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Diagnostics
Diagnostic

PM 7/124 (1) *Spodoptera littoralis, Spodoptera litura, Spodoptera frugiperda, Spodoptera eridania*

Specific scope

This Standard provides guidance for the identification of *S. littoralis, S. litura, S. frugiperda* and *S. eridania*.

Specific approval and amendment

This Standard was approved in 2015-09.

Contents

1. Introduction

Spodoptera littoralis, S. litura, S. frugiperda and *S. eridania* are important Noctuid pest species that are highly polyphagous. More than 100 species of host plants are reported, many of which are of economic importance (EPPO/CABI, 1997, Robinson *et al.*, 2010). *S. littoralis* is present in the Palearctic region from Africa and Southern-Europe, the Arabian peninsula into Iran. *S. litura* has its range covering Oriental and Australasian areas with some Palearctic overflows in the region of Iran. *S. eridania* occurs in the South Eastern United States from Maryland south to Florida and west to Kentucky and Texas; in the Neotropics it ranges from Mexico, throughout the Caribbean, and south through Central America to Argentina. *S. frugiperda* is widely distributed in the Americas, occurring from South Central to Eastern Canada, coast to coast in the United States, south to Argentina and throughout the Caribbean (Pogue, 2002). For detailed distributions see EPPO Global Database (https://gd.eppo.int/) and for host ranges see the EPPO Data Sheet and CABI Crop Protection Compendium (CABI, 2015). Figure 1 below describes the diagnostic procedure for *Spodoptera* species.

2. Identity

Name: *Spodoptera littoralis* (Boisduval, 1833)
Synonyms (including former names): *Hadena littoralis* Boisduval
Taxonomic position: Insecta, Lepidoptera, Noctuidae
EPPO code: SPODLI
Phytosanitary categorization: EPPO A2 no. 120; EU annex I/A2

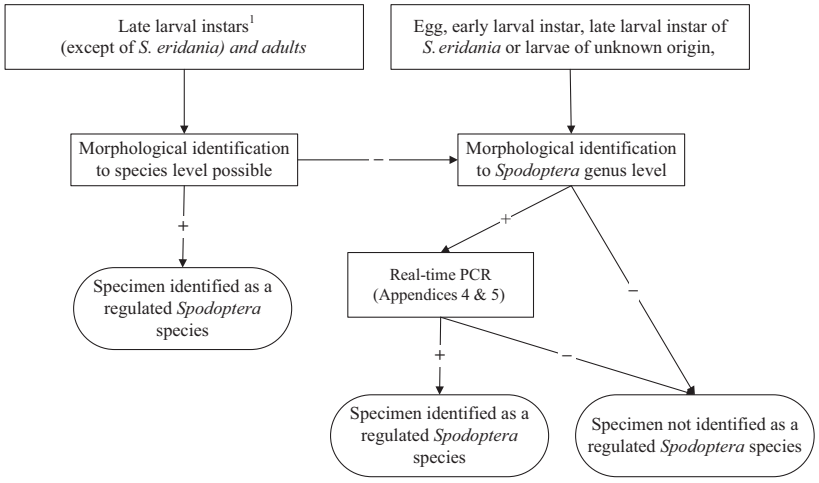
Name: *Spodoptera litura* (Fabricius, 1775)
Synonyms (including former names): *Prodenia litura* Fabricius
Taxonomic position: Insecta, Lepidoptera, Noctuidae
EPPO code: PRODLI
Phytosanitary categorization: EPPO A1 no. 42; EU annex I/A1

Name: *Spodoptera frugiperda* (J.E. Smith, 1797)
Synonyms (including former names): *Laphygma frugiperda* (J.E. Smith)
Taxonomic position: Insecta, Lepidoptera, Noctuidae
EPPO code: LAPHFR
Phytosanitary categorization: EPPO A1 no. 197; EU annex I/A1

Name: *Spodoptera eridania* (Cramer, 1782)
Synonyms (including former names): *Laphygma eridania* (Cramer), *Prodenia eridania* (Cramer), *Xylomyges eridania* (Cramer). This species has many other synonyms, see Todd & Poole (1980).
Taxonomic position: Insecta, Lepidoptera, Noctuidae
EPPO code: PRODER
Phytosanitary categorization: EPPO A1 no. 196; EU annex I/A1

3. Detection

Due to their polyphagous nature *Spodoptera* species can be found on almost all types of commodities of plants or above ground plant parts. Fruits can also be infested by eggs or, more often by, larvae. For host lists see EPPO/CABI (1997). All stages of the pest can be detected visually, with a hand lens for early stages, and specimens can be collected by hand or a sweep net (adults). In the field and in production-, storage-, handling- and other facilities adults can also be detected with the aid of light traps and pheromone baited traps. Pheromone baited traps allow adult males to be caught and light traps catch both female and male adults. Adults can sometimes be found and collected by hand, especially in a commodity that is



[1]When the origin (and host plant) is certain; see main text for specification.

Fig. 1 Diagnostic procedure for *Spodoptera* species.

transported or stored in cool conditions. Eggs can be found on all above ground plant parts, mostly on the underside of leaves. The larvae are mainly external feeders but will occasionally bore into plant parts (EPPO/CABI, 1997). Symptoms of the presence of larvae are holes in fruits or leaves along with the presence of excrements. Early stages are likely to be found scraping the epidermis of the underside of the leaves, but for example in cut flowers such as *Rosa*, larvae tend to migrate to the flowers very soon after hatching. Larvae never tie leaves together. Symptoms caused by the larvae are not specific to *Spodoptera* but generic for most primarily foliage feeding Lepidoptera species. Under natural conditions pupation takes place in the soil where the pupae are difficult to detect. However pupae can incidentally be found in commodities without soil, since larvae will always start pupating when fully grown, regardless of the presence of soil.

4. Identification

4.1. Morphological identification

Equipment needed for the morphological identification is a stereomicroscope (binocular), preferably with a minimum magnification of 25×. For the preparation of genital dissections 70–80% ethanol and potassium hydroxide solution (approximately 10%) and standard dissection tools are needed.

A reliable morphological identification is best carried out on adult stages. Experts with experience on this genus may make an identification to species level based on the morphology of immature stages (in particular larvae), given consideration of context.

4.1.1 Eggs

Eggs of all *Spodoptera* species are laid in groups, ranging from 20 (authors' observation) to over 350 eggs per batch (Peterson, 1964). They are usually laid in one layer, but sometimes in two (partial) layers. Typically *Spodoptera* egg batches are covered with hairs from the females abdomen, coloured creamy white to light camel with tufts of darker hairs in some species (Fig. 2); these hairs are often obvious, but sometimes only a few are present on the egg batch, so careful observation is needed. The eggs are typically noctuid, having ribs and a flat micropyllar rosette (Fig. 3). The eggs are wider than they are high (width × height is approximately 0.45 mm × 0.35 mm). The number of ribs for eggs of species which have been studied is between 37 and 65 (Peterson, 1964; Valverde, 2007; Zenker *et al.*, 2007b; Korycinska, 2012). Since there is much overlap in the number of ribs between the species and the morphology of the eggs of the majority of *Spodoptera* species is unknown, molecular methods are needed for reliable identification of this stage to species level (see 4.2).

Fig. 2 Eggs of *Spodoptera littoralis.* Photo M. vd Straten © NPPO, The Netherlands.

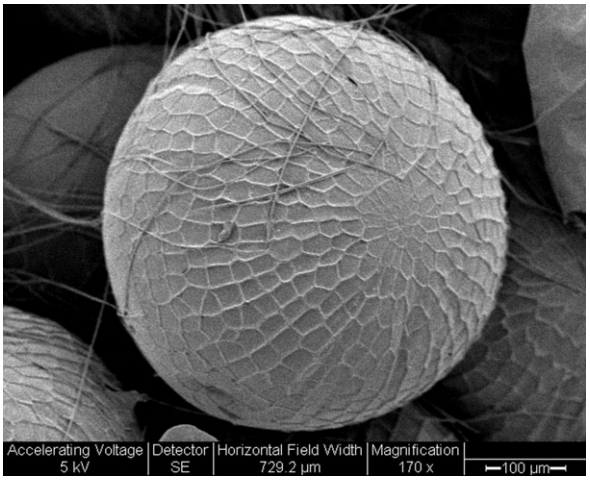


Fig. 3 Detailed SEM image of the egg of *Spodoptera littoralis* Fera © British Crown Copyright.

4.1.2. Larvae

4.1.2.1. Introduction

Several authors have published descriptions of larvae of selected *Spodoptera* species. Descriptions of African species and species from the Near East are given by Brown & Dewhurst (1975). Passoa (1991) describes the larvae of economically important *Spodoptera* species from Honduras and developed a field key to the larvae of *Spodoptera* of America north of Mexico (Passoa, 2008). Zenker *et al.* (2007) describe the larva of *S. cosmioides*. Pogue (2002) describes the larvae of 21 of the 31 species of *Spodoptera*. It should be noted that larvae of *Spodoptera* species are highly variable regarding overall colouration and colour patterns, both between populations but also within populations and, for some species, these can also depend on population density. Furthermore, the colouration strongly fades close to each moulting and in the prepupal stage. Therefore, descriptions given in this protocol include often words such as 'often', 'usually' etc. Due to this variability and the overlap in characteristics between species a reliable morphological identification of all species of the genus *Spodoptera* is not

possible. However, when the origin is known, fully grown larvae of the quarantine *Spodoptera* species, except for *S. eridania,* can be distinguished from other Noctuid species, at least those moved in trade, when careful attention is paid to the details.

Similarly, younger larvae of *S. littoralis, S. litura* and *S. frugiperda* can be distinguished from other species. However, molecular identification is recommended for earlier stages, especially when experience is lacking and when the origin of the larva is unknown. Information for morphological identification is nevertheless provided below for younger stages as well, as it facilitates distinguishing the possible quarantine species from most non-quarantine Noctuid species.

The descriptions given below all are based on live specimens, because colours are important for morphological identification. Nevertheless, the most important features, allowing a separation of the quarantine species from the non-quarantine species, will also be valid for preserved specimens. General information on identification of larvae (morphological structures, chaetotaxy) and identification keys to family level are given in Stehr (2005). Descriptions given below follow the terminology used in Stehr.

Illustrations showing the location of features of larvae are provided in Appendix 1.

4.1.2.2. General description Spodoptera larvae. Spodoptera larvae have the following characters which they share with most other Noctuid larvae:

- 3 pairs of true legs;
- 4 pairs of abdominal prolegs;
- 1 pair of anal prolegs;
- Chaetotaxy is typically Noctuid with two L-setae on the first thorax segment (=prothorax);
- Head with 6 ocelli (stemmata) on both sides;
- Crochets unilateral, arranged in a mesoseries (one line);
- No anal comb.

L1-stage larvae

First stage larvae (egg-larvae) of *Spodoptera* species are 1–2.5 mm long, whitish with a black head and dark pinacula (Fig. 4). They cannot be distinguished morphologically from other *Spodoptera* species nor from most other species of Noctuidae in most cases (and are described under *S. littoralis/litura*). Molecular methods are recommended for identification to species level (see 4.2).

Fully grown Spodoptera *larvae*

In addition to the general characters for the *Spodoptera* larvae given above fully grown *Spodoptera* larvae have the following characters:

- Length is 35–45 mm depending on species
- Overall colour brown or green (younger larvae of all species usually more whitish to greenish; older brown larvae often change colour to dark greenish shortly before moulting). Several species develop black patches and bright orange or yellow longitudinal stripes (Fig. 5).

Fig. 4 Neonate larvae of *Spodoptera eridania.* Photo L. Buss © Insect Identification Lab., Entomology & Nematology Dept., University of Florida.

Fig. 5 Fully grown larva of *Spodoptera littoralis*. © E. Roditakis (Plant Protection Institute Heraklion, GR).

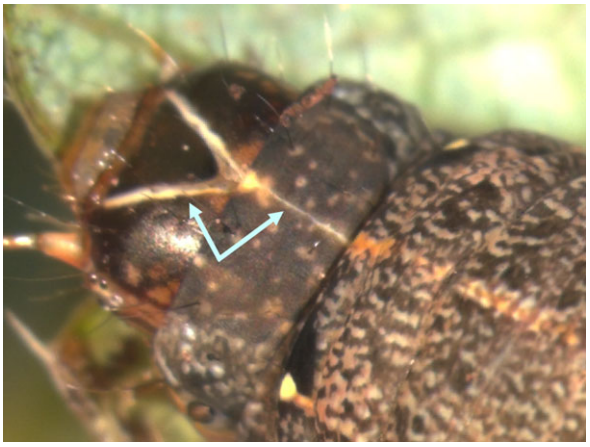


Fig. 6 Y-shaped form on head/prothorax of *Spodoptera litura*. Photo M. vd Straten © NPPO, The Netherlands.

The ground colour, but especially the brighter patterns and black patches present in several species, can change dramatically during development. They also vary greatly between individual specimens even within one population.

- White or pale middorsal stripe and often two white dorsolateral stripes on thoracic shield (=dorsal plate on prothorax).
- Older larvae of most species have white ecdysial lines on the head forming a V-shape, which continues into the white middorsal stripe on the thoracic shield, together forming a Y-shape (Fig. 6).
- The adfrontal sutures do not reach the epicranial notch, as they do with e.g. *Agrotis* species (Passoa, 1991).

The above characters are however not unique for *Spodoptera;* and can be found in several other species of Noctuid larvae. No general characters are known to separate larva of all species of the genus *Spodoptera* from all other Noctuidae larvae.

