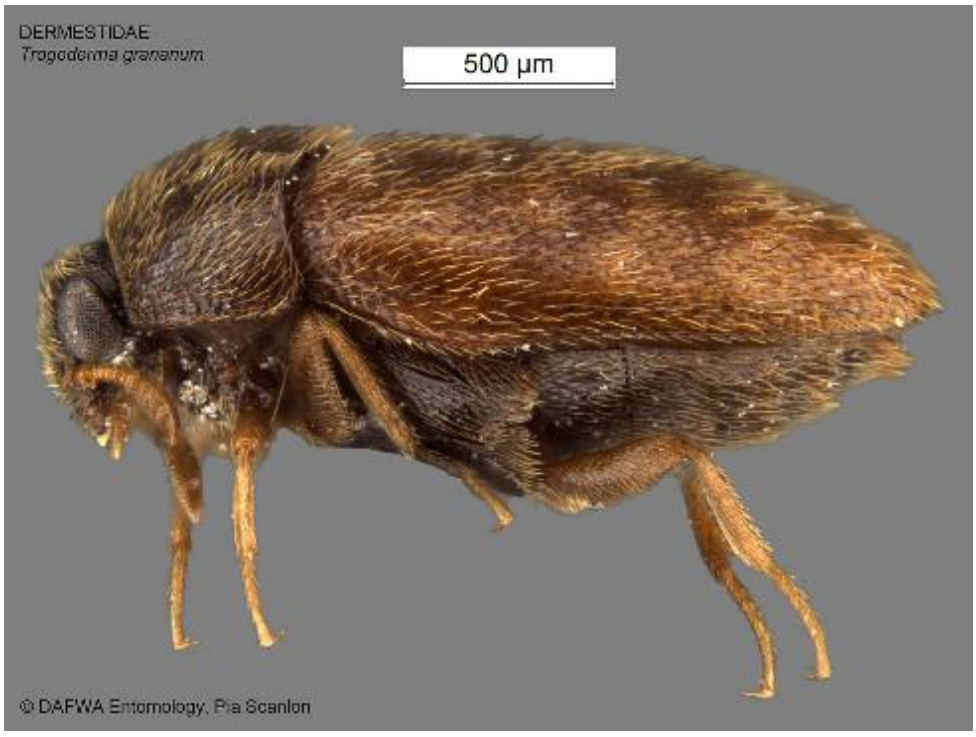


**Figure 12** Adult *Trogoderma granarium*, lateral view (DPIRD, P Scanlon)


**Figure 13** Adult *Trogoderma granarium*, ventral view (DPIRD, P Scanlon)

**Figure 14** Adult *Trogoderma granarium*, head and pronotum dorsal view. Setae are distinctive (DPIRD, P Scanlon)


**Figure 15** Adult *Trogoderma granarium*, head view. Median ocellus clearly visible (DPIRD, P Scanlon)


**Figure 16** *Trogoderma granarium*, male genitalia (DPIRD, P Scanlon)

### *8.3.3 Images of* Trogoderma variabile *Ballion – Warehouse beetle*


**Figure 17** Adult *Trogoderma variabile*, dorsal view (DPIRD, P Scanlon)


**Figure 18** Adult *Trogoderma variabile*, lateral view (DPIRD, P Scanlon)


**Figure 19** Adult *Trogoderma variabile*, ventral view (DPIRD, P Scanlon)


**Figure 20** Adult *Trogoderma variabile* in stored grain (DPIRD, P Scanlon)


**Figure 21** *Trogoderma variabile* larvae in stored grain (DPIRD, P Scanlon)

### *8.3.4* Trogoderma glabrum *Herbst*

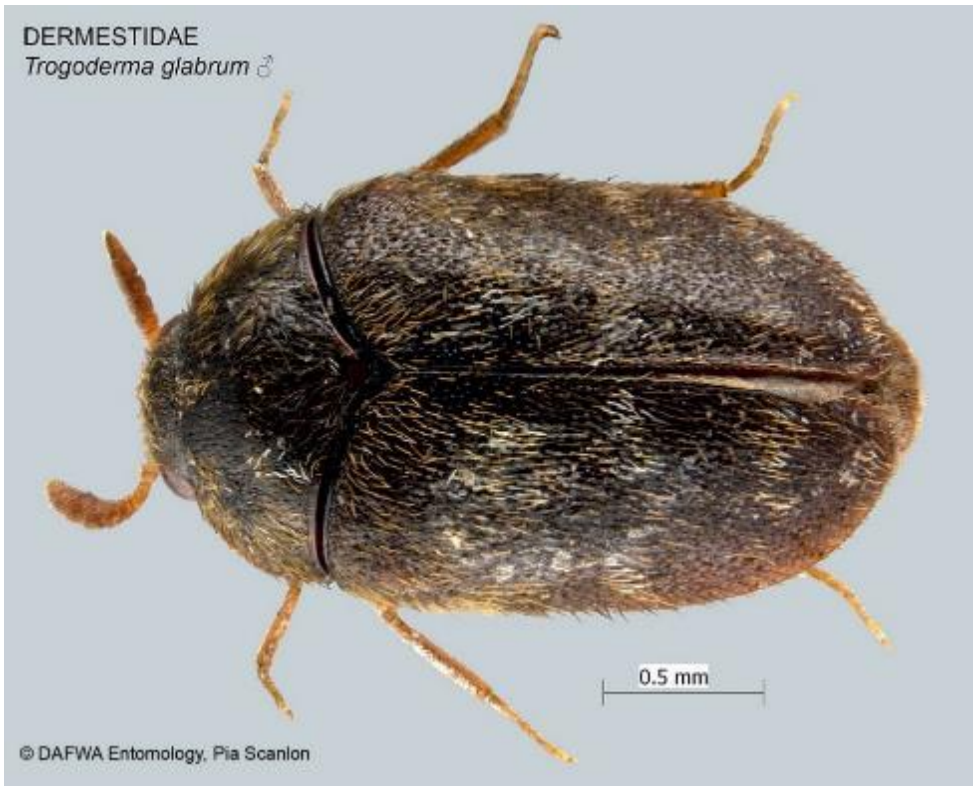


**Figure 22** Adult *Trogoderma glabrum,* dorsal view (DPIRD, P Scanlon)

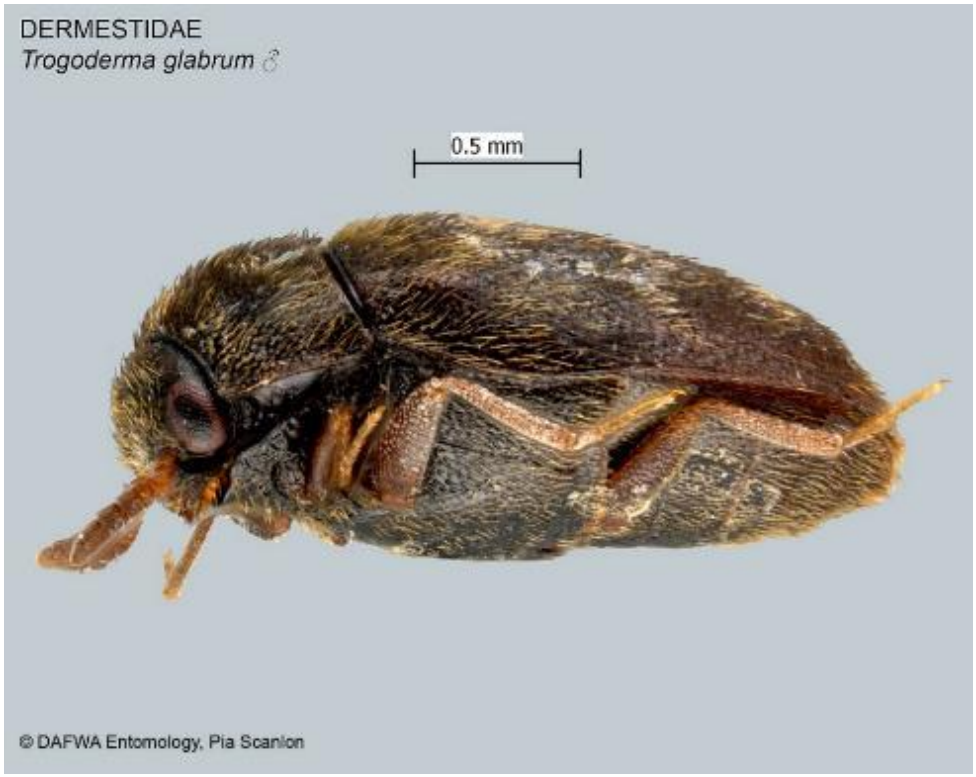


**Figure 23** Adult *Trogoderma glabrum*, lateral view (DPIRD, P Scanlon)


**Figure 24** Adult *Trogoderma glabrum*, ventral view (DPIRD, P Scanlon)

### *8.3.5 Dermestid beetles that commonly occur in Australian stores and households*

***Anthrenocerus* spp.**

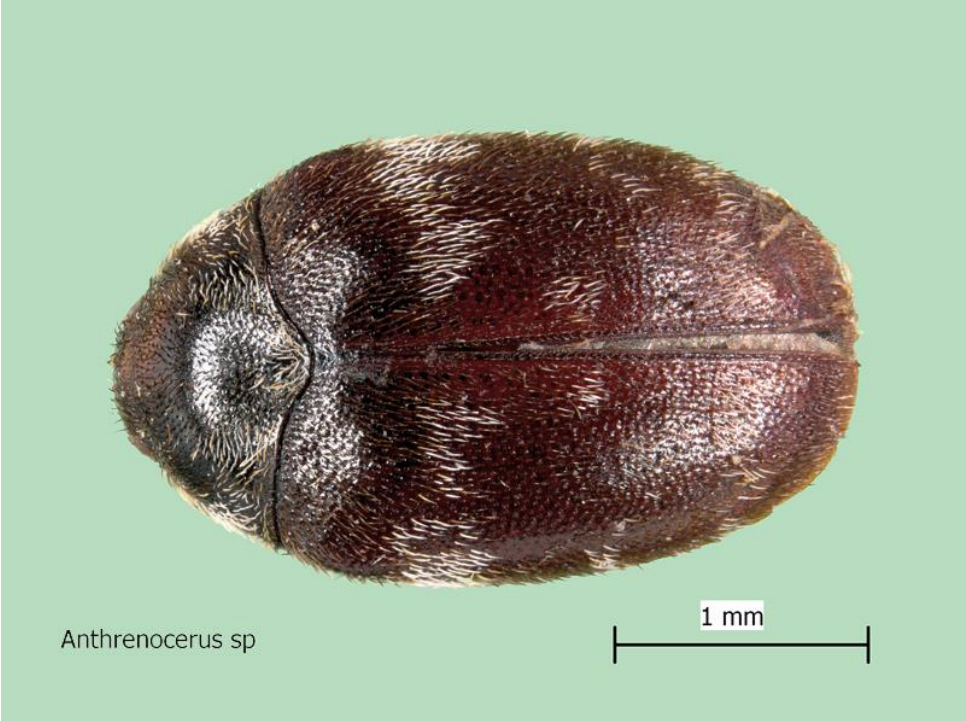