4.1.2.3. Spodoptera littoralis *and* S. litura. The larvae of *S. littoralis* and *S. litura* cannot be distinguished from each other for most of the stages. The description therefore concerns both species. Only the later instars can be distinguished.

L1-stage larva

First stage larvae (egg larvae) for all *Spodoptera* species conform to the general description for this stage (see above) and cannot be identified morphologically for most of this stage. However, shortly before the first moult an amber-brown spot develops right below SD1 (see Appendix 1) at the first abdominal segment (Figs 7 and 8); this spot will later develop into a larger dark lateral spot on this segment (see Later instars). This lateral spot, and therefore also the amber-brown spot below SD1 in the young larva, is absent in at least *S. exigua* and *S. triturata* (authors' personal observations) and likely to be absent in the other African and Asian species, since they lack the large lateral patch on abdominal segment 1 in the later instars (Pogue, 2002). Therefore, if the larva is known to come from Africa or the Near East, and the cutting edge of the mandible is serrate (see Later instars) and an amber-brown spot is present on the first abdominal segment, it can be identified as *S. littoralis*. Note that young larvae of *S. exempta* also have a serrate cutting edge (Dewhurst, 1999), but the amber-brown spot is likely to be absent. If the larva is known to come from Asia and the spot is present, this can be identified as *S. litura.* Larvae of *S. picta* however strongly resemble those of *S. litura,* especially regarding the presence of a large black lateral patch on the first abdominal segment. Therefore it is likely, that there will be an amber-brown spot in young larvae of *S. picta* as well. It should be noted that *S. picta* is only known from plants of the family Liliaceae.

If the first stage larva does not show an amber-brown spot on the first thorax segment, or if the origin is unknown (see also *S. eridania*), or if the larva was found on a host plant of the family Liliaceae, molecular identification is needed.

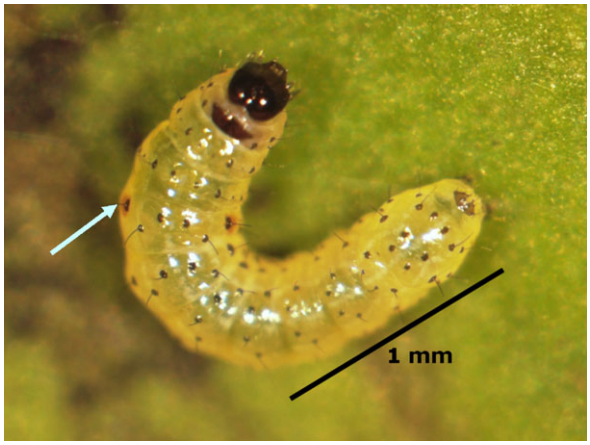


Fig. 7 *Spodoptera litura* 1st instar Photo M. vd Straten © NPPO, The Netherlands.

Fig. 8 *Spodoptera littoralis* 1st (right) and early 2nd instar Photo M. vd Straten © NPPO, The Netherlands.

L2/L3 stage larva

The subsequent stages of instars of *S. littoralis* and *S. litura* can be recognized by the increasing size of the dark spot at the SD1-position on the first abdominal segment. It turns black in early L2-stage (Fig. 8), and becomes more prominent during development into the third larval stage (Figs 9 and 10). Dark spots also develop at the D1 position (see Appendix 1) on the second and third thorax segment and at the eighth abdominal segment, and often smaller black dorsal spots develop on the other segments as well. By the end of the third stage a yellow to white dot is present in the black dorsal spots on the second and third thorax segments (=meso- and metathorax; see Later instars) and in many specimens the middorsal line becomes prominent often turning bright yellow or orange, especially in *S. litura*. The L2 instar is approximately 2.5–8 mm long and the L3-instar approximately 9–20 mm.

Fig. 9 *Spodoptera littoralis* 2nd (left) and early 3rd instar Photo M. vd Straten © NPPO, The Netherlands.

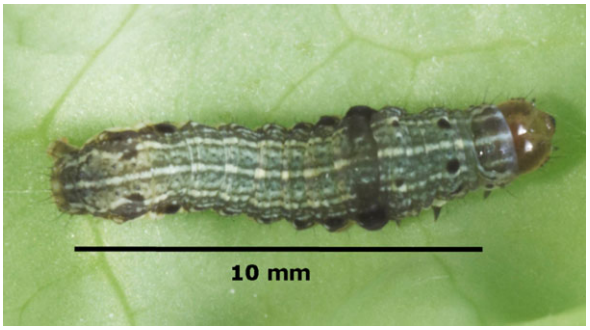


Fig. 10 *Spodoptera littoralis* early 3rd instar. Fera © British Crown Copyright.

Later instars (L4, L5/L6)

The ground colour of the larvae is brown or sometimes greenish. The head is brown to black with a reticulate pattern on the lateral side; the colour gets darker and the reticulation more intense as the larva develops. Typically, older larvae have the Y-shape on head/thorax shield as described earlier. Conspicuous features are the dark patches on the dorsum, most prominently on abdominal segments 1 and 8. Larvae often have a bright yellow or orange middorsal line, but also a less conspicuous subdorsal line marked by yellow or orange spots or dashes. The spiracular line often has a yellowish or orange-pink reticulate colouration with more intense orange or yellow accents. There is a small white or light coloured spot caudal to the spiracle on the abdominal segments. On thorax segments 2 and 3 there are often black dorsal patches, the one on segment 2 is usually the largest; both patches are however clearly smaller than the one on the first abdominal segment. Black dorsal patches on abdominal segments 2–7 can be absent, but when present they are always smaller, or at most equal in size, to the one on abdominal segment 1 (Figs 5 and 11–13). This is a key element in separating *S. littoralis* and *S. litura* from non-quarantine African and Asian Noctuid species: in the latter species, except for

Fig. 11 4th instar of *Spodoptera littoralis.* Photo M. vd Straten © NPPO, The Netherlands.

Fig. 12 4th instar of *Spodoptera litura*. Photo M. vd Straten © NPPO, The Netherlands.

Fig. 13 Fully grown larva of *Spodoptera litura.* Photo M. vd Straten © NPPO, The Netherlands.

Figs 14–17 From the left to the right and top to bottom. *Spodoptera litura*: Yellow dots on 2nd and 3rd thorax segment (arrows). Figs 15–17 Black patches fade before moulting/pupation; yellow dots faint but detectable. Photos M. vd Straten © NPPO, The Netherlands.

S. picta (see below), the black patch on abdominal segment 1 is never larger then on the other abdominal segments. (Note: Pogue's key and description (2002) of *S. littoralis* and *S. litura* state that the dorsal spot on abdominal segment 7 is larger than on abdominal segment 1. Based on the authors' observations this is incorrect: the spot on abdominal segment 7 is at most equal in size but usually much smaller than on abdominal segment 1).

An important characteristic of *S. littoralis* and *S. litura* are small yellow to white dots at the base of the black patches on the second and third thoracic segment (Figs 14–17); these dots distinguish both *Spodoptera* species from the non-quarantine African and Asian *Spodoptera* species.[1] In the later instars (and for all instars shortly before moulting) the black patches on the segments may fade completely, even those on the thorax and abdominal segments 1 and 8. The yellow to white dots on the second and third thoracic segment however remain visible, although sometimes faintly (Figs 15–17). Last instar larvae are 40–45 mm long.

Often, but not always, the larva of *S. litura* can morphologically be distinguished from that of *S. littoralis* by the fact, that in *S. litura* the dark lateral spot on the first abdominal segment extends laterally in such a way, that it strongly interrupts the spiracular line (Fig. 18; Passoa S., pers. comm.). In *S. littoralis* the dark spot also extends laterally, but the interruption of the spiracular line is not as pronounced (Fig. 19). This feature also distinguishes *S. litura* larvae from those of *S. picta*: in *S. picta* the lateral spot of the first abdominal segment does not, or not as sharply, interrupt the spiracular line. Furthermore the middorsal stripe in *S. picta* is distinct and broad, as are the prominent white subdorsal stripes. According to Pogue (2002) in *S. picta* the white to yellow dots on thorax segments 2 and 3 are lacking.

Another characteristic that may add to the morphological separation of *S. littoralis* and *S. litura* from the non-quarantine African and Asian *Spodoptera* species is the cutting edge of the mandible: this is serrate (=separate, pointy teeth) in *S. littoralis* and *S. litura,* and chisel-like (=blunt,

[1]The larva of *S. apertura* is unknown (Pogue, 2002), but it is a relatively rare species so far only known from *Nicotiana tabacum* (Brown & Dewhurst, 1975) and *Drosera* (Robinson, 2010).

Fig. 18 3rd instar of *Spodoptera litura*, interruption of the spiracular line pronounced. Photos M. vd Straten © NPPO, the Netherlands.

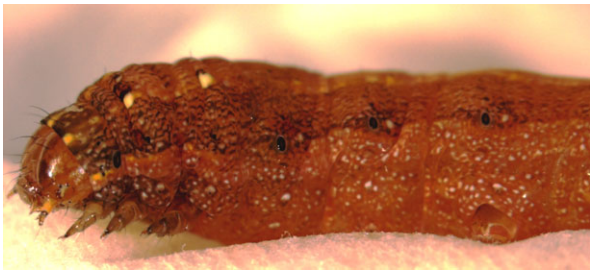


Fig. 19 3rd instar of *Spodoptera littoralis*, interruption of the spiracular line faint. Photo M. vd Straten © NPPO, The Netherlands.

somewhat fused, teeth) in the other African species, except for *S. exigua* (Brown & Dewhurst, 1975). It is also chisel-like in most other Asian species, except for *S. picta, S. pectinicornis* (Pogue, 2002) and *S. apertura* (Kergoat *et al.*, 2012). Further detailed information on the larva of *S. littoralis* & *S. litura*, including setal maps, can be found on respectively http://idtools.org/id/leps/lepintercept/littoralis.html and http://idtools.org/id/leps/lepintercept/litura.html (Gilligan & Passoa, 2014).

4.1.2.4. Spodoptera eridania.
Earlier instars

Observations of colonies reared in the laboratory show that developing larvae are very similar to those of *S. littoralis* and *S. litura*, and are the same size as *S. littoralis*. However in *S. eridania* the dorsal pinacula D1 of first A1 (Fig. 20) and later also A8 (Fig. 21) become enlarged, darker, and slightly raised (Figs. 20 & 21). Also a dark dorsolateral spot develops on A7 (Fig. 20), which is obscured in the later stages. However, since even later instars are difficult to distinguish from many other American *Spodoptera* spp., molecular methods are consequently required for a positive identification of earlier instars (Figs 20 and 21).

Fig. 20 *Spodoptera eridania* 1st instar. Photo L. Buss © Insect Identification Lab., Entomology & Nematology Dept., University of Florida.

Fig. 21 *Spodoptera eridania* 2nd instar. Photo L. Buss © Insect Identification Lab., Entomology & Nematology Dept., University of Florida.

Fig. 22 *Spodoptera eridania* approximately 2nd instar. Photo: L. Buss © Insect Identification Lab., Entomology & Nematology Dept., University of Florida.

Fig. 23 *Spodoptera eridania* approximately 3rd or 4th instar. Photo: L. Buss © Insect Id. Lab., Entomology & Nematology Dept., Univ. of Florida.

Later instars (L4, L5/L6)

The larva of *S. eridania* shows a strong resemblance to the larva of *S. littoralis* and *S. litura* (Figs 22–29). Large dark dorsal patches are present on at least abdominal segments 1 and 8 and usually smaller ones on thorax segments 2 and 3. Also on the other abdominal segments dark dorsal patches can be present but always smaller than the ones on abdominal segment 1 and 8. The yellow to white dot at the base of the patches on the thorax is however missing (Passoa, 1991; Pogue, 2002, authors' pers. obs. on two laboratory colonies). In general the ground colour of *S. eridania* is more greyish than brown, although it can also be greenish. There is also a dark larval form which is predominantly brown.

The fully grown larva is about 35–38 mm in length with a reddish-brown reticulate head (Pogue, 2002).

The normal larval form of *S. eridania* has a prominent dark lateral spot on the first abdominal segment, as in *S. littoralis* and *S. litura*. This spot passes through the spiracular line, but not as pronounced as in *S. litura*; in *S. eridania* this spot obscures the spiracular line on and sometimes in front of the first abdominal segment (Passoa, 1991). However, this lateral spot is present in several other American *Spodoptera* species as well and is therefore not a unique character for identification on its own. According to Passoa *S. eridania* can be distinguished from the other species nevertheless, because in the other species this lateral spot does not pass through the spiracular line. The authors' personal observations show however that the dark lateral marking on abdominal segment 1 can extend into and obscure the spiracular line at that position more or less in other American *Spodoptera* species as well. This character is considered very difficult to interpret by the authors and therefore unsuitable for reliable identification of *S. eridania*. Furthermore the dark larval form of *S. eridania* lacks the lateral spot. According to Passoa this form can still be seperated from at least species present in Honduras, by the fact that the dorsal patch on abdominal segment 1 is always larger than the one on abdominal segment 4.

The cutting edge of the mandibles is serrate, which is the same in all other American *Spodoptera* species (Kergoat *et al.*, 2012), making this character irrelevant for identification.

The most consistent character that identifies *S. eridania* in the authors' opinion, if the origin is America, is the presence of the large dorsal marking on abdominal segment 1 which is larger than the ones, if present, on segments 2–7. However, Zenker reports that at least in the younger stages of *S. cosmioides* there is also a large dark spot/band on the first abdominal segment, which gives it a very similar appearance to *S. eridania*.

Given the high variability of the larvae within the species, the overlap with many characteristics of other American *Spodoptera* species, and the fact that a description of the larvae of several species is lacking, it is clear that a reliable positive morphological identification of *S. eridania* is very difficult. The descriptions given above can help with

Figs 24–29 From the left to the right and top to bottom. *Spodoptera eridania* late instars. Photos: L. Buss © Insect Identification Lab., Entomology & Nematology Dept., University of Florida.

the identification, especially to rule out *S. eridania,* but for a reliable positive identification molecular methods are highly recommended (see 4.2).

4.1.2.5. Spodoptera frugiperda.
Earlier instars

Young larvae of *S. frugiperda* are hard to identify morphologically because early instars of several other Noctuids are very similar. Nevertheless pictures and a description are given here, assisting identification of possible *S. frugiperda* specimens. For a final positive identification molecular diagnostics are required.

Young larvae are greenish or brownish with white longitudinal stripes. They are about the same size as *S. littoralis*. Around SD1 on abdominal segment 1 a pinkish shade often develops, comparable to the amber-brown shade on the same segment in young larvae of *S. littoralis and S. litura* (Fig. 30). Above the spiracles a pinkish line develops, especially on the posterior abdominal segments (Figs 32–33). Also the white middorsal and lateral stripes appear on the thoracic shield. The pattern on the head can be slightly pinkish and is already quite distinct (see picture of the fully grown larva). Starting from L3-stage on the dorsal pinacula on abdominal segment 8 and 9 are larger than on the other abdominal segments (see Later instars); in L1 and L2 stage larvae these dorsal pinacula hardly differ in size. On the anal shield a row of dark brown platelets has developed.

In approximately the L3-stage *S. frugiperda* differs from *S. exigua* by the large dark pinacula and this pink line: in *S. exigua* there can be a pinkish colouration under/around the spiracles, but not a pronounced pinkish line above the spiracular line. Also the pattern on the head is less pronounced in *S. exigua.*

In the young larvae (late L1 to L2/L3) a conspicuous character is the sclerotized base of SD2 on the second and third thorax segment and of SD1 of the ninth abdominal segment, like described and illustrated for the later instars (see below).

Later instars (L4, L5/L6)

The larva of *S. frugiperda* is very different from the other *Spodoptera* larvae. The fully grown larva is about 30

Figs 30–33 Young larva of *Spodoptera frugiperda* (from the left to the right and top to bottom). Upper left: late L1 instar right before moulting; Upper right: early L3 instar. Lower: early L2 instar frontal part (left) and caudal part (right). Photos M. vd Straten © NPPO, the Netherlands.

Figs 34–35 Mid stage instars of *Spodoptera frugiperda,* (top left and right) approximately L3 and L4. Photos M. vd Straten © NPPO, the Netherlands.

Figs 36–41 Second, third and forth line, from the left to the right and top to bottom. Last instar of *Spodoptera frugiperda.* Photos M. vd Straten © NPPO, the Netherlands. Fig. 41: Seta MD1, MD2, MSD1 and MSD2 present on 2nd and 3rd thorax segments; M1 and SD2 present on all abdominal segments (2); a heavily sclerotized spot at ventral margin of SD1- & SD2-pinaculum (3); on 2nd and 3rd thorax segments seta L3 about half the length of L1, L2 is hairlike and situated on ventral margin of the spiracular line; on 1st thorax segment setae SD1 and SD2 on a joint pinaculum positioned ventral of the thorax shield (5); MXD1 is present at posterior margin of thoracic shield.

(Pogue, 2002) to 40 mm (EPPO, 2004) in length and variable in colour, ranging from pinkish, through yellowish, olivaceous, brown and dull grey to almost black (Pogue, 2002; Figs 34–36). The head has a reticulate pattern and is variable in colour, from yellowish to very dark brown; the thoracic shield is the same colour as the head (Figs 34–35, 37). It has no dark dorsal or subdorsal segmental patches. It has conspicuous dark pinacula[2] and the texture of the skin is granulose (at magnification of at least 25×). The dorsal pinacula on abdominal segment 8 and especially 9 are large and have a typical arrangement: on segment 8 they are arranged in a square, on segment 9 in a trapezoid (Fig. 38). The anal plate has dark elongate patches. A further conspicuous element is the hairlike seta SD1 on the second and third thorax segment and on the ninth abdominal segment, which is implanted on a pinaculum with a ringshaped dark sclerotization (Figs 37 and 39). In between the segments two (partial) rows of small dark platelets are visible (Fig. 40). Pogue (2002) states that a lateral patch can be present on the first abdominal segment. In the authors' experience this patch is only very faint, however it is clearly present in the younger stages. The cutting edge of the mandible is serrate, as in all other American *Spodoptera* species (Kergoat *et al.*, 2012). Note that some other Noctuid species also have a granulate skin, e.g. *Agrotis* species; the latter can be distinguished from *Spodoptera* species by the length of the adfrontal sutures (see general description of *Spodoptera* larvae).

Further detailed information on the larva of *S. frugiperda*, including a setal map, can be found on http://idtools.org/id/leps/lepintercept/frugiperda.html (Gilligan & Passoa, 2014).

4.1.2.6. Possible confusion with Spodoptera exigua.

Spodoptera exigua is a cosmopolitan species present in most EPPO countries, either outdoors or in protected cultivation. It is also one of the most frequently intercepted species in trade in EU countries. Therefore some notable characters of this species are given here. Figures of larvae are presented in Figs 42–45.

The larva is green or brownish with thin white interrupted lines ('waves'). The larva usually has no conspicious dark spots, but sometimes dark dashes of about equal size are present on the segments of the posterior part of the body. This dark dash/patch is always smaller on abdominal segment 1 as on abdominal segment 8. It lacks the yellow dots on the 2nd and 3rd thorax segment. Older instars may have a very small dark spot on the 2nd thorax segment just above the spiracular line (Fig. 45). Last instar larvae are 25–30 mm long. Further detailed information on the larva of *S. exigua*, including a setal map, can be found on http://idtools.org/id/leps/lepintercept/exigua.html (Gilligan & Passoa, 2014).

[2]Passoa (1991) reports an overall green form on which the pinacula are concolorous with the skin; it is easily confused with *S. exigua*. A close look will reveal the size of the pinacula and the typical arrangement on abdominal segments 8 and 9, as well as the granulate skin.

Fig. 42 *Spodoptera exigua* second instar (Thailand). Photo M. vd Straten © NPPO, the Netherlands.

Fig. 43 *Spodoptera exigua* fully grown larvae. Photo M. vd Straten © NPPO, the Netherlands.

Fig. 44 *Spodoptera exigua* (Israel): brown form with dark spots. Photo M. vd Straten © NPPO, the Netherlands.

Fig. 45 *Spodoptera exigua* (right below): dark spot on 2nd thorax segment. Photo S.C. Passoa, © USDA-APHIS-PPQ.

Fig. 46–47 Pupa of *Spodoptera littoralis*, dorsal view. Pupa of Spodoptera littoralis, pits on dorsum. Photos M. vd Straten © NPPO, the Netherlands.

4.1.3. Pupae

4.1.3.1. General description. The pupa is brown, about 15–22 mm in length, with a cremaster consisting of 2 spines of about 0.5 mm long (Figs 46–51); these spines can easily break off or are sometimes poorly developed, which makes it an unreliable diagnostic character. There is a ring of pits on the anterior part of abdominal segments 5–8 (Fig. 47). Many other Noctuidae species share these same features of the pupa. Brown & Dewhurst (1975) studied the majority of the African *Spodoptera* species resulting in a key, of which they themselves state 'that the key cannot be very reliable in view of the intraspecific variety and limited material available. . .'. They showed however, that *S. exigua* has an extra pair of very short, thin spines dorsally from the more prominent spines (see 4.1.3.2). This is consistent with the authors' own observations on *S. exigua* and the four *Spodoptera* quarantine species, in which this extra pair of spines is lacking. Therefore with this character pupae of the frequently intercepted *S. exigua*, can be distinguished from pupae of all quarantine *Spodoptera* species. In all other cases however, at the moment, pupae cannot reliably be identified morphologically to species or genus-level. Therefore it is recommended to rear pupa to the adult-stage.

4.1.3.2. Possible confusion with Spodoptera exigua.

The pupa of *S. exigua* can be separated from the pupa of *S. littoralis, S. litura, S. eridania* and *S. frugiperda* by the fact that the cremaster of the pupa of *S. exigua* has an extra pair of short spines implanted dorsally from the long caudal spines (Fig. 52).

4.1.4. Adults

4.1.4.1. Introduction

Identification of *Spodoptera* to genus level is not straightforward; Pogue (2002) gives a few characteristics of the genus based on wing colour and wing pattern, but a reliable description that separates all *Spodoptera* species from all other genera is not available. A comparison with the pictures given in this protocol is the best way to determine, if a specimen looks like the suspected quarantine species. The key given in Appendix 2 can then be used to verify the characters for the species. After that a dissection of the genitalia is needed for reliable identification.

This protocol gives descriptions of the four quarantine species and the characteristics needed for a positive identification. The adults of the majority of species of the genus *Spodoptera* are well known and described. A complete overview of the genus is provided by Pogue (2002) giving extensive descriptions of all species included in the genus. Subsequently one more species was transferred into the genus (Pogue, 2011). Brown & Dewhurst (1975) give an extensive overview of the African species. Information on

Fig. 48–49 Pupa of *Spodoptera littoralis,* cremaster dorsal view. Pupa of *Spodoptera littoralis,* cremaster lateral view. Photos M. vd Straten © NPPO, the Netherlands

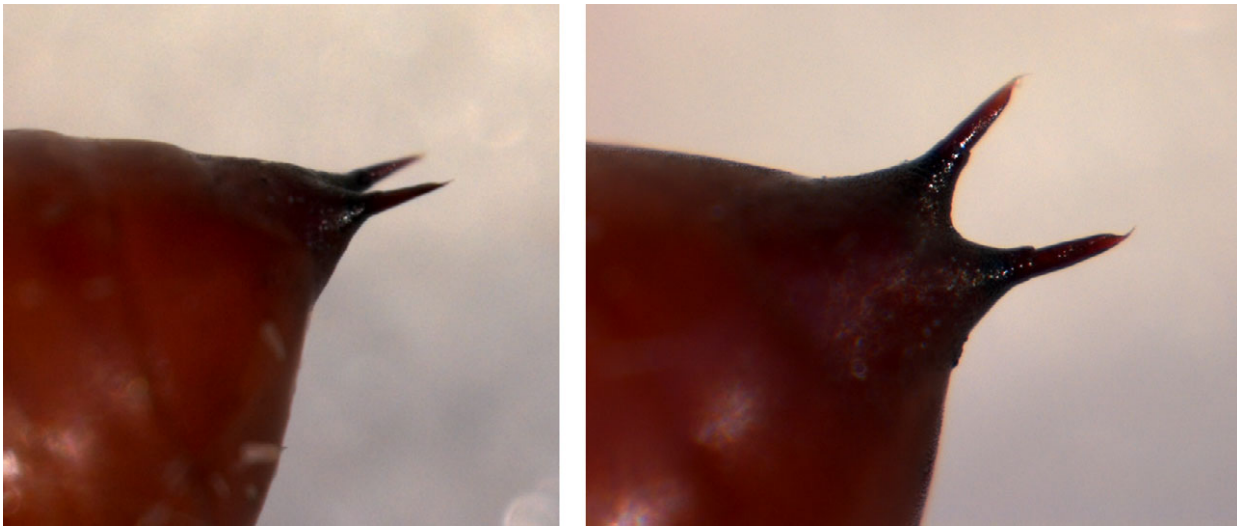


Fig. 50–51 Pupa of *Spodoptera frugiperda,* cremaster dorsal view. Pupa of *Spodoptera frugiperda,* cremaster lateral view. Photos M. vd Straten © NPPO, the Netherlands.

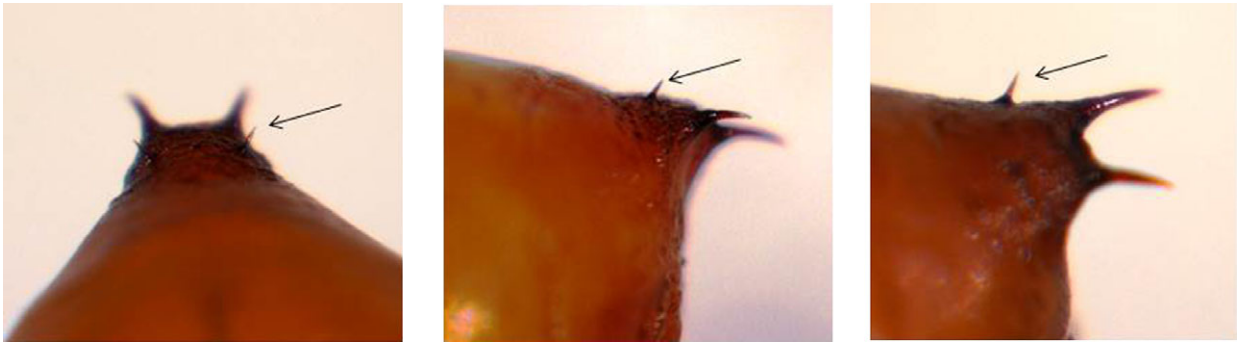


Fig. 52 Cremaster of pupa of *Spodoptera exigua* showing dorsal extra pairs of spines (see arrow) Left: dorsal view. Middle: ventrolateral view. Right: dorsolateral view. Photos M. vd Straten © NPPO, the Netherlands.

moths of Borneo, including most Asian *Spodoptera* species including *S. litura,* is provided by Holloway (1989). Todd & Poole (1980) describe all American *Spodoptera* species. Descriptions and good pictures on common American species, including *S. frugiperda* and *S. eridania*, are provided by Heppner (1995, 1998). Good diagnostic pictures of several American species, including *S. frugiperda* and *S. eridania* are provided online by Zagatti *et al.* (1995–2006). Lalanne-Cassou *et al.* (1994) describe a new species from South-America.