**Figure 25** Adult *Anthrenocerus* sp., dorsal view (DPIRD, P Scanlon)


**Figure 26** Adult *Anthrenocerus* australis, ventral view (DPIRD, P Scanlon)

**Figure 27** *Anthrenocerus* sp., larvae (DPIRD, P Scanlon)

### *Anthrenus* spp.

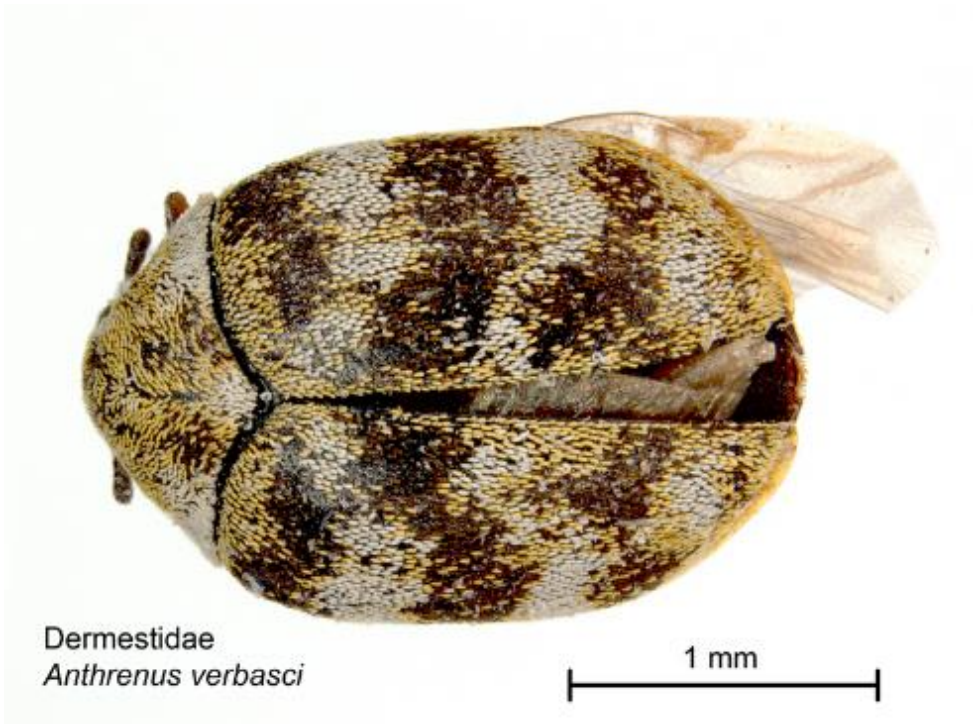


**Figure 28** Adult *Anthrenus verbasci*, dorsal view (DPIRD, P Scanlon)

**Figure 29** *Anthrenus verbasci*, adult and larva (DPIRD, P Scanlon)

### *Attagenus* spp.

**Figure 30** *Attagenus* sp., larva – European carpet beetle (DPIRD, P Scanlon)

### *8.3.6 Native Australian* Trogoderma *spp.*


**Figure 31** Adult native Australian *Trogoderma* sp. (DPIRD, P Scanlon)


**Figure 32** Adult native Australian *Trogoderma* sp. (DPIRD, P Scanlon)

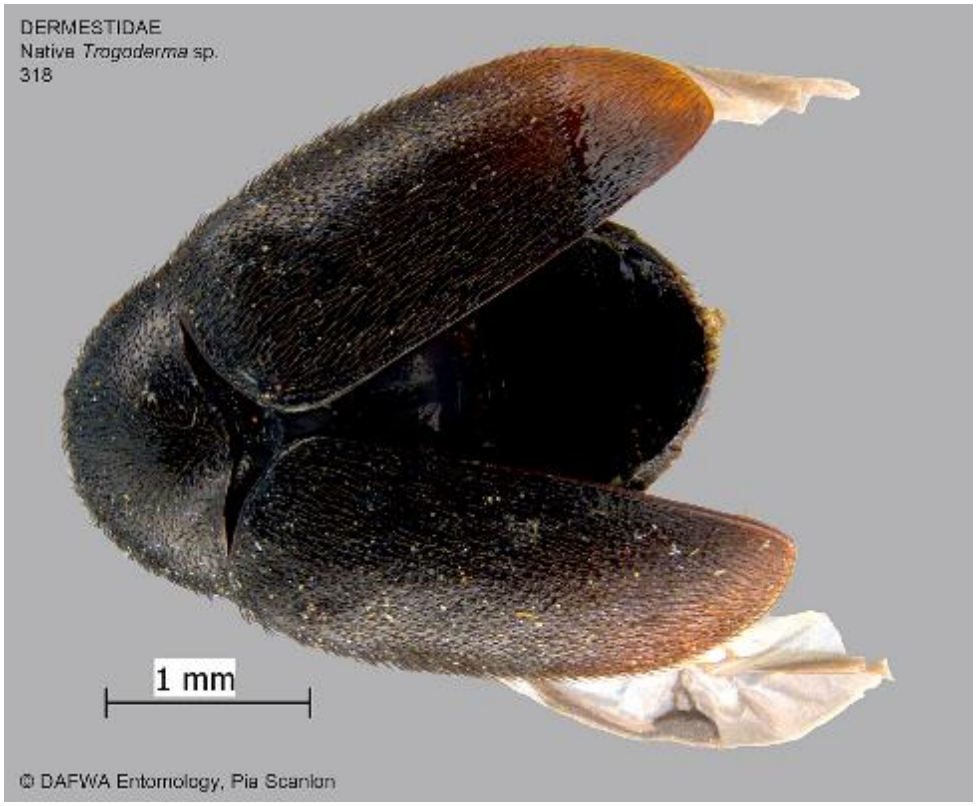


**Figure 33** Adult native Australian *Trogoderma* sp. (DPIRD, P Scanlon)

**Figure 34** Native Australian *Trogoderma* sp., larva (DPIRD, P Scanlon)

**Figure 35** Native Australian *Trogoderma* sp. larva (DPIRD, P Scanlon)

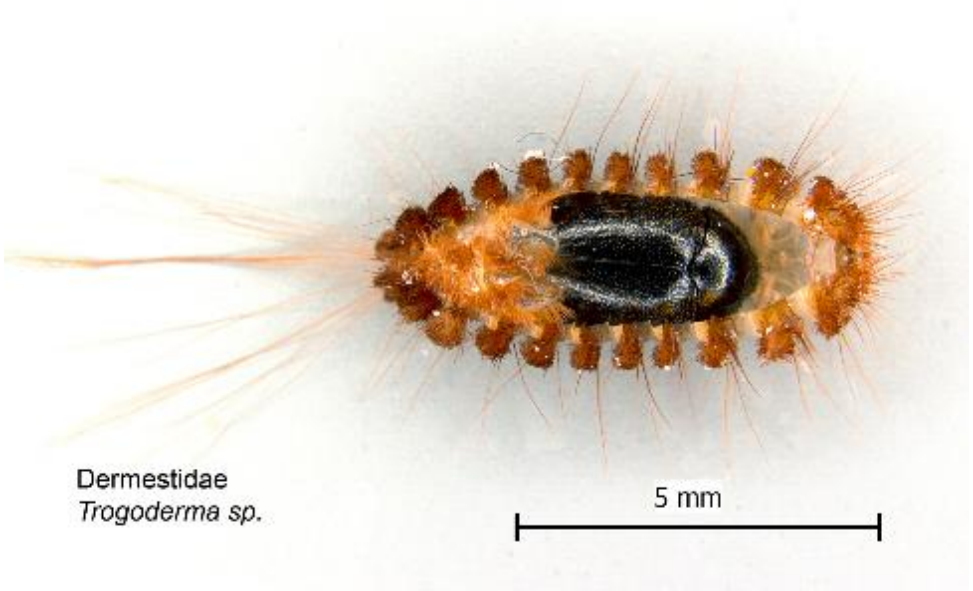


**Figure 36** Native Australian *Trogoderma* sp. (DPIRD, P Scanlon)

## 8.4 Annex X to ISPM 27:2006 *Trogoderma granarium* Everts [figures]

Source: https://onlinelibrary.wiley.com/doi/full/10.1111/epp.12080#pane-pcw-figures

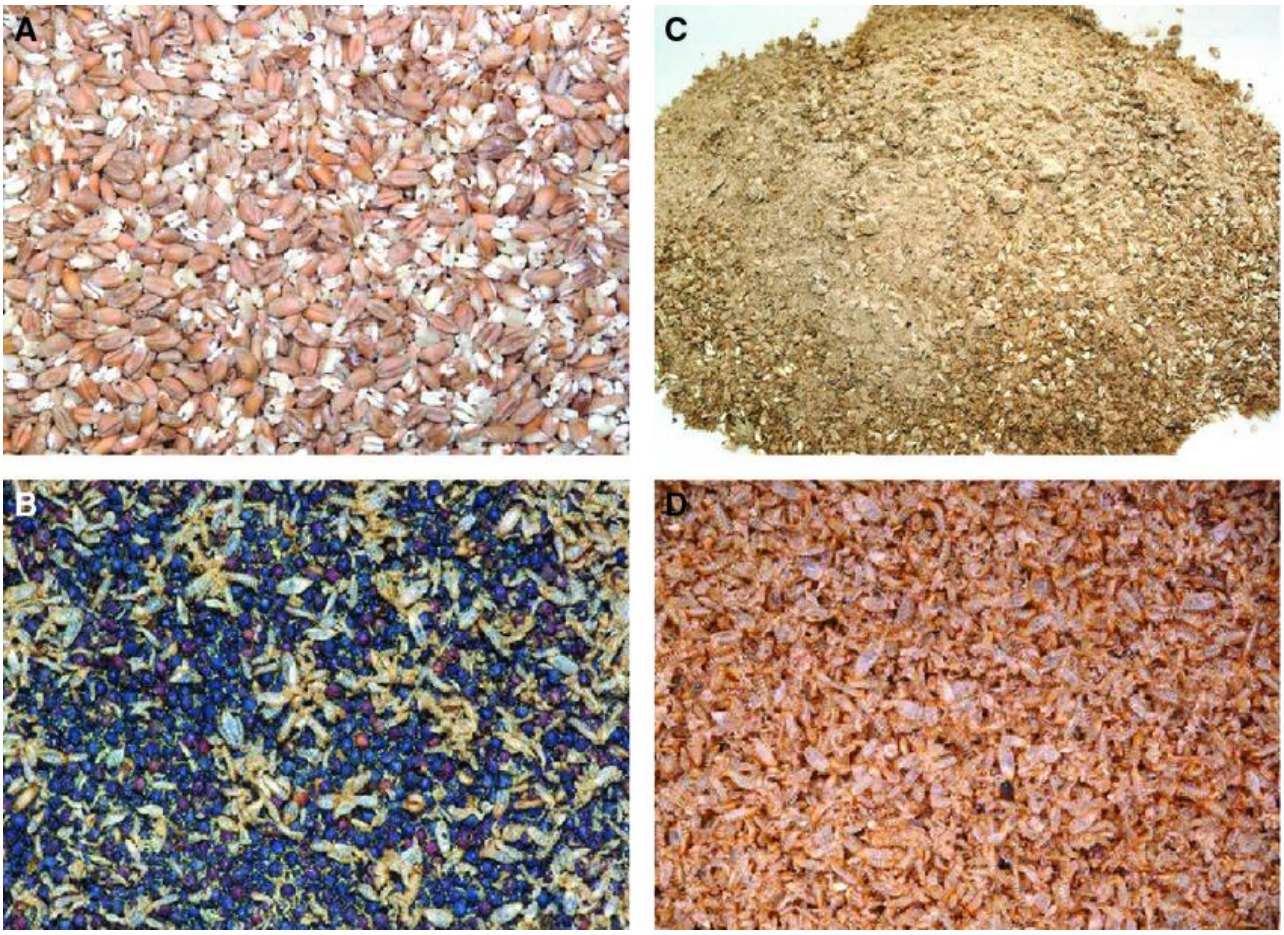


**Figure 1** Symptoms of infestation of stored products with *Trogoderma granarium*: (A) damaged wheat grain; (B) infested rape seeds; (C) totally destroyed wheat grain (dust and remains of grains); (D) larval exuviae (cast skins) contaminating stored product (Paweł Olejarski, Instytut Ochrony Roślin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

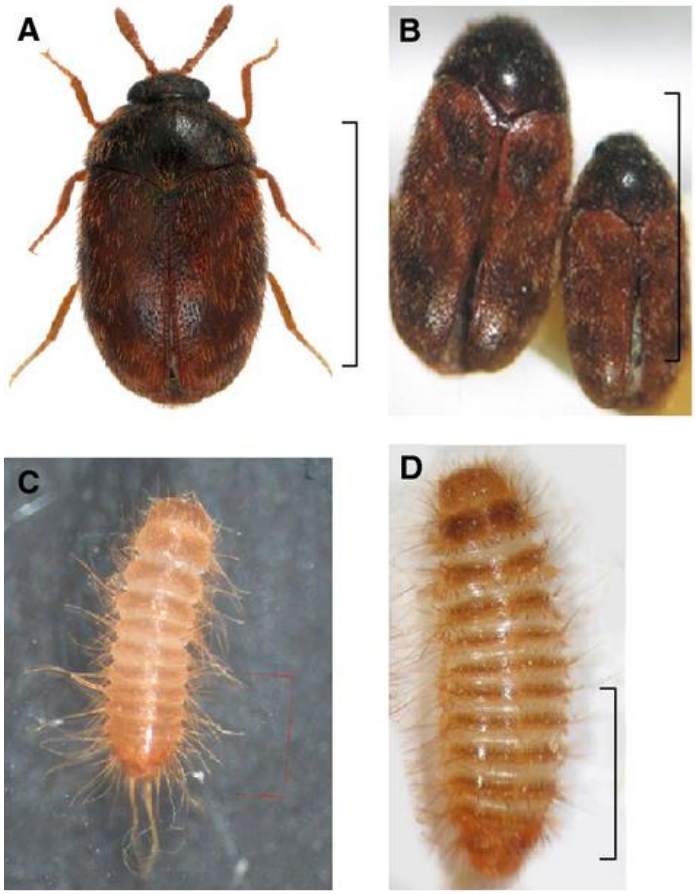


**Figure 2** *Trogoderma granarium*: (A) adult, female; (B) comparison of shape of female (left) and male (right); (C) young larva; (D) mature larva. Scale bar: (A), (B), (D) = 2 mm; (C) = 1 mm. ((A), Tomasz Klejdysz, Instytut Ochrony Roślin - Państwowy Instytut Badawczy, Poznań, Poland; (B), (D), Ya.B.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