Pogue (2002) gives keys for identification based on external morphological characters (wings, antenna, legs), but for old and new world species separately. Brambila (2009a,b), who developed diagnostic protocols for selected *Spodoptera* species, also gives diagnostic characteristics based on wing patterns, but for species of concern for the USA only. Adults of some of the quarantine *Spodoptera* species, such as *S. eridania* are variable in the overall colouration and colour patterns, both between populations but also within populations. Males and females also differ, some of them dramatically. Therefore, for a fully reliable morphological identification of the quarantine *Spodoptera* species, genital dissection is required, or alternatively, molecular identification can be used.

Nevertheless, the wing pattern is a first and very useful way to facilitate distinguishing the possible quarantine species from most non-quarantine Noctuid species, especially when the origin of the specimen is known. Therefore their descriptions and diagnostic characteristics are given, as well as keys based on the wing pattern. Further descriptions and figures of both male and female genitalia are included. The descriptions are largely based on the work of Pogue (2002) and the work of Brambila (2009a,b), Brambila & Buss (2009). Appendix 3 gives a glossary of the terminology used and an overview of main characteristics of the genitalia of all *Spodoptera* species (with the exception of *S. hipparis*) enabling easy comparison of the quarantine species with the non-quarantine species. In general morphological identification of adults using dissection is not complicated; identification of males is easier than of females in some species.

The procedure for genital dissection can be found in EPPO diagnostic protocol PM 7/72(1). The time needed for *Spodoptera* abdomen or genitalia to soften in potassium hydroxide is about 30 min, depending on how fresh the specimen is. Special care is needed not to damage the coremata, which is a thin, hyaline tissue that is easily ruptured.

For the identification at family level see Arnett (1993) and Delvare & Aberlenc (1989).

4.1.4.2. Spodoptera littoralis.

Habitus (Fig. 53)

Forewings length: 12–16 mm in males and 13–16 mm in females. Five characters in combination could help to distinguish this species but because of the variability in colour they are not sufficient to ensure the identification. They are: reniform spot light brown outlined in white; because of its triangular shape it appears to be a titled letter 'A'; orbicular spot elongate, oblique, narrow, light brown and outlined with a white margin; a row of dark brown or black hour-glass markings along outer margins (between terminal and adterminal lines); a white fork in the median area of wing, the fork is made of veins M3, CuA1 and CuA2; a large yellowish or light brown area on the median area along the inner margin, females lack this light brown patch.

Male genitalia (Fig. 54)

The combination of genitalic characters at valve level allows the identification of this species: large opening at the base of valve (similar to *S. frugiperda*); clavus in the shape of small bump; costal process small, elongate, narrow and curved; ampulla elongate and curved; cucullus truncate (nearly square-edged); juxta quadrate with two ventrolateral projections. This is the most important character, but often difficult to see if it is folded or torn; coremata with two lobes.

Female genitalia (Fig. 55)

Ventral plate of ostium bursa with height greater than width, distal margin straight; ventrolateral invaginated pocket of 8th sternite absent. Ductus bursae short (length less than twice width); completely sclerotized. Appendix bursae membranous. Corpus bursae bulbous, length less than twice width; striate convolutions. Signum in apical half of corpus bursae; short, length <0.65 mm; forming greater than a 45 degree angle to vertical axis of corpus bursae.

Differences with similar species

There are no African species that will be confused easily with *S. littoralis*; only *S. apertura* shows some resemblance, especially with female *S. littoralis*. A simple difference in the male genitalia is, that *S. apertura* has no coremata. In the female genitalia the signum in *S. apertura* is elongate and the ventral plate of the ostium bursae is not wider than the ductus bursae. In *S. littoralis* this is clearly broader then the ductus bursae and the signum is less elongate. See Appendix 3 for more details.

The juxta is the simplest character of the male genitalia to distinguish between *S. littoralis* and *S. litura*, because it is triangular in the latter (see Fig 57). Also in several, but not all, New World species this juxta is not quadrate, but more triangular or much higher. Females of *S. littoralis* can be distinguished from *S. litura* by the ductus bursae, which is elongate in *S. litura* and much shorter in *S. littoralis*. Furthermore the

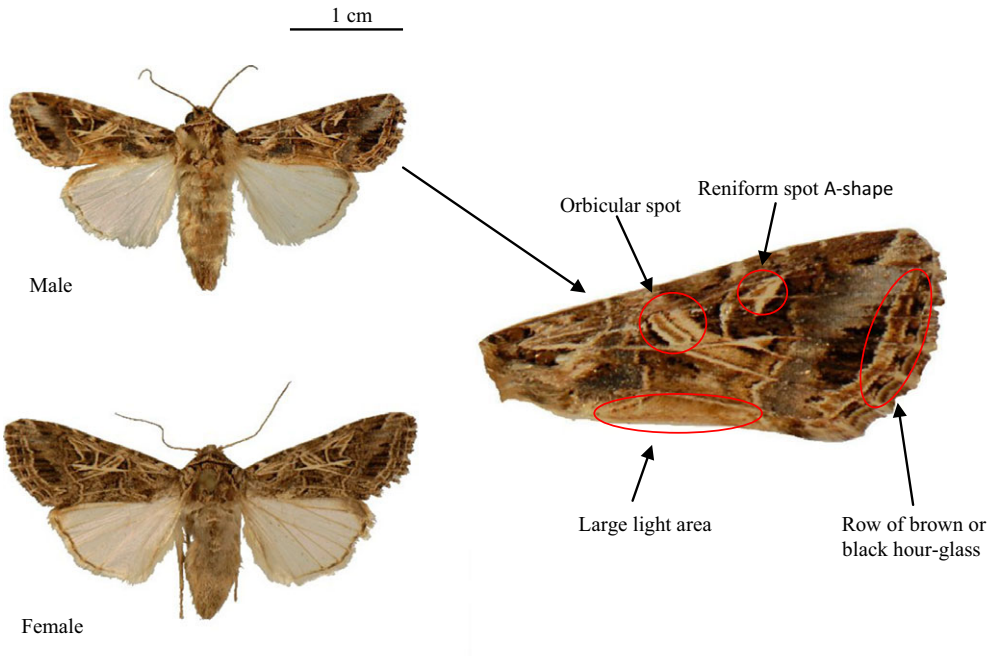


Fig. 53 Adult *Spodoptera littoralis*. Photos © J.-F. Germain, Anses, FR.

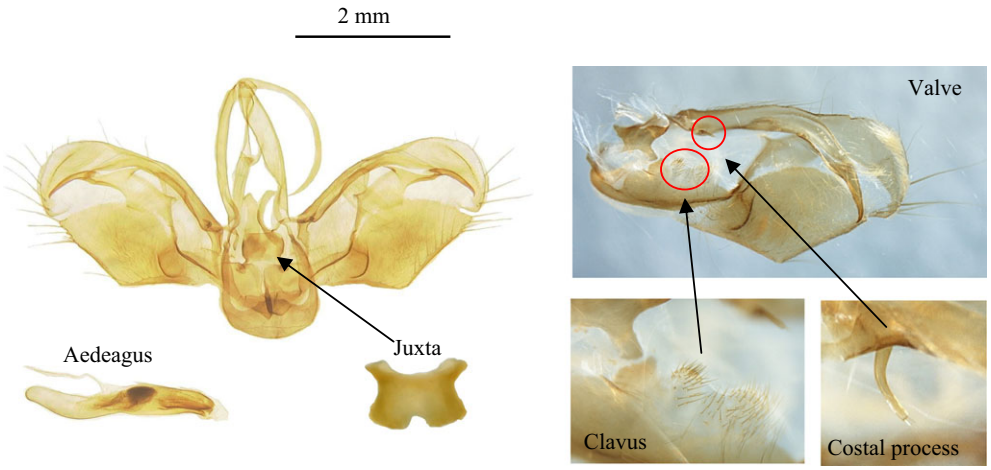


Fig. 54 Male genitalia *Spodoptera littoralis.* Note: when mounted in a slide the costal process can direct either upwards or downwards. Photos © J.-F. Germain, Anses, FR and J. Brambila, USDA-APHIS-PPQ.

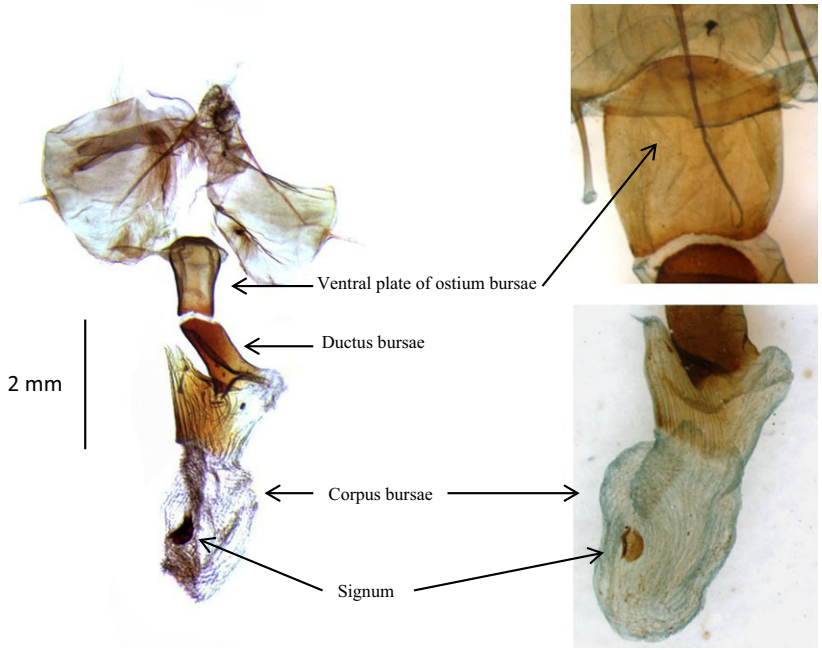


Fig. 55 Female genitalia *Spodoptera littoralis.* Photos on left © J.-F. Germain, Photos on right R. vd. Biggelaar NPPO, NL.

ventral plate of *S. littoralis* is straight, while it has a deep V-shaped notch in *S. litura,* which is also the case for several New World species. See Appendix 3 for more details.

4.1.4.3. Spodoptera litura.

Habitus (Fig. 56)

Forewings length: 14–17 mm in males, 15–18 mm in females. Colour and pattern almost indistinguishable from *S. littoralis.* Sexual dimorphism is present. The male usually has an ochreous forewing patch between the antemadial and postmedial lines below vein M., but not all specimens show sexual dimorphism; some males have no ochreous patch. The orbicular spot is more solid in the male. There are five characters that in combination could help to distinguish this species. These are: reniform spot brown, outlined in white, followed by black, with a white-margined light brown area at apex; orbicular spot elongate, narrow, oblique, light brown and outlined with a white margin; a row of dark brown or black hour-glass markings along outer margins (between terminal and adterminal lines, and between veins); a white fork in the median area of the wing, the fork is made of veins M3, CuA1 and CuA2; a large

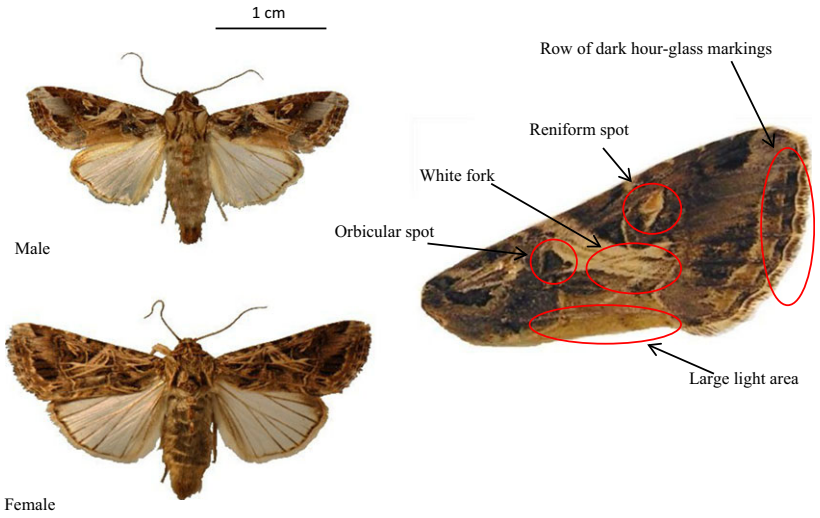


Fig. 56 Adult *Spodoptera litura*. Photos © J.-F. Germain, Anses, FR.

yellowish or light brown area at the median area along the inner margin. Because of the variability in colour, however, a genital dissection is needed for reliable identification.

Male genitalia (Fig. 57)

The combination of genitalic characters at valve level allows the identification of this species: two windows, one triangular and one rectangular separated by a right angle in the center of the valve; clavus small; costal process small, elongate, narrow and curved; ampulla slightly curved; cucullus truncate (nearly square-edged); juxta triangular with a narrow base and a pointed process; coremata with two lobes (one shorter, one elongate).

Female genitalia (Fig. 58)

Ventral plate of ostium bursa with height greater than width; distal margin with a broad V-shaped notch; ventro-lateral invaginated pocket of 8th sternite absent. Ductus bursae elongate (length >3 times width); completely sclerotized. Appendix bursae membranous. Corpus bursae bulbous, length less than twice width; striate convolutions. Signum in apical half of corpus bursae; short, length <0.65 mm; almost vertical.