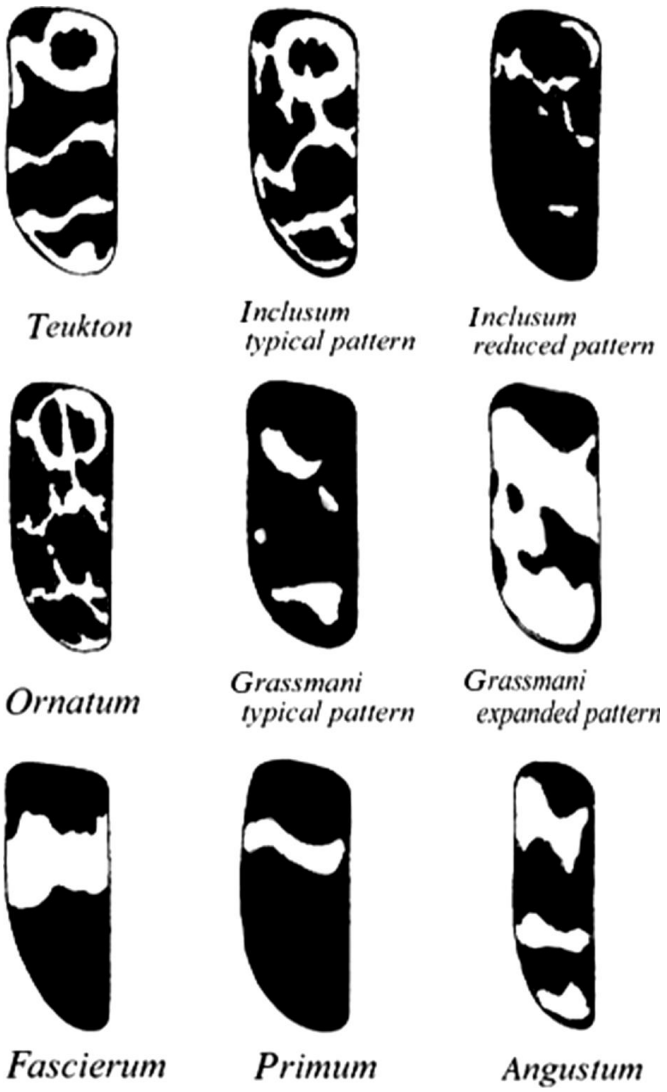


**Figure 3** *Trogoderma* spp. elytral pattern (Beal, 1954).

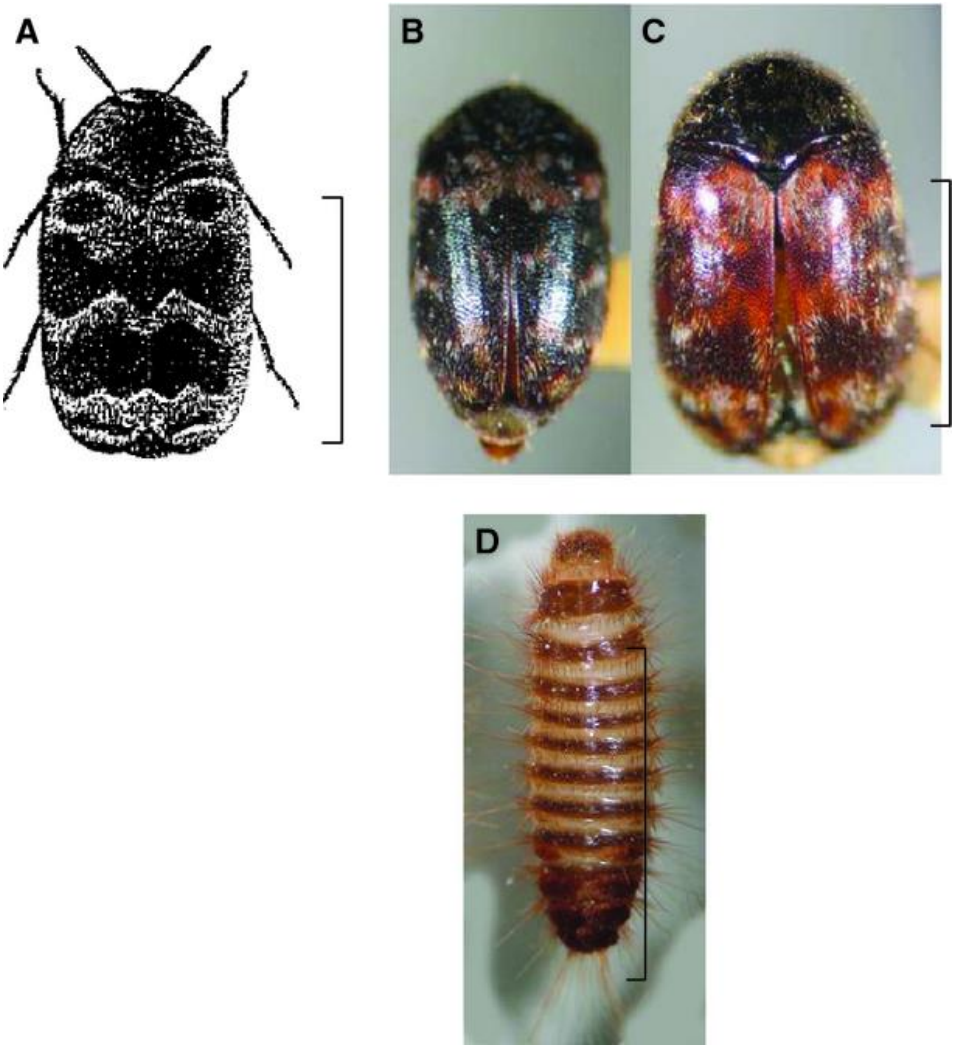


**Figure 4** *Trogoderma variabile*: (A) schematic drawing of the adult; (B) male; (C) female; (D) larva. Scale bar = 2 mm. ((A), OIRSA (1999b); (B)–(D), Ya.B. Mordkovich and E.A. Sokolov, All-Russian Plant Quarantine Centre, Bykovo, Russia).

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

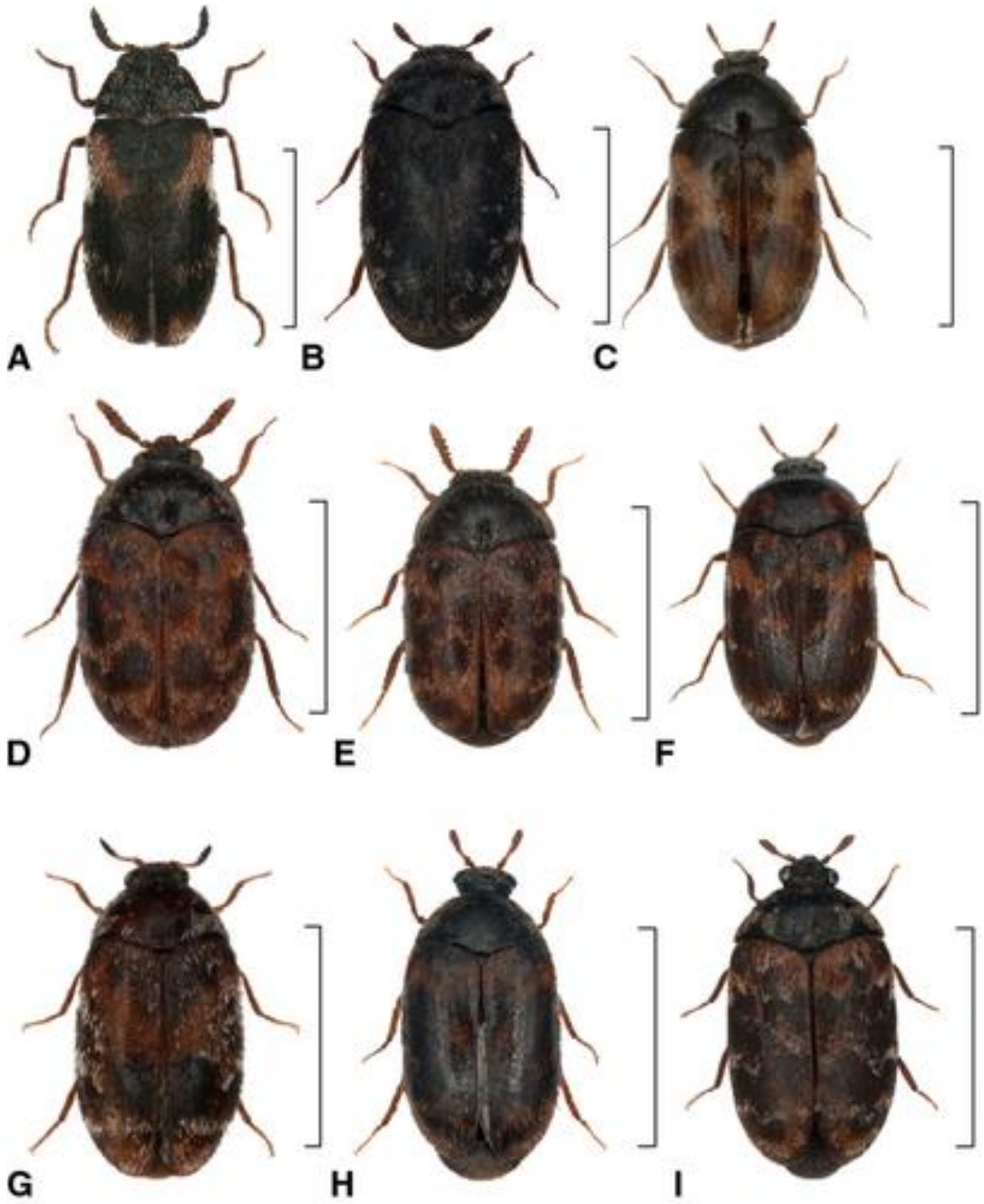


**Figure 6** Comparison of females of some *Trogoderma* non-granarium species: (A) *T. angustum*; (B) *T. glabrum*; (C) *T. grassmani*; (D) *T. inclusum*; (E) *T. ornatum*; (F) *T. simplex*; (G) *T. sternale*; (H) *T. variabile*; (I) *T. versicolor*. Scale bar = 2 mm. (Tomasz Klejdysz, Instytut Ochrony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

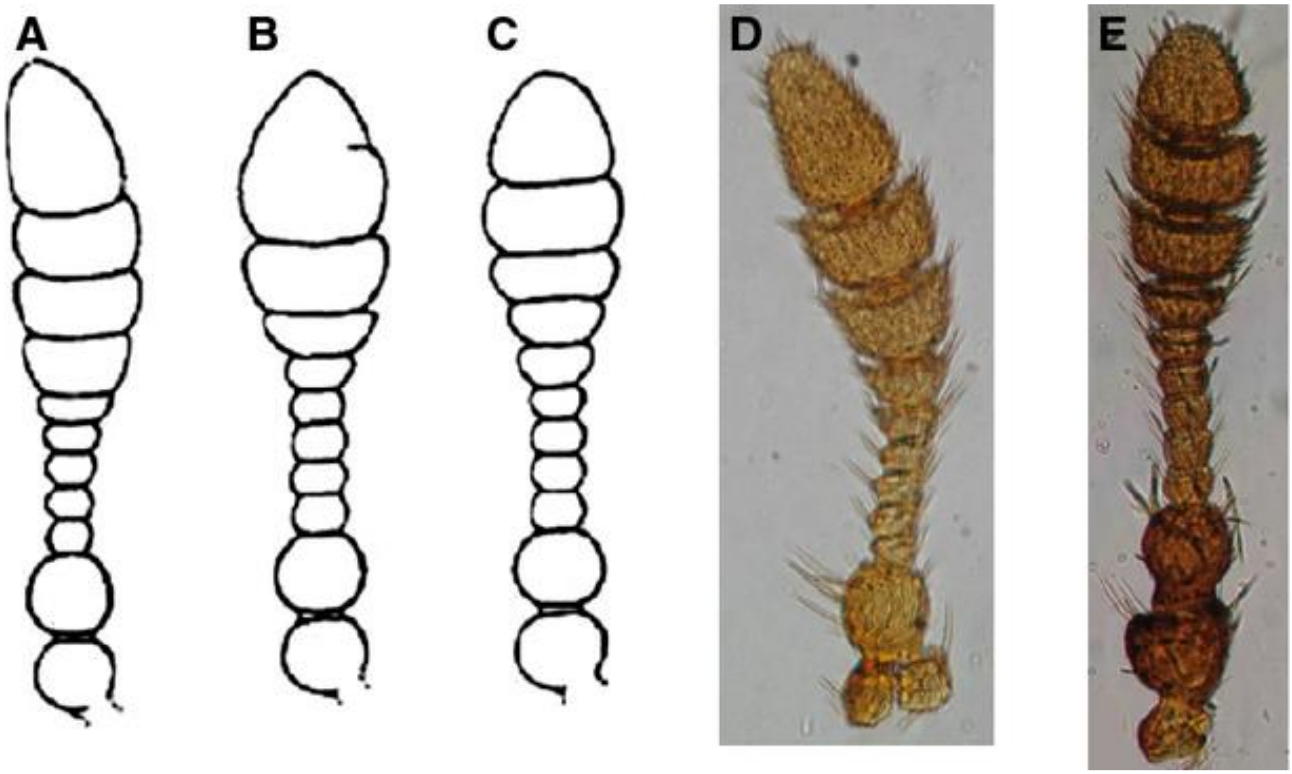


**Figure 7** Antennae of *Trogoderma granarium*: (A), (D) male antenna with normal number of segments; (B) female antenna with reduced number of segments; (C), (E) female antenna with normal number of segments ((A)–(C), Beal (1956); (D), (E), Ya.B. Mordkovich and E.A. Sokolov, All-Russian Plant Quarantine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

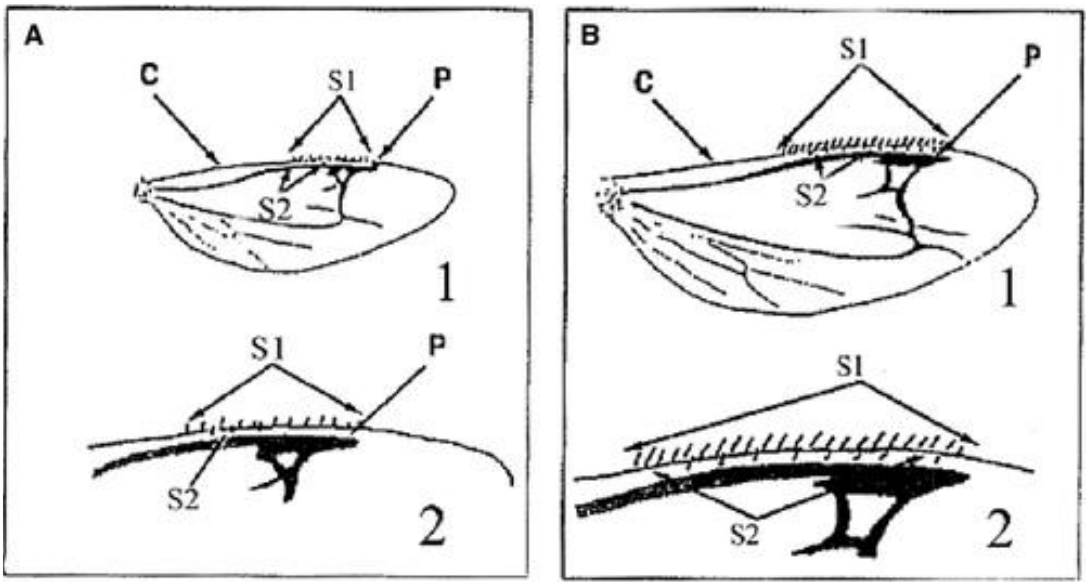


**Figure 9** Schematic representation of the morphology of the hind wing: (A) *Trogoderma granarium* (Maximova, 2001), with up to 14 S1 setae on costal vein (mean = 10 S1), and 2–5 S2 setae, or with no S2 setae, between costal vein and pterostigma (mean = 2 S2); (B) *Trogoderma variabile* and *T. glabrum* 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.

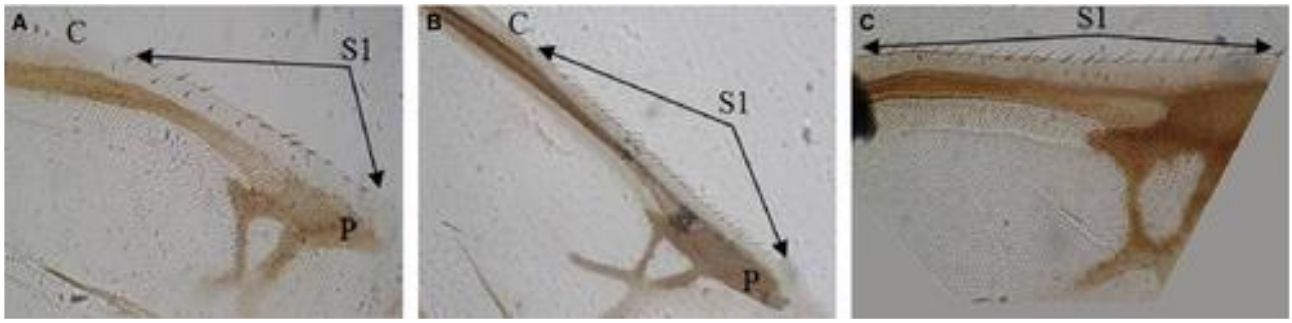


**Figure 10** Morphology of hind wings: (A) *T. granarium*; (B) *T. glabrum*; (C) *T. variabile* (Ya.B. Mordkovich and E.A. Sokolov, All-Russian Plant Quarantine Centre, Bykovo, Russia).

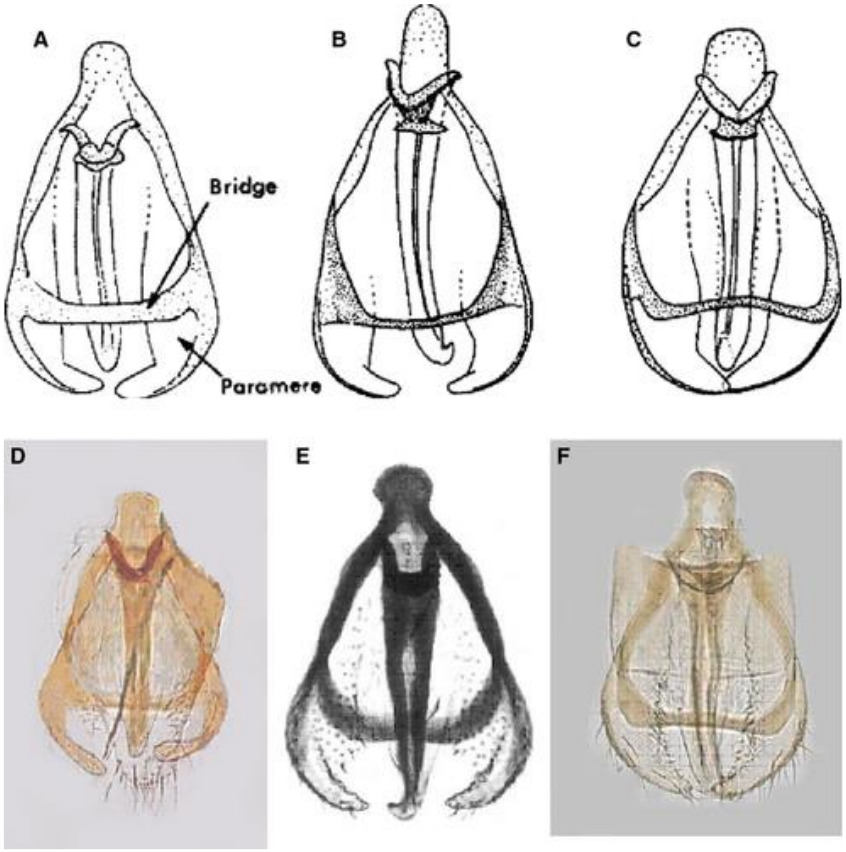


**Figure 11** Male genitalia: (A), (D) *Trogoderma granarium*; (B) *T. inclusum*; (C), (F) *T. variabile*; (E) *T. glabrum* ((A)–(C), Green (1979); (D)–(F), Ya.B. Mordkovich and E.A. Sokolov, All-Russian Plant Quarantine Centre, Bykovo, Russia.

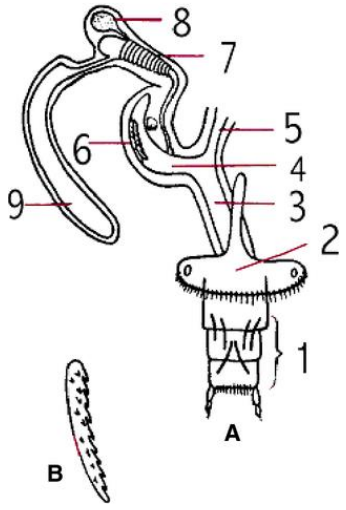


**Figure 12** Female genitalia of *Trogoderma granarium*: (A) general view of genitalia; (B) one of the serrate sclerites from the bursa copulatrix (Varshalovich, 1963). Details: 1, ovipositor; 2, 7th abdominal sclerite; 3, vagina; 4, bursa copulatrix; 5, oviduct; 6, two serrate sclerites on bursa copulatrix;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

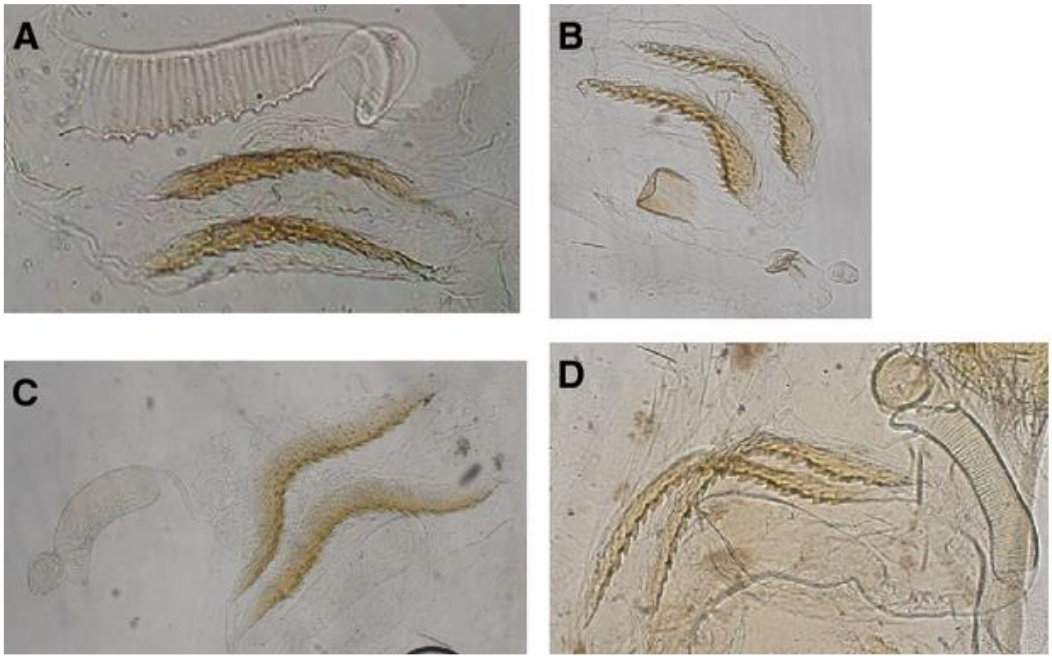


**Figure 13** Serrate sclerites from the bursa copulatrix of female genitalia of various *Trogoderma* species: (A) *T. granarium*; (B) *T. variabile*; (C) *T. glabrum*; (D) *T. teukton* (Ya.B. Mordkovich and E.A. Sokolov, AllRussian Plant Quarantine Centre, Bykovo, Russia).