Differences with similar species

Spodoptera litura will not easily be confused with other Asian species, except *S. apertura* (see *S. littoralis* for a description of the relevant, distinguishing, characters of *S. apertura*). In general both *S. litura* and *S. littoralis* are most similar to several New World species.

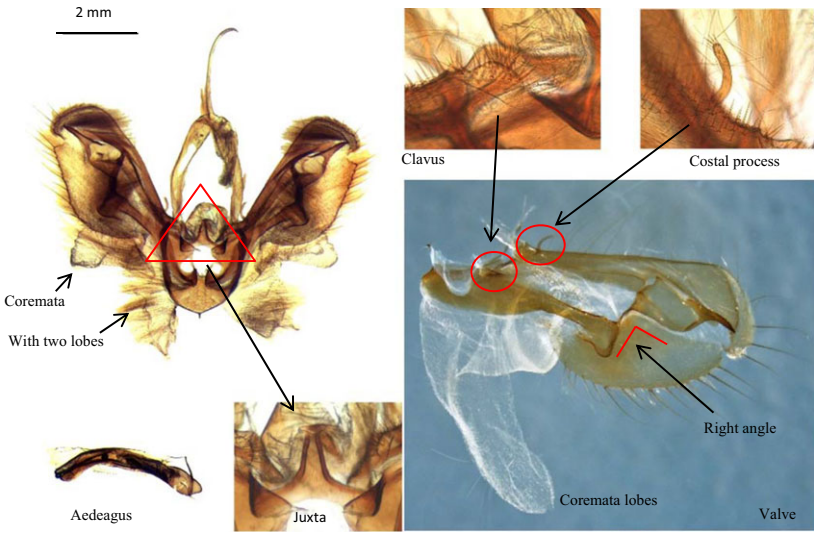


Fig. 57 Male genitalia *Spodoptera litura*. Note: when mounted in a slide the costal process can direct either upwards or downwards. Photos © J.-F. Germain, Anses, FR, lower right photo © J. Brambila, USDA-APHIS-PPQ.

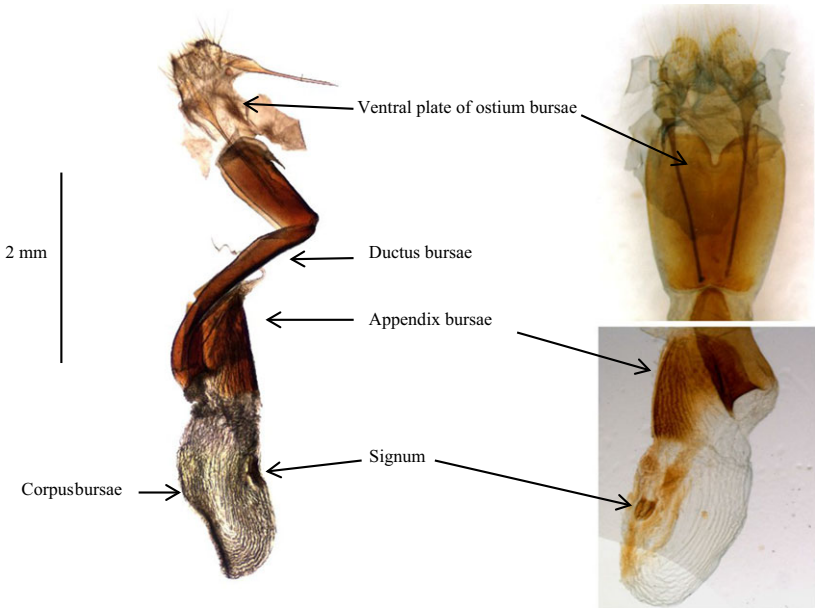


Fig. 58 Female genitalia *Spodoptera litura*. (Photo on left © J.-F. Germain, Anses, FR, Photos on right R. vd. Biggelaar NPPO, NL.).

4.1.4.4. Spodoptera eridania.
Habitus (Fig. 59)

Forewings: length 12–15.5 mm in males and 13–16 mm in females. This species is quite variable. The forewings ground colour is most commonly cream, but can become grey. The pattern on the forewing can also vary; specimens can have a faint or obscure reniform spot, a distinct, a distinct one or an elongate horizontal bar extending from the reniform spot to wing margin. There is no evident sexual dimorphism. The most common forms have background without bold black, brown and white stripes; reniform spot of a black or dark brown streak, or at least in the form of a brown spot. The primary character to notice is a short black dash near the base of wing along the inner margin (Fig. 59, arrow). Note that *S. albula* is highly similar, but you can easily distinguish the two species by the fact that *S. albula* also has a black dash, usually narrow, but that starts in the middle of the wing instead of near the base (see http://www.inra.fr/papillon/noctuid/amphipyr/texteng/s_albula.htm). *S. ochrea* is more or less similar, but this also lacks the black dash near the base of the wing.

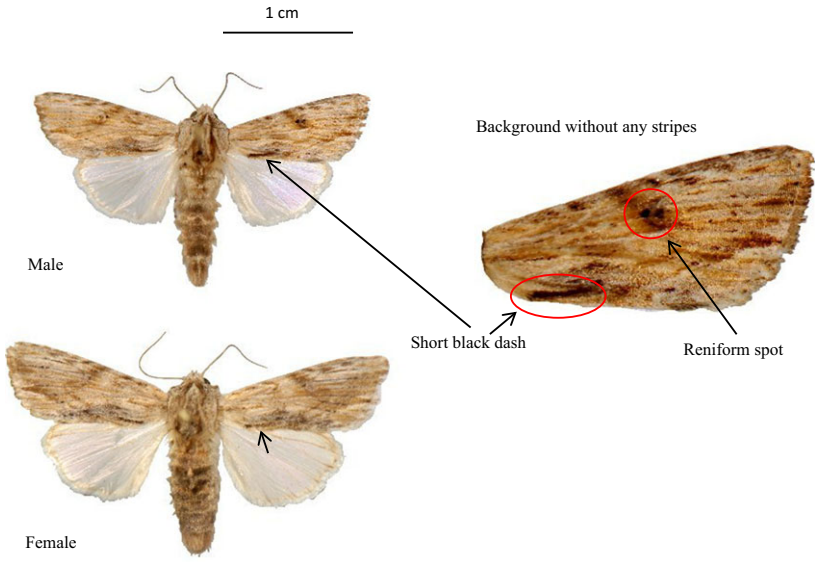


Fig. 59 *Adult Spodoptera eridania*. Photos © J.-F. Germain, Anses, FR.

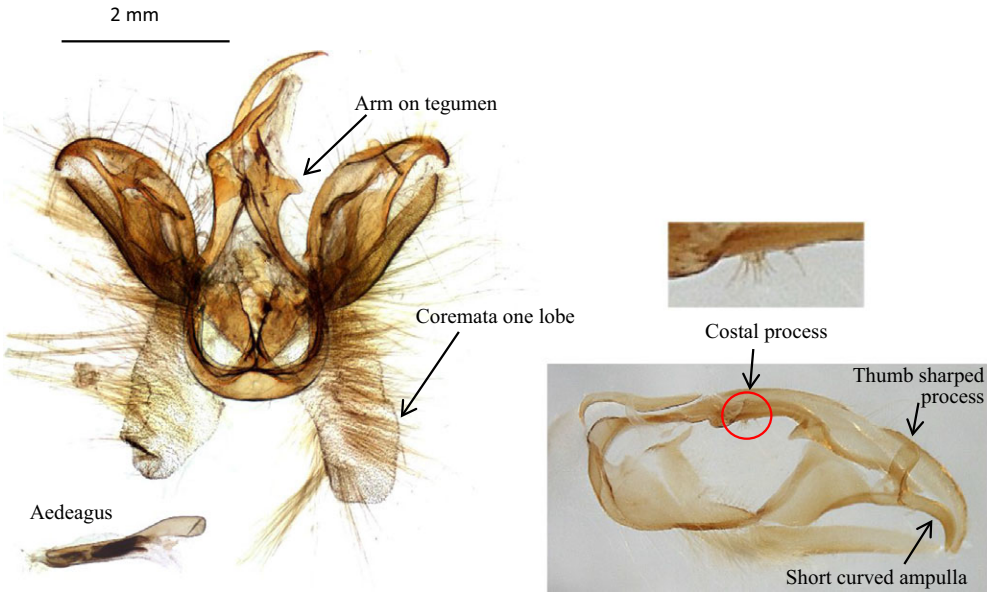


Fig. 60 Male genitalia *Spodoptera eridania*. Photos on left © J.-F. Germain, Anses, FR, photos on right © J. Brambila, USDA-APHIS-PPQ.

Male genitalia (Fig. 60)

The combination of genitalic characters at valve level allows the identification of this species: clavus absent; costal process a very small bump; ampulla short, curved, and with a large thumb-shaped process; juxta angular at base with a dorsal process broad at base, tegumen with 2 arms, coremata composed of single lobe.

Female genitalia (Fig. 61)

Female genitalia: ventral plate of ostium bursa wider than high; distal margin with a bifurcate median projection; ventrelateral invaginated pocket of 8th sternite absent. Ductus bursae short (length less than twice width); completely sclerotized. Appendix bursae membranous. Corpus bursae elongate, length greater than twice width; straight convolutions. Signum at middle of corpus bursae; elongate, length >1.15 mm; forming less than a 45 and greater than a 30 degree angle to vertical axis of corpus bursae, oriented left to right.

Differences with similar species

The male genitalia of *S. eridania* differ from those of *S. abula* in the costal process, that is very short in *S. eridania* and longer in *S. albula*. The male genitalia of *S. ochrea* lack the arms on the tegumen, which are present in *S. eridania*.

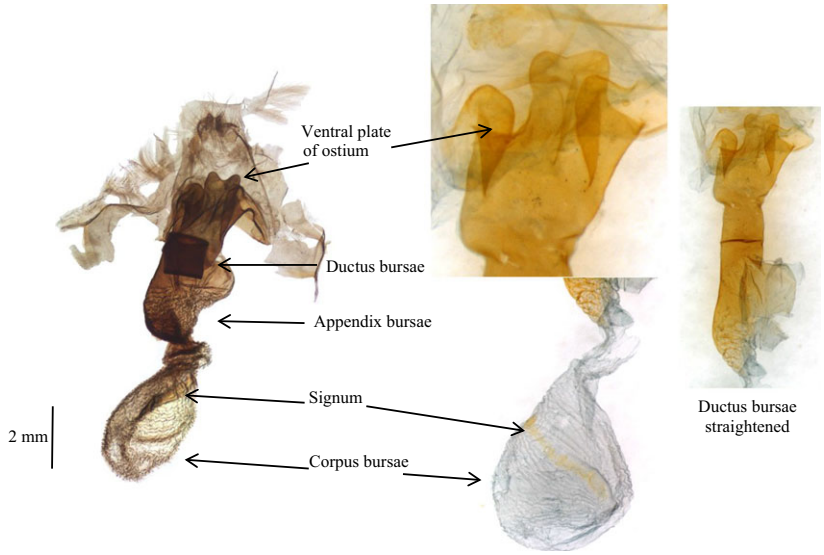


Fig. 61 Female genitalia *Spodoptera eridania*, Photo on left Fera © British Crown Copyright photos on right © R. vd. Biggelaar, NPPO, NL.

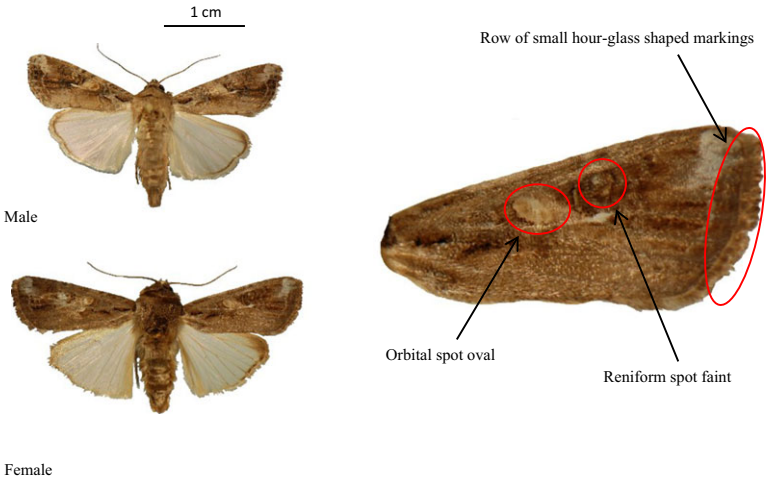


Fig. 62 Adult *Spodoptera frugiperda* (Photos © J.-F. Germain, Anses, FR).

In the female genitalia the distal margin of the ostium bursae has one long median projection in *S. abula* and one short and broad projection in *S. ochrea*; in *S. eridania* the distal margin has a bifurcate projection.

4.1.4.5. Spodoptera frugiperda.

Habitus (Fig. 62)

Forewings length: 10.5–15 mm in males and 11–18 mm in females. The species has some white and brown transverse line markings but they are not as contrasting as in other species. The ground colour is variable from greyish brown to rust brown. Furthermore, males and females differ strongly in that females have indistinct wing markings. This species is smaller than the other species that have the bold forewing markings. Notice that black dashes or bars are not present at the base of the wing. Males can have light brown orbital spots, oval, oblique, and not as narrow as in other species with strongly contrasting transverse lines; reniform spot indistinct, partially outlined in black, with a small, sideways v-shaped marking; row of small hour-glass shapes markings near apical margin of the wing.