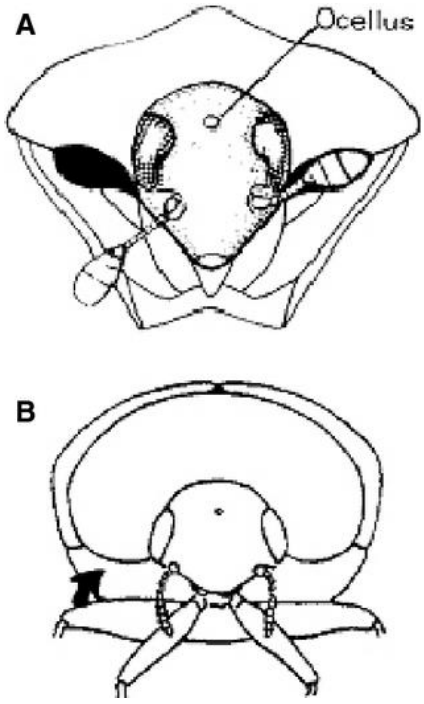


**Figure 14** Antennal cavity: (A) antennal cavity clearly visible in anterior view (*Anthrenus*), antennae fully filling the cavity; (B) antennal cavity not visible in anterior view (*Trogoderma*), antennae loosely fit in the cavity ((A), Mound (1989), copyright: Natural History Museum, London, UK;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

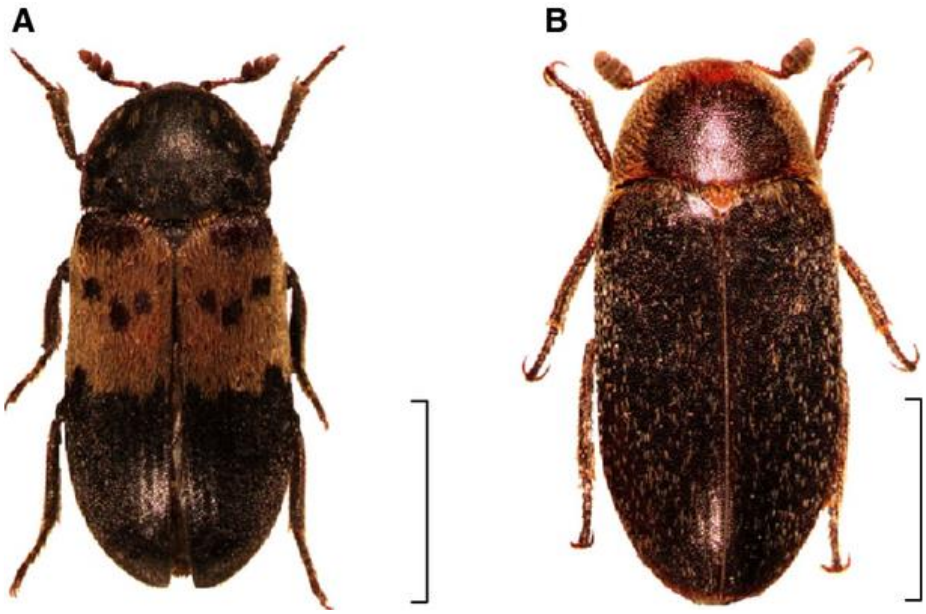


**Figure 15** Adults of *Dermestes* species: (A) *D. lardarius*; (B) *D. maculates*. Scale bar = 2 mm. (Marcin Kadej, Instytut Zoologiczny, Uniwersytet Wrocławski, Wrocław, Poland).

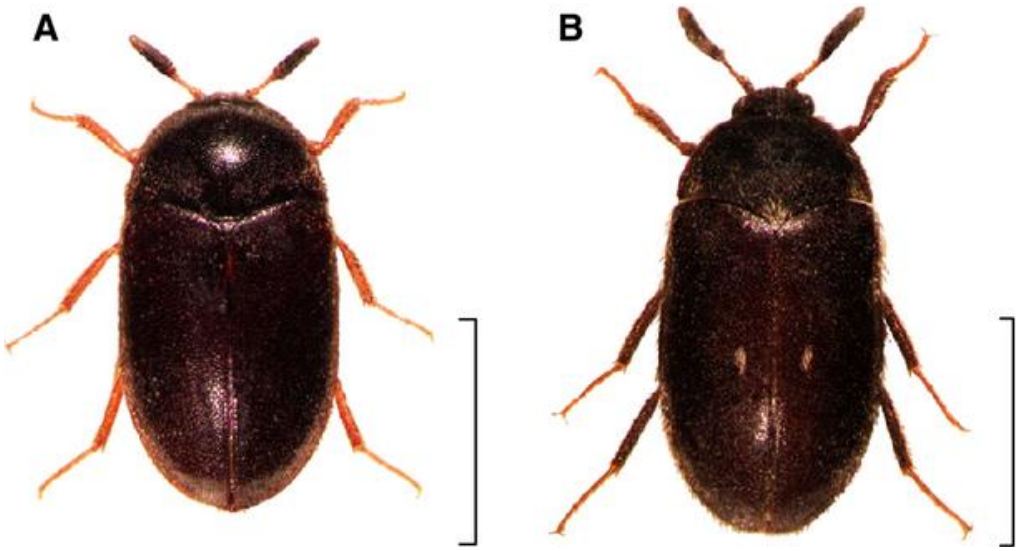


**Figure 16** Adults of *Attagenus* species: (A) *A. unicolor*; (B) *A. pellio*. Scale bar = 2 mm. (Marcin Kadej, Instytut Zoologiczny, Uniwersytet Wrocławski, Wrocław, Poland)

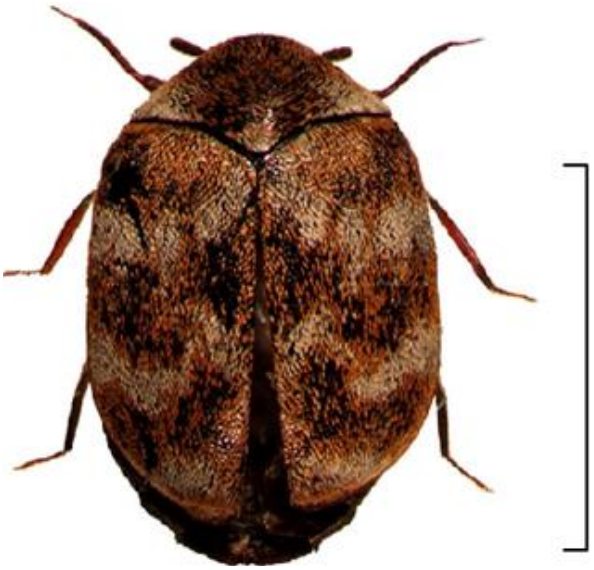

**Figure 17** Adult of *Anthrenus verbasci*: Scale bar = 2 mm. (Marcin Kadej, Instytut Zoologiczny, Uniwersytet Wrocławski, Wrocław, Poland).

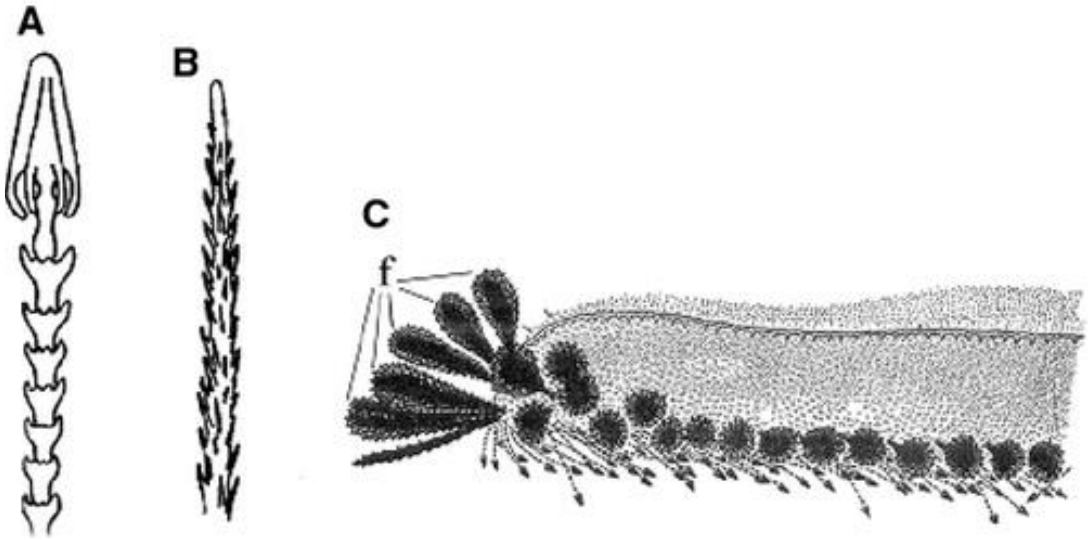


**Figure 18** Larval setae: (A) hastiseta; (B) spiciseta; (C) fiscisetae (f) on first abdominal tergum of *Trogoderma carteri* larva ((A), (B), Varshalovich (1963); (C), Beal (1960)).

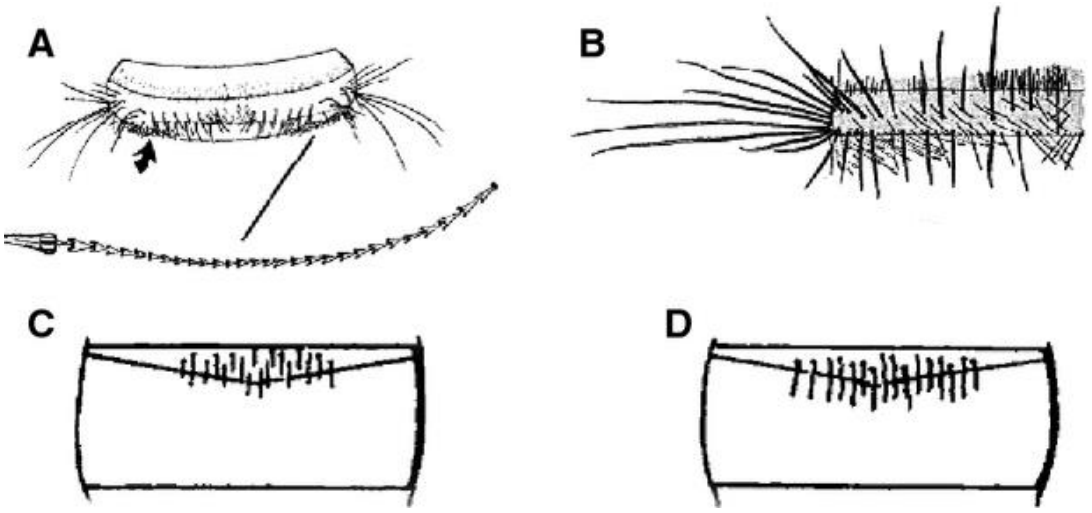


**Figure 19** Abdominal tergite and setae: (A) abdominal tergite of *Trogoderma variabile* larva with enlarged hastiseta; (B) first abdominal tergite of *T. variabile* larva; (C) setae of the anterior portion of first abdominal tergite not long enough to extend caudally over the antecostal suture (*T. 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)).

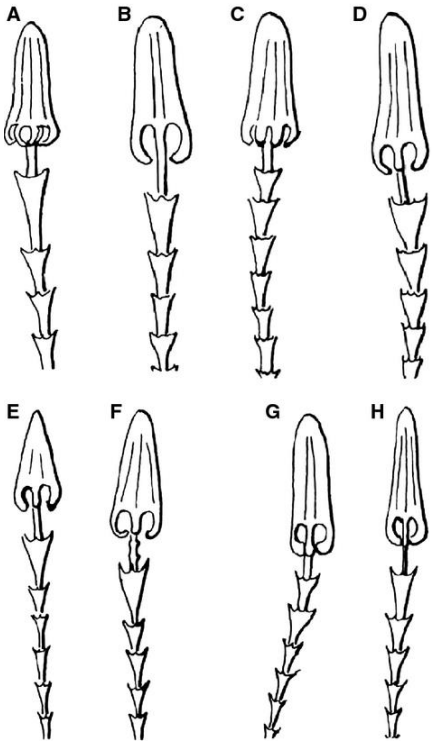


**Figure 20** Comparison of hastisetae morphology of various *Trogoderma* larvae: (A), (B) *T. granarium*; (C), (D) *T. glabrum*; (E), (F) *T. variabile*; (G), (H) *T. inclusum*; copyright: Natural History Museum, London, UK (Peacock, 1993).

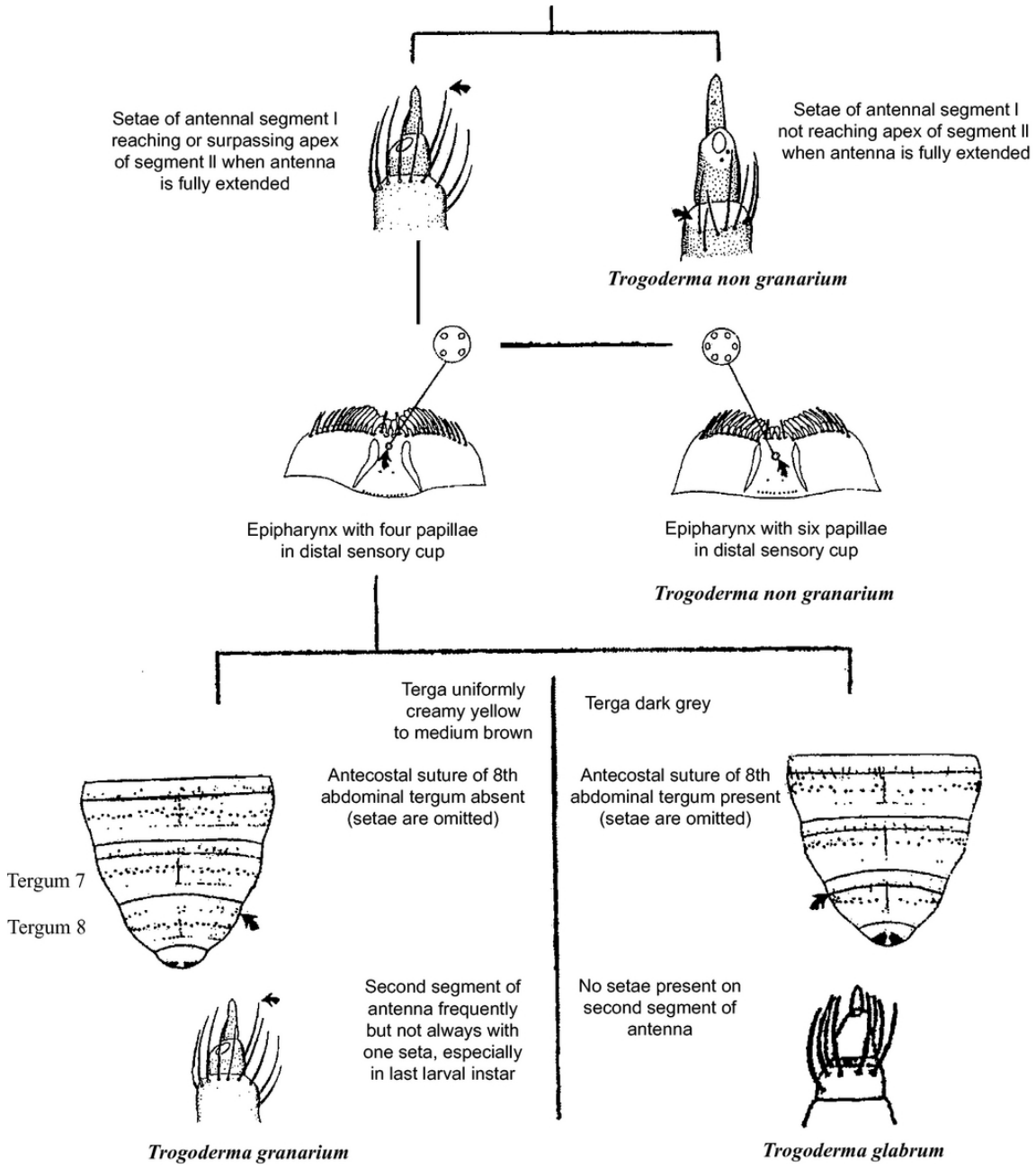


**Figure 21** Pictorial key for distinguishing larvae of *Trogoderma granarium* from other species of *Trogoderma* (Kingsolver, 1991; OIRSA, 1999a).

**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).

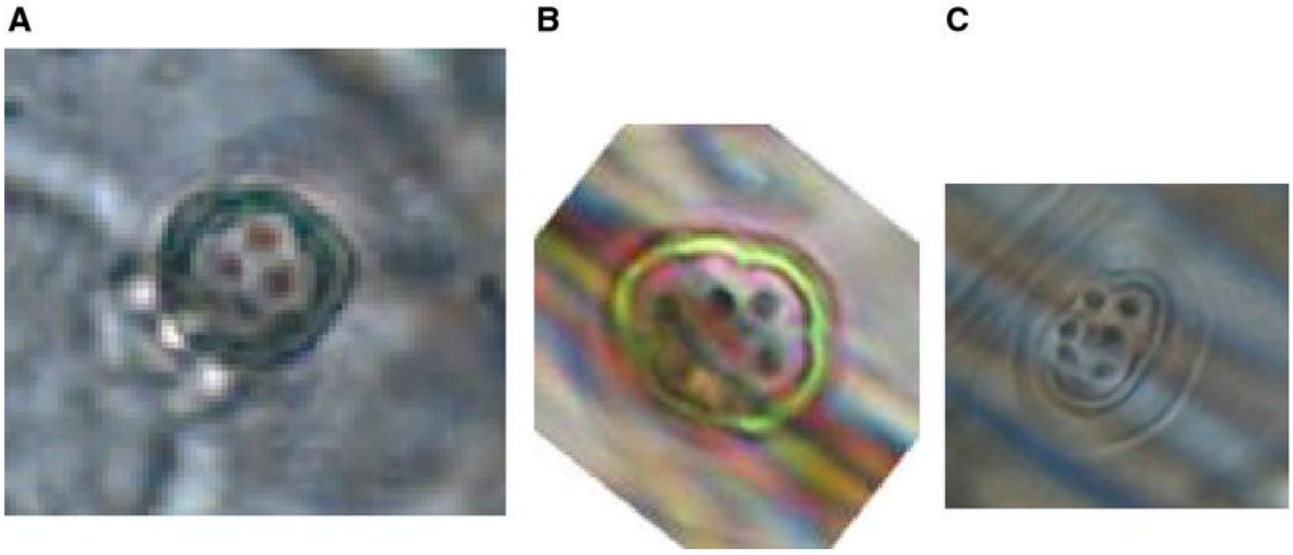


**Figure 23** Distal papillae: (A) four distal papillae in sensory cup of *T. granarium* larva; (B) six distal papillae in *T. variabile*; (C) six distal papillae in *T. glabrum*. (Ya.B. Mordkovich and E.A. Sokolov, All-Russian Plant Quarantine Centre, Bykovo, Russia).

## 8.5 Equipment and reagents used for diagnostics in this protocol

Where applicable, manufacturer details have been provided, although substitution with a similar product/application is acceptable. However, substitution of these products requires in-house verification on the platforms available within the laboratory.