Male genitalia (Fig. 63)

The combination of genitalic characters at valve level allows the identification of this species: the valve is very broad, almost quadrate; clavus short; costal process narrow, elongate, straight, inclined; ampulla only slightly curved; juxta concave at base and with a dorsal process; coremata composed of a single lobe. The base of the valve resembles that of *S. littoralis*.

Female genitalia (Fig. 64)

Ventral plate of ostium bursa with height greater than width; distal margin straight; ventrolateral invaginated pocket of 8th sternite absent. Ductus bursae short (length less than twice width); completely sclerotized. Appendix bursae partially sclerotized. Corpus bursae bulbous, length less than twice width; striate convolutions. Signum in basal half of corpus bursae; short, length <0.65 mm; forming <45 and >30 degree angle to vertical axis of corpus bursae, oriental left to right.

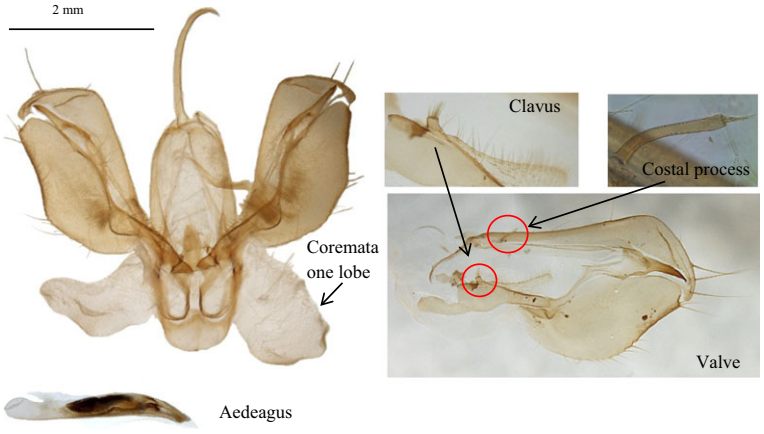


Fig. 63 Male genitalia *Spodoptera frugiperda*. Photos © J. Brambila, USDA-APHIS-PPQ.

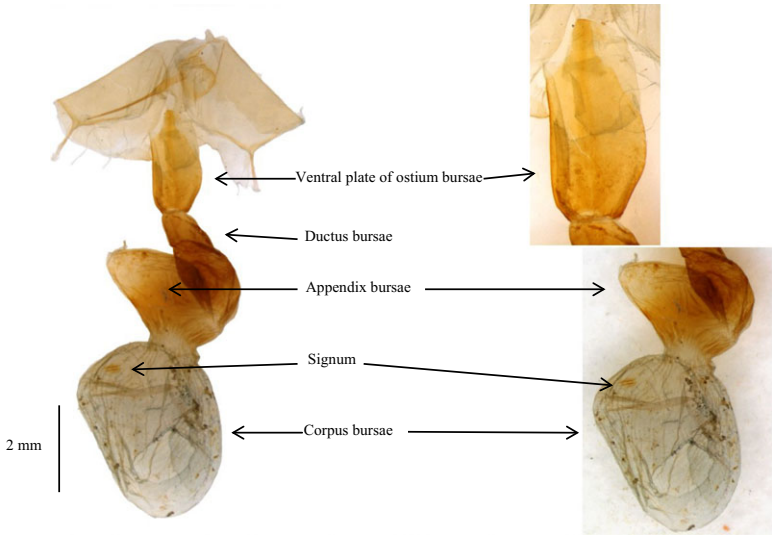


Fig. 64 Female genitalia *Spodoptera frugiperda.* Photos R. vd. Biggelaar © NPPO, the Netherlands.

Differences with similar species

Males of *S. frugiperda* can be confused with *S. ornithogalli* and females with *S. exigua.* The male genitalia of *S. ornithogalli* differ from *S. frugiperda* by the double lobed coremata (one lobe small!) and the bigger, broader clavus. The valve is also less broad then in *S. frugiperda*. The female genitalia of *S. exigua* differ from *S. frugiperda* in the elongate corpus bursae and the elongate signum.

4.2. Molecular methods

For a reliable positive identification of *S. eridania, S. frugiperda, S. littoralis* and *S. litura* by the molecular tests described in this protocol, the specimen should have been identified to the genus level based on morphology. A molecular identification can be relatively time-consuming as opposed to a morphological identification by an experienced person (e.g. a larvae of *S. littoralis* known to originate from Africa, can morphologically be identified within minutes). Another advantage is that morphological identification can be done with relatively simple equipment and requires no or only very few chemicals.

In this protocol, when a molecular test is recommended, this is indicated in the morphological identification sections for the different stages and species.

The identification of *S. eridania, S. frugiperda, S. littoralis* and *S. litura* can be performed using four simplex real-time PCR tests based on TaqMan chemistry (Van De Vossenberg & Van Der Straten, 2014). To cover the overlap in geographical distribution the *S. eridania* and *S. frugiperda* tests, and the *S. littoralis* and *S. litura* tests are combined in a single test. The *S. eridania/S. frugiperda* test is described in Appendix 4 and the *S. littoralis/S. litura* test is described in Appendix 5.

5. Reference material

Reference material of larvae, pupae and adults of all species in this protocol can be obtained from:

– MJ van der Straten (National Plant Protection Organisation, dep. NRC, NL)
– JF Germain (Anses, Laboratoire de la Santé des Végétaux, FR)

DNA reference material of all species in this protocol can be obtained with:

– BTLH van de Vossenberg (National Plant Protection Organisation, dep. NRC, NL)

Sequences for several specimens, including those used for the development of the molecular test, are registered in Q-bank (http://www.q-bank.eu/).

6. Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM 7/77 (1) Documentation and reporting on a diagnosis.

7. Performance criteria

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

8. Further information

Further information on these organisms can be obtained as follows:

Morphological identification from:

– MJ van der Straten (National Plant Protection Organisation, dep. NRC, NL m.j.vanderstraten@nvwa.nl)
– JF Germain (Anses, Laboratoire de la Santé des Végétaux, FR jean-francois.germain@anses.fr)

Molecular tests from:

– BTLH van de Vossenberg (National Plant Protection Organisation, dep. NRC, NL b.t.l.h.vandevossenberg@nvwa.nl)

9. Feedback on this diagnostic protocol

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

10. Protocol revision

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols identified as needing revision are marked as such on the EPPO website. When errata and corrigenda are in press, this will also be marked on the website.

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– Marja J. van der Straten (National Plant Protection Organisation, the Netherlands, dep. NRC);
– Jean-Francois Germain (Anses, Laboratoire de la Santé des Végétaux, FR);
– Bart T.L.H. van de Vossenberg (Plant Protection Service, dep. NRC, NL).

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General web sites

www.eppo.int
http://www.cabi.org/cpc

Appendix 1 – Features of Lepidoptera larvae

Figure 65 presents the general features, setal nomenclature of body segments, arrangements of crochets on prolegs and type of setae described.

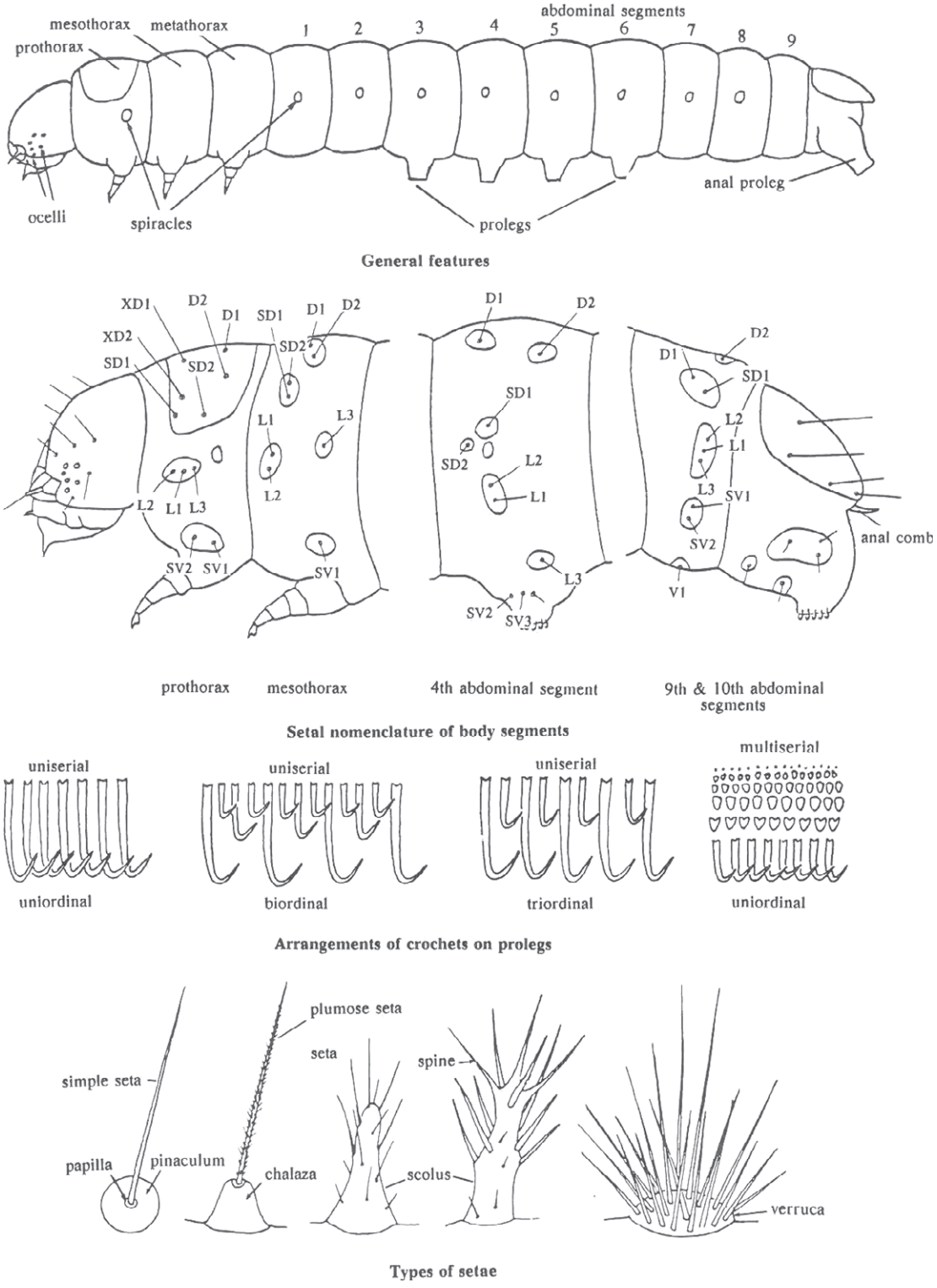

Fig. 65 Location of features of Lepidoptera larva (from Holloway *et al.*, 1987).

Appendix 2 – Keys to adults based on external morphology

Note: The keys are intended for first screening of a specimen. Guidelines for correct and reliable positive identification of quarantine species are given in the main text.

Key to adults of selected *Spodoptera* species of the Old World including the quarantine species (after Pogue, 2002):

1	Antenna serrate or bipectinate	Other species
1′	Antenna filiform	2
2	Prothoracic tibia with lateral scale tufts extending beyond first tarsal segment	Other species
2′	Prothoracic tibia with lateral scale tufts shorter than first tarsal segment	3
3	Forewing ground colour cream with an irregular patch of rose scale; forewing length 18–22 mm; Oriental, Australasia, and Oceania regions	*S. picta* ♀ ♂
3′	Forewing ground colour not cream and no rose scales present	4
4	Forewing length <10 mm	*S. pectinicornis* ♀
4′	Forewing length >10 mm	5
5	Forewing with orbicular spot round	Other species
5′	Forewing with orbicular spot oval or absent	Other species
5′’	Forewing with orbicular spot an oblique trapezoid	6
6	Forewing with reniform spot brown outlined in white on proximal border, distal apex with a triangular light brown spot outlined in white, a white distal border extending to middle	7
6′	Forewing with reniform spot crescent-shaped, lacking light brown triangular spot outlined in white at apex	Other species
7	Hindwing veins infuscated with brown scales apically; Oriental, Australasia, and Eastern Palearctic regions	*S. litura* ♀ ♂
7′	Hindwing veins not infuscated with brown scales near apex; Western Palearctic and Ethiopian regions	*S. littoralis* ♀ ♂

Key to adults of selected *Spodoptera* species of the New World including the quarantine species (after Pogue, 2002):

1	Antenna serrate; endemic to the Galapagos Islands	*S. roseae* ♂
1′	Antenna filiform	2
2	Forewing orbicular spot round or slightly oval	3
2′	Forewing with orbicular spot oval or an oblique trapezoid extending from costal margin to Cu vein	4
3	Forewing with orbicular spot cream, with pale rufous centre; forewing length 11–14 mm	*S. exigua* ♀ ♂
3′	Forewing with orbicular spot cream, with buff to brown centre, outlined in black; forewing length 20–22 mm, endemic to the Galapagos Islands	*S. roseae* ♀
4	Forewing with no white scales along CU vein under orbicular and reniform spots	5
4′	Forewing with white scales along Cu vein under orbicular and reniform spots extending to M3 and Cu1 veins, continuing down CuA2 vein to postmedial line	Other species
5	Forewing ground colour rufous	Other species
5′	Forewing ground colour pale grey or cream	6
5′’	Forewing ground colour brown, or dark brown	8
6	Forewing with a narrow, black longitudinal dash at base of Cu vein; Southern USA to Northern Argentina and Southern Brazil	*S. albula* ♀ ♂
6′	Forewing lacking a narrow, black longitudinal dash at base of Cu vein	7
7	Hindwing with some contrasting scales along the margin; forewing of some specimens with a distinct broad longitudinal black streak from apex of discal cell to margin; Southern USA to Argentina	*S. eridania* ♀ ♂
7′	Hindwing with no contrasting scales along margin, completely white; never with a distinct broad longitudinal black streak from apex of discal cell to margin; Western Peru and Ecuador	*S. ochrea* ♀ ♂
8	Forewing with a rufous area between antemedial and post medial lines ventral to Cu and CuA2 veins that extends to outer margin	*S. latifascia* ♂
8′	Forewing lacking a rufous area between antemedial and postmedial lines to Cu and CuA2 veins that extends to outer margin	9
9	Forewing ground colour brown with contrasting markings, a small conspicuous white spot at junction of M3 and CuA1 veins, and a white patch at apex	*S. frugiperda* ♂
9′	Forewing ground colour dark brown without contrasting markings	*S. frugiperda* ♀

Appendix 3 – Information on genitalia

Glossary on terminology of genitalia (after Gordh & Headrick, 2001, modified)

Male

Ampulla: a process (often finger-like) arising from the medial part of the valve, near the base and extending more-or less-dorsad
Clavus: a rounded or finger-liked process, usually setose, arising from the sacculus of the valve.
Coremata: a pair of long, eversible, tubes with glandular cells.
Costal process: finger-like process (sometimes very short) opposite of clavus
Cucullus: the terminal part of the valve
Juxta: a sclerite beneath the aedeagus and to which it may be hinged or fused
Uncus: curved hook directed downward from a dorsal triangular dorsal sclerite, shielding the aedeagus.
Valve: genitalic clasper.

Female

Bursa copulatrix: the female genital chamber that is divided into the ductus bursae and the corpus bursae
Corpus bursae: membranous sac-like structure
Ductus bursae: duct which extends from the ostium bursae to the corpus bursae
Ostium bursae: the opening of the bursa copulatrix
Plate: any broad, flat surface, such as sclerite
Signum: one or more heavily sclerotized and often elaborated structures in the wall of the corpus bursae

Overview characteristics of male genitalia of Spodoptera species (excluding *S. hipparis*)
[based on Pogue (2002), Brambila (2009a,b), Lalanne-Cassou (1994), pers. obs., van der Straten (2011/2012)]

	Character						
Species	Valve	Juxta	Coremata	Clavus	Costal process	Ampulla	Miscellaneous
exigua	Broad, elongate oval	Narrow eliptical band	Moderately elongate (no distinct lobes)	Absent	Absent	Elongate, slightly curved, apex not split in two	Apex cucullus round, sacculus not well differentiated
cilium	Broad, oval	Rectangular band	Reduced (no distinct lobes)	Absent	Indistinct swelling with setae	Apex split in two (like deer antler), clasper proper present	
depravata	Medium, tapering, costa sinuate	Rectangular band	Reduced (no distinct lobes)	Absent	Minute knob in middle of costa	Apex split in two (like deer antler), clasper proper absent	

(continued)

Overview characteristics of male genitalia of *Spodoptera* species (excluding *S. hipparis) (Continued)*

	Character						
Species	Valve	Juxta	Coremata	Clavus	Costal process	Ampulla	Miscellaneous
pecten	Medium, tapering	Rectangular band	Reduced (no distinct lobes)	Absent	Minute knob in middle of costa	Elongate, slightly curved, apex not split in two	
umbraculata	Medium, tapering, costa sinuate	Rectangular band	Reduced (no distinct lobes)	Absent	Narrow, elongate, at base of costa	Broad with 'double' apex (also broad at the apex)	
pectinicornis	Narrow, tapering	Rectangular band	Reduced (no distinct lobes)	A small bump	Narrow, elongate, at base of costa	Elongate, slightly curved, pointed apex	
apertura	Narrow, quadrate-like	Narrow rectangular band	Absent, no lobes	A small bump	An elongate, big bump	Elongate, extends beyond apex of valve	Genitalia similar to malagasy, hindwing white
malagasy	Narrow, quadrate-like	Narrow rectangular band	Reduced (no lobes)	A small bump	An elongate, big bump	Elongate, extends beyond apex of valve	Genitalia similar to apertura, hindwing brown
compta			Reduced, appressed to sacc., no lobes	Absent	Short but elongate, at inner margin	Broken, clasper proper present	Tegumen with 2 bumps, scaphium sclerot.
eridania		Narrow rectangular band	One lobe	Absent	Very short, at inner margin	Straight, clasper proper present	Tegumen with 2 arms, scaphium sclerot.
albula		Narrow rectangular band	One lobe	Absent	Short but elongate, at inner margin	Broken, clasper proper present	Tegumen with 2 arms, scaphium sclerot.
ochrea		Narrow rectangular band	One lobe	Absent	Short but elongate, at inner margin	Broken, clasper proper present	Tegumen with 2 bumps, scaphium weak
exempta	Narrow, rectangular	Narrow eliptical band, median process triangular	Single lobe, ≤0.5 length valve	Absent	Minute knob	Elongate, bent in middle downwards; very small clasper proper pointing downwards	
mauritia	Narrow, tapering	Narrow eliptical band, median process triangular	Single lobe, ≤0.5 length valve	A small bump	Small, elongate, narrow, and curved	Elongate, slightly curved downwards	
triturata	Narrow, tapering, costa sinuate	Narrow eliptical band, median process triangular	Single lobe, ≤0.5 length valve	Absent	Indistinct swelling, medium length	Elongate, slightly curved downwards (longer then mauritia)	

(continued)

Overview characteristics of male genitalia of *Spodoptera* species (excluding *S. hipparis*) *(Continued)*

	Character						
Species	Valve	Juxta	Coremata	Clavus	Costal process	Ampulla	Miscellaneous
frugiperda	Very broad, 'quadrate'	Narrow rectangular band	Single lobe, elongate >0.5 length valve	Minute round projection	Narrow, elongate, at base of costa	Elongate, curved with decurved apex, extends just beyond apex of valve	Apex cucullus truncate (angular), sacculus well differentiated
dolichos	Broad, rounded	Quadrate, broader at base	Two lobes	A bent thumb	Small, elongate, narrow, and bent	Big: (less) elongate, slender, and curved	
androgea	Very broad, rounded	Narrow rectangular band, triangular base	Two lobes, 0.5 length of valve	Elongate, finger shaped	Narrow, elongate, at base of costa	Big: elongate, slender, and curved	Like dolichos, but no dark band on tegula
littoralis	Broad, quadrate, ventral margin dentate	Broad, quadrate	Two lobes, one 0.5 length of valve, other very short	A small bump	Small, elongate, narrow, and curved	Relatively short (medium length), curved with decurved apex, extends beyond apex of valve	
litura	Broad	Triangular	Two lobes	A small bump	Small, elongate, narrow, and curved	Relatively short (medium length), curved	
latifascia	Broad	More or less high triangular	Two lobes	Clublike and bent.	Very large: like a spine	Big: elongate, broad, and curved	
cosmioides	Broad	Narrow rectangular band	Two lobes: upper 0.5 length lower	Clublike and bent.	Elongate, broad, heavily sclerotized	Big: elongate, broad, and curved, apex de-curved	Like latif. & descoinsi
descoinsi	Broad	Narrow high triangular	Two lobes, >0.5 length of valve	Clublike and bent.	Elongate, broad, heavily sclerotized	Big: elongate, broad, and curved, apex de-curved	
praefica	Broad, quadrate	Narrow rectangular band	Reduced (no lobes)	Short, round, hairy	Medium length and medium broad	Medium length, extending beyond apex of valve	
ornithogalli	Broad	High, broad triangular	Two lobes, upper very small	A hairy toe (not bent)	Small, elongate, narrow, slightly curved		
pulchella	Broad	Broad triangular	Two lobes, both >0.5 length valve	A hairy toe (not bent)	Small, elongate, narrow, slightly curved		Spine in vesica (hard to see)
marima	Broad, quadrate, ventral margin hardly dentate	Narrow rectangular band	Two lobes, 1 > 0.5 length of valve	Short, round, hairy	Narrow, elongate, at base of costa	Medium length, slightly curved with decurved apex, extends beyond apex of valve	
evanida	Broad, tapering	Narrow rectangular band	Single lobe, ≤0.5 length valve	Short clublike, not bent	Elongate, broad, heavily sclerotized	Big: elongate, broad, and curved, apex de-curved	Scaphium weakly developed
picta							Similar to no other species

Overview characteristics of female genitalia of *Spodoptera* species (excluding *S. hipparis)* [based on Pogue (2002), Brown & Dewhurst (1975), pers. obs., van der Straten (2011/2012)]

	Character					
Species	Corpus bursae	Ductus bursae	Signum	Diverticulum (Brown) (=appendix bursae)	Ventral plate of ostium bursae	Miscellaneous
exigua	Elongate: length >2× width	Short (length <2× width), sclerotized	Elongate >1.15 mm, in apical half of bursa, <30° angle to vertical axis	Not sclerotized	Height > width, distal margin straight	
cilium	Bulbous, but relative elongate	Short (length <2× width), sclerotized	Medium elongate 0.6–1.1 mm, in apical half of bursa, <30° angle to vertical axis	Not sclerotized	Height > width, distal margin long central groove	
depravata	Bulbous	Medium length (2–3× width), sclerotized	Short, <0.65 mm, in apical half bursa, <30° angle to vertical axis	Not sclerotized	Height > width, distal margin straight	
pecten	Bulbous	Elongate (length >3× width), sclerotized	Elongate >1.15 mm, in apical half of bursa, <30° angle to vertical axis	Partially sclerotized	Height > width (looks triangular), distal margin straight and bent outwards	Wings of ♀ pecten with faint pattern
umbraculata	Bulbous	Short (length <2× width), sclerotized	Elongate >1.15 mm, in apical half of bursa, <30° angle to vertical axis	Sclerotized	Height < width, distal margin lightly depressed (sinuate)	Wings of ♀ umbraculata with distinct pattern
pectinicornis	Elongate: length >2× width	Medium length (2–3× width), sclerotized	Short, <0.65 mm, in apical half bursa, almost vertical	Not sclerotized	Height > width, distal margin straight	
apertura	Bulbous, few striae	Short (length <2× width), sclerotized	Elongate > 1.15 mm, in apical half of bursa, >45° angle to vertical axis		Height > width, distal margin straight	Genitalia similar to malagasy, hindwing white
malagasy	Bulbous, few striae	Short (length <2× width), sclerotized	Elongate >1.15 mm, in apical half of bursa, >45° angle to vertical axis		Height > width, distal margin straight	Genitalia similar to apertura, hindwing brown
compta	Bulbous	Short (length <2× width), not sclerotized	Medium elongate 0.6–1.1 mm, in apical half of bursa, almost vertical		Width > height, distal margin with one elongate median projection ('upwards tongue')	
eridania	Elongate: length >2× width	Short (length <2× width), sclerotized	Elongate >1.15 mm, in middle of bursa, >30° and <45° angle to vertical axis	Not sclerotized	Width > height, distal margin wide with bifurcate median projection ('two lobed')	

(continued)

Overview characteristics of male genitalia of *Spodoptera* species (excluding *S. hipparis) (Continued)*

	Character					
Species	Corpus bursae	Ductus bursae	Signum	Diverticulum (Brown) (=appendix bursae)	Ventral plate of ostium bursae	Miscellaneous
albula	Elongate: length >2× width, smooth	Short (length <2× width), sclerotized	Medium elongate 0.6–1.1 mm, in apical half of bursa, <30° angle to vertical axis	Not sclerotized	Width > height, distal margin with one elongate median projection (‘upwards tongue’)	
ochrea	Bulbous	Medium length (2–3× width), sclerotized	Elongate >1.15 mm, in apical half bursa, almost vertical	Not sclerotized	Width > height, distal margin with wide/low median projection (one ‘lobe’: smooth curve upwards)	
exempta	Bulbous	Medium length (2–3× width), sclerotized	Elongate >1.15 mm, in middle of bursa, almost vertical	Not sclerotized	Width > height, distal margin with wide/low median projection (one ‘lobe’: smooth curve upwards)	
mauritia	Bulbous, caudally abruptly constricted (looks like part of diverticulum)	Short (length <2× width), sclerotized	Medium elongate 0.6–1.1 mm, in basal half of bursa, almost vertical	Membranous	Height > width, distal margin straight (apex abdomen with big black hairtufts)	Corpus bursae and appendix bursae of mauritia and triturata highly similar: difference: striae on corpus bursae of mauritia extent into upper constricted part of appendix bursae; in triturata the corpus and appendix bursae are separated by rounded striae in the appendix
triturata	Bulbous, caudally gradually narrowing	Medium length (2–3× width), sclerotized	Medium elongate 0.6–1.1 mm, in apical half of bursa, almost vertical	Sclerotized	Height > width, distal margin straight but ‘pinched’ lateral	
frugiperda	Bulbous	Short (length <2× width), sclerotized	Short, <0.65 mm, in basal half of bursa, >30° and <45° angle to vertical axis	Partially sclerotized	Height ≫ width, distal margin straight	
dolichos	Elongate: length >2× width	Short (length <2× width), sclerotized	Elongate >1.15 mm, in apical half of bursa, almost vertical	Partially sclerotized	Width > height, distal margin straight	
androgea	Bulbous	Medium length (2–3× width), distinct sclerotized midventral band	Medium elongate 0.6–1.1 mm, in apical half of bursa, <30° angle to vertical axis	Sclerotized	Width > height, distal margin straight	

(continued)

Overview characteristics of male genitalia of *Spodoptera* species (excluding *S. hipparis) (Continued)*

	Character					
Species	Corpus bursae	Ductus bursae	Signum	Diverticulum (Brown) (=appendix bursae)	Ventral plate of ostium bursae	Miscellaneous
littoralis	Bulbous	Short (length <2× width), sclerotized	Short, <0.65 mm, in apical half bursa	Short and wide	Height > width, distal margin straight Height > width, distal margin with broad V-shaped notch	
litura	Bulbous	Elongate (length >3× width), sclerotized	Short, <0.65 mm, in apical half bursa	Short and wide		
latifascia	Elongate: length >2× width	Medium length (2–3× width), distinct sclerotized midventral band	Elongate >1.15 mm, in apical half of bursa, >45° angle to vertical axis	Partially sclerotized	Height > width, distal margin with wide rectangular notch (at base a little wider)	
cosmioides	Elongate: length >2× width	Medium length (2–3× width), sclerotized	Medium elongate 0.6–1.1 mm, in apical half of bursa, almost vertical	Sclerotized	Height > width, distal margin narrow U-shaped	
descoinsi	Bulbous	Medium length (2–3× width), distinct sclerotized midventral band	Medium elongate 0.6–1.1 mm, in apical half of bursa, >45° angle to vertical axis	Sclerotized	Height > width, distal margin narrow U-shaped	
praefica	Bulbous	Short (length <2× width), sclerotized	Short, <0.65 mm, in apical half bursa, >45° angle to vertical axis	Partially sclerotized	Height > width, distal margin slightly concave (downwards depression)	
ornithogalli	Bulbous	Short (length <2× width), sclerotized	Medium elongate 0.6–1.1 mm, in middle of bursa, almost vertical		Height > width, distal margin U-shaped	
pulchella	Bulbous	Short (length <2× width), sclerotized	Elongate >1.15 mm, in apical half of bursa, <30° angle to vertical axis	Partially sclerotized	Height > width, distal margin U-shaped	
marima	Bulbous; smooth	Short (length <2× width), sclerotized	Short, <0.65 mm, in apical half bursa, almost vertical	Sclerotized	Height > width, distal margin U-shaped	
evanida	Bulbous; smooth	Elongate (length >3× width), partially sclerotized	Short, <0.65 mm, in apical half bursa, almost vertical	Sclerotized	Height > width, distal margin U-shaped	
picta						

Appendix 4 – Identification of *Spodoptera eridania* and *S. frugiperda* using real-time PCR

1 General Information

1.1 Identification of adults, larvae and eggs of *Spodoptera eridania* and *S. frugiperda* using real-time PCR based on TaqMan chemistry.

1.2 Development, optimisation and validation of this test by BTLH van de Vossenberg and MJ van der Straten (National Plant Protection Organization – NL) was finalised September 2010.

1.3 72 bp of the Cytb gene are amplified for *S. eridania*, and 110 bp of the Cytb gene are amplified for *S. frugiperda*.

1.4 *S. erid*-fw: 5′ CCG TAT TTA TTA GGG GAT CCT GAT 3′, *S. erid*-rv: 5′ AGG TTG AAT ATG TAC AGG AG 3′, *S. frug*-fw: 5′ CAT TTA AGG AYT TAA TTG GAT T 3′, *S. frug*-rv: 5′ AGG ATT TGC TGG GAT AAA ATT ATC A 3′, *S. eridania*-T: 5′ VIC – CCT GCT AAC CCC CTT GT – MGB 3′, *S. frugiperda*-T: 5′ FAM – ATC TCC TAA TAA ATA AGG ATT AGT TAA A – MGB 3′. Note that the bold/underlined nucleotides are Locked Nucleic Acids (LNA).

1.5 The test is validated on a 7900HT real-time PCR system (Applied Biosystems) and CFX96 (Bio-Rad) using automatic baseline settings. For the 7900HT, a fixed threshold was used (0.2). For the CFX96, an automatic threshold was used in combination with a drift control.

2 Methods

2.1 Nucleic Acid Extraction and Purification

2.1.1 (Single) Egg(s), parts of larvae and parts of adults (e.g. single leg) of *Spodoptera* sp. serve as input for DNA extraction.

2.1.2 DNA is extracted using the High Pure PCR Template Preparation Kit (Roche) according to the mammalian tissue protocol or the Blood & Tissue kit (Qiagen) according to the animal tissue protocol.

2.1.3 Crushing of the insect in lysis buffer (provided by manufacturer) prior to DNA extraction is required.

2.1.4 For both the Roche-kit and the Qiagen-kit, DNA is eluted in 50 µL preheated elution buffer (provided).

2.1.5 After DNA extraction, no DNA clean-up is required.

2.1.6 Either use extracted DNA immediately or store it at −20°C until use.

2.2 Real-time Polymerase Chain Reaction – real-time PCR

2.2.1 The master mixes for both the *S. eridania* and *S. frugiperda* test are displayed in Table 1. The master mixes are based on the qPCR core kit from Eurogentec.

Table 1. Master mixes for the *Spodoptera eridania* and *Spodoptera frugiperda* real-time PCR tests

	Spodoptera eridania			*Spodoptera frugiperda*		
Reagent	Working concentration	Volume per reaction (µL)	Final concentration	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	N.A.	16.0	N.A.	N.A.	16.0	N.A.
10× reaction buffer (Eurogentec)	10×	2.5	1×	10×	2.5	1×
$MgCl_2$ (Eurogentec)	50 mM	2.5	5 mM	50 mM	2.5	5 mM
dNTPs (Eurogentec)	5 mM	1.0	0.2 mM	5 mM	1.0	0.2 mM
S. erid-fw	10 µM	0.75	0.3 µM	–	–	–
S. erid-rv	10 µM	0.75	0.3 µM	–	–	–
S. frug-fw	–	–	–	10 µM	0.75	0.3 µM
S. frug-rv	–	–	–	10 µM	0.75	0.3 µM
S. eridania-T	10 µM	0.25	0.1 µM	–	–	–
S. frugiperda-T	–	–	–	10 µM	0.25	0.1 µM
Hot Goldstar Taq (Eurogentec)	5 U μL^{-1}	0.25	1.25 U	5 U μL^{-1}	0.25	1.25 U
Subtotal		24.0			24.0	
DNA dilution: 10^0		1.0			1.0	
Total		25.0			25.0	

*Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.45 µm filtered) and nuclease-free.

2.2.2 Real-time PCR cycling conditions: 95°C for 10 min, 40 × (95°C for 15 s, 60°C for 60 s)

3 Essential Procedural Information

3.1 Controls

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

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: Empty eppendorf tube introduced before crushing of the sample material and processed as a normal sample.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: a single egg from a *S. eridania* or *S. frugiperda* egg package.
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. Two DNA extracts (PAC I and PAC II) of both *S. eridania* and *S. frugiperda*; PAC I: 10 ng μL^{-1} (above LOD) and PAC II 0.1 ng μL^{-1} (close to LOD).

3.2 Interpretation of results

To prevent false positive results caused by late C_t values obtained with *Spodoptera mauritia*, a cycle cut off value for *the S. frugiperda* test is set at 30. The cycle cut off value needs to be verified in each laboratory when implementing the test for the first time.

Verification of the controls:

- The PIC, PAC I and PAC II amplification curves should be exponential.
- PIC, PAC I and PAC II should have a C_t value below 40 for the *S. eridania* test and 30 for the *S. frugiperda* test.
- NIC and NAC should be negative (C_t equal or above 40 for the *S. eridania* test and 30 for the *S. frugiperda* test).

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve, and a C_t value below 40 for the *S. eridania* test or 30 for the *S. frugiperda* test.
- A test will be considered negative, if it does not produce an exponential amplification curve or it gives a C_t value equal to or above 40 for the *S. eridania* test or 30 for the *S. frugiperda* test.

4 Performance criteria available

4.1 Analytical sensitivity data: *S. eridania* and *S. frugiperda* 0.2 pg and 100 pg DNA respectively.

4.2 Analytical specificity data: analytical specifcity was determined by testing both target and nontarget species in the four specific *Spodoptera* tests, i.e., 114 *Spodoptera* specimens covering 18 of the 31 *Spodoptera* species and 12 non-*Spodoptera* specimens (4 *S. albula,* 1 *S. androgea* (Cramer), 1 *S. cilium,* 1 *S. cosmioides* (Walker), 10 *S. dolichos,* 7 *S. eridania,* 1 *S. exempta,* 15 *S. exigua,* 12 *S. frugiperda,* 6 *S. latifascia,* 18 *S. littoralis,* 10 *S. litura,* 5 *S. mauritia,* 2 *S. ochrea,* 1 *S. ornithogalli* (Guenée), 2 *S. pecten,* 4 *S. pulchella,* 2 *S. triturata,* 2 *Apospasta fuscirufa,* 5 *Copitarsia corruda* Pogue & Simmons, 1 *Elaphria agrotina* (Guenée), 3 *E. nucicolora,* 1 *Periodroma saucia*). Target organisms yielded the following *Ct* values: *S. eridania* 19.5 ± 2.8 and *S. frugiperda* 20.4 ± 2.1. One *S. mauritia* specimen produced late C_t values (32.8 and 32.7) in the *S. frugiperda* test, which is considered to be negative applying the C_t cut-off value of 30.

4.3 Data on Repeatability: In total, 11 samples (2 *S. eridania*, 6 *S. frugiperda*, 1 *S. littoralis* and 2 *S. litura*) were divided in three equal parts from which 22 samples were tested as biological duplicates. All 44 results (11 samples × 2 biological duplicates × 2 technical duplicates) obtained a positive signal with the corresponding specific real-time PCR assay, and no signal with the other tests. The real-time PCR tests for the identification of *S. eridania*, *S. frugiperda*, *S. littoralis* and *S. litura* were found to be 100% repeatable.

4.4 Data on Reproducibility: In total, 11 samples (2 *S. eridania*, 6 *S. frugiperda*, 1 *S. littoralis* and 2 *S. litura*) divided in three equal parts were tested in technical duplicate. All 66 results (11 samples × 3 parts × 2 technical duplicates) obtained a positive signal with the corresponding specific real-time PCR assay, and no signal with the other tests. The real-time PCR tests for the identification of *S. eridania*, *S. frugiperda*, *S. littoralis* and *S. litura* were found to be 100% reproducible.

Appendix 5 – Identification of *Spodoptera littoralis* and *S. litura* using real-time PCR

1 General Information

1.1 Identification of adults, larvae and eggs of *Spodoptera littoralis* and *S. litura* using real-time PCR based on TaqMan chemistry.

1.2 Development, optimisation and validation of this test by BTLH van de Vossenberg and MJ van der Straten (National Plant Protection Organization – NL) was finalised September 2010.

1.3 70 bp of the Cytb gene are amplified for *S. littoralis* and *S. litura*.

1.4 *S. litt_S. litu*-fw: 5′ TAC TAG GA**G** AT**C** CT**G** ATA ATT TT 3′, *S. litt_S. litu*-rv: 5′ TCA TT**C** AG**G** TT**G** AA**T** AT**G** AAY 3′, *S. littoralis*-T: 5′ FAM–TCC AGC TAA TCC ACT AGT AA-MGB

3′ and *S. litura*-T : 5′ VIC-CCC TGC TAA CCC ATT AG-MGB 3′. Note that the bold/underlined nucleotides are Locked Nucleic Acids (LNA).

1.5 The test is validated on a 7900HT real-time PCR system (Applied Biosystems) and CFX96 (Bio-Rad) using automatic baseline settings. For the 7900HT, a fixed threshold was used (0.2). For the CFX96, an automatic threshold was used in combination with a drift control.

2 Methods

2.1 Nucleic Acid Extraction and Purification

2.1.1 (Single) Egg(s), parts of larvae and parts of adults (e.g. single leg) of *Spodoptera* sp. serve as input for DNA extraction.

2.1.2 DNA is extracted using the High Pure PCR Template Preparation Kit (Roche) according to the mammalian tissue protocol or the Blood & Tissue kit (Qiagen) according to the animal tissue protocol.

2.1.3 Crushing of the insect in lysis buffer (provided by manufacturer) prior to DNA extraction is required.

2.1.4 For both the Roche-kit and the Qiagen-kit, DNA is eluted in 50 µL preheated elution buffer (provided).

2.1.5 After DNA extraction, no DNA clean-up is required.

2.1.6 Either use extracted DNA immediately or store it at −20°C until use.

2.2 Real-time Polymerase Chain Reaction – real-time PCR

2.2.1 The master mixes for both the *S. littoralis* and *S. litura* test are displayed in Table 2. The master mixes are based on the qPCR core kit from Eurogentec.

2.2.2 real-time PCR cycling conditions: 95°C for 10 min, 40 × (95°C for 0:15 min, 66°C for 1:00 min)

3 Essential Procedural Information

3.1 Controls

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

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: Empty eppendorf tube introduced before crushing of the sample material processed as a normal sample;
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: a single egg from a *S. littoralis* or *S. litura* egg package;
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix;
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. Two DNA extracts (PAC I and PAC II) of both *S. littoralis* and *S. litura*; PAC I: 10 ng µL^{-1} (above LOD) and PAC II 0.1 ng µL^{-1} (close to LOD).

3.2 Interpretation of results

Verification of the controls:

- The PIC, PAC I and PAC II amplification curves should be exponential;
- PIC, PAC I and PAC II should have a C_t value below 40;

Table 2. Master mixes for the *Spodoptera littoralis* and *Spodoptera litura* real-time PCR tests

	Spodoptera littoralis			*Spodoptera litura*		
Reagent	Working concentration	Volume per reaction (µL)	Final concentration	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	N.A.	16.0	N.A.	N.A.	16.0	N.A.
10× reaction buffer