**General laboratory requirements**

- 0.2mL and 1.5mL molecular grade microcentrifuge tubes (mct)
- Microcentrifuge tube racks
- 2.0 mL screw cap tubes with O-ring
- Cryoboxes (suitable for 2.0 mL screw cap tube)
- PCR 8-well strip tubes
- Adjustable pipettes (0.5–10µL, 20–200µL and 100-1000µL)
- Sterile filter tips (suitable for volumes above)
- Disposable gloves (latex or nitrile); lab coat
- Vortex mixer
- PCR hood/cabinet

**DNA extraction**

- Toothpicks
- Forceps and scalpel blades
- Fine gauge needle/syringe (e.g., Insulin syringe)

**For HotShot4 extraction method:**

- Thermocycler
- 1M NaOH
- 0.5M EDTA
- 1M Tris-HCl
- Nuclease-free water
- Ice
- 15mL Falcon tubes

**Quick Extract (QE) extraction method**

- Thermocycler
- QuickExtract™ DNA Extraction Solution (Epicentre, USA)
- Ice

**Alkaline lysis KOH extraction method**

- Thermocycler
- 0.3M KOH
- Ice

**Spin column extraction method**

- Heating block, preferably with shaker i.e., thermomixer
- Benchtop centrifuge
- Optional - QIAcube DNA extraction robot (Qiagen)

Follow the manufacturers protocol for any of the following commercially available DNA extraction kits:

| DNA extraction kit | Manufacturer |
|---|---|
| DNeasy® Tissue Kit | Qiagen |
| GenElute™ Mammalian Genomic DNA Extraction Kit | Sigma |
| ISOLATE II Genomic DNA Kit | Bioline |

**Conventional PCR**

- Conventional PCR machine. Suggested instruments include: ProFlex™ (Thermo Fisher Scientific), Mastercycler® nexus (Eppendorf), T100 Thermal cycler (Bio-Rad).
- Conventional PCR master mix; any of the following commercial mixes are recommended:

| Master mix | Manufacturer |
|---|---|
| MangoMix | Bioline |
| MyFi Mix | Bioline |
| HotStarTaq Master Mix Kit | Qiagen |
| GoTaq® G2 Green Master Mix | Promega |

- Primers, as detailed in Section 4.6.2 Universal conventional PCR and Sanger sequencing
- Positive control DNA
- TBE/TAE (0.5/1.0x) buffer
- Molecular grade agarose
- DNA gel stain, e.g., SYBR Safe (Invitrogen), GelRed (Biotium)
- 100bp DNA ladder
- Loading dye
- Gel electrophoresis system
- UV transilluminator/Gel documentation imaging system
- Geneious Prime software for sequence analysis (Biomatters Ltd.)

**Loop-mediated isothermal amplification (LAMP)**

- Genie®III (8 sample capacity) or Genie®II (16 sample capacity) portable real-time fluorometer (Manufactured by OptiGene, UK; supplied by GeneWorks, Australia).
- Genie Explorer software (OptiGene, UK), latest version.
- Genie strip tubes and caps, 150 µl (GeneWorks; OPG-OP-0008-50).
- Isothermal Master Mix (Geneworks OPG-ISO-DR001 or OPG-ISO-DR004) or Fast Isothermal Master Mix (Geneworks OPG-ISO-DR005).
- Primers and probes, as detailed in Section 4.6.3 Loop-mediated isothermal amplification (LAMP) assay.
- Positive control DNA.

**Real-time PCR**

- Real-Time PCR machine and corresponding latest software version. Suggested instruments include: Rotor-Gene Q (Qiagen), MIC (Bio Molecular Systems).
- Real-time PCR master mix; any of the following commercial mixes are recommended:

| Master mix | Manufacturer | Suitable for probe assays | Suitable for multiplex assays |
|---|---|---|---|
| Rotor-Gene Multiplex Kit | Qiagen | Yes | Yes |
| QuantiTect Probe PCR Kit* | Qiagen | Yes | Yes |
| PerfeCTa qPCR ToughMix | Quantabio | Yes | Yes |
| PerfeCTa SYBR FastMIx** | Quantabio | No | No |

* Tested only with Olson and SARDI assay. Should work across all assays.
** Suitable when running singleplex, intercalated dye assays.

- Primers and probes, as detailed in each Real-time PCR assay Section (4.6.4-4.6.6)
- Positive control DNA

# 9 DIAGNOSTICS RPOCEDURES TO SUPPORT SURVEILLANCE

## 9.1 Introduction

The diagnostic techniques described earlier in this National Diagnostic Protocol are appropriate for the identification or detection of *Trogoderma granarium* when the sample matrix is good quality or when there is abundant DNA from a single source (e.g.: from a well-preserved adult or larva). As indicated by the decision matrix (Table 3), as the sample matrix becomes poorer quality and/or DNA quantity and quality declines (e.g. larval skins, single legs in vacuum material) morphology and conventional PCR are unlikely to satisfactorily detect *T. granarium.*

## 9.2 Sampling

Ideally, sampling as part of surveillance to support diagnostics should target larva or adult beetles, which can be achieved using wall or dome traps. Due to the messy feeding nature of *T. granarium* and to maximise detection opportunities, surveillance sampling often focuses on the collection of a dust sweeping which is sifted and examined microscopically for insect material. Samples presented for molecular assessment are frequently single larval skins or insect parts. These trace tissue samples yield low quantities of poor quality, fragmented DNA with high contamination from other DNA sources. This matrix complexity introduces difficulties in data interpretation and definitive assessment of whether *T. granarium* DNA is present in the sample.

## 9.3 In field tests

No in field tests currently exist that can reliably detect *T. granarium*. Work is underway to develop tests for this purpose.

## 9.4 Laboratory tests

### *9.4.1 Recommended methods for detecting* T. granarium *in surveillance samples*

If complete or mostly complete larvae or adults are found during surveillance, any of the methods described earlier in the National Diagnostic Protocol are likely sufficient to detect *T. granarium*. Conventional PCR and Sanger sequencing of surveillance sample DNA extracts is problematic for detecting trace quantities of *T. granarium* when the template contains DNA from multiple sources. In this situation the Trog-tRNA-TyrF and Trog-JerR primers developed by Gopurenko may be the optimum choice since they are quite specific to *T. granarium* and were specifically developed for this situation. Sanger sequencing of real-time PCR product is of limited value because of the amplicon's short length and the unavailability of reference sequences for some gene regions targeted by real-time PCR.

When trace tissue samples are presented for diagnostics, only the LAMP and real-time PCR protocols have suitable sensitivity to detect *T. granarium.* In situations where the DNA is quite degraded, real-time PCR is the most likely option to detect *T. granarium.* The purpose of surveillance testing from trace tissue samples is to detect the presence of DNA consistent with *T. granarium*, usually in a mixed DNA sample.

The quantity of DNA extracted from single larval skin samples is typically very low. Assays will need to be run in simplex to maximise sensitivity and the use of endogenous and exogenous controls will be required to indicate whether assayable quantities of extracted DNA are present. The LAMP assay (section 4.6.3) includes an insect specific 18S ribosomal DNA (18S) LAMP control to confirm presence of assayable insect DNA. The multiplex species-specific *T. granarium* real-time PCR (Furui protocol) (section 4.6.4) employs an 18S control targeting eukaryotic DNA. An amplification of these control assays indicates sufficient amplifiable DNA has been extracted. It should be noted that the 18S eukaryotic internal control in the real-time PCR protocol will amplify any eukaryotic DNA in a sample including contamination from humans. While internal controls may detect significant quantities of total DNA from these mixed sources, the quantity of *T. granarium* DNA (if present) may be much lower. As a result, the 18S eukaryotic internal control can only be used as an indication of PCR inhibition.

The LAMP and real-time PCR protocols described in this NDP are highly specific for and sensitive to *T. granarium* DNA. Extracted DNA from surveillance samples can be highly fragmented due to specimens being collected through sweeping, physically damaged, desiccated or in exposed situations for long periods. Highly fragmented DNA will fail to amplify in the LAMP assay and the arthropod 18S internal control will test for this feature. For real-time PCR, only the 16S, Tg/Tv and KBdetII probe assays described in the National Diagnostic Protocol are recommended for use. Ring testing has demonstrated that they remain highly sensitive on fragmented DNA due to the short length of their DNA target sequences. Initially, it is recommended that all real-time PCR assays be trialled, in simplex, with surveillance sample DNA. If multiplexing assays is of interest, then validation using dilution series of surveillance samples should be conducted to ensure assay sensitivity is not compromised.

### *9.4.2 Interpretation and reporting of molecular results for surveillance samples*

Interpretation of results for material assessed using the LAMP assay described in section 4.6.3 should be as described in that section of the protocol.

Any real-time PCR assay showing amplification in the 'no template control' (NTC) should be dismissed and the assay repeated. Generally, the results from real-time PCR assays will be as described for sections 4.6.4 to 4.6.6. When interpreting results derived from trace tissue samples, a high Cq value (>36) in any *T. granarium* specific real-time PCR assay should not be discounted as a non-detection but should be replicated and investigated further. A high Cq value can be a result of a low copy number of the target (i.e. weak positive). When target copy numbers are low, interpretation is difficult because the detection limit of the assay is approached. Sample replication within an assay will not always yield consistent results. Additionally, consistency of results between different assays is not assured. Differences in gene regions targeted and assay efficiencies, combined with a potentially fragmented DNA template, can lead to ambiguous results between assays.

Due to the difficulties in interpretation of results within assay replicates and between different assays, it is suggested that reporting of real-time PCR results for surveillance samples be restricted to the following four categories and satisfy the specified criteria:

1. **Detection** of DNA consistent with *Trogoderma granarium*
    - Detection in one or more *T. granarium* specific assays, replicated at least twice.
2. **No detection** of DNA consistent with *Trogoderma granarium*
    - No detection in *T. granarium* specific assays AND,
    - Detection of assayable DNA in internal controls.
3. **Inconclusive**
    - Replicates in any *T. granarium* specific assays give inconsistent results.
4. **Indeterminate**
    - When a result cannot be reliably determined, e.g. sample contamination

## 9.5 Acknowledgements

The section on diagnostics to support surveillance was first drafted by Ros Mirrington and Peter Gillespie (NSW Department of Primary Industries) and was reviewed by the authorship of the main protocol (see Section 6).

## 9.6 References

See Section 7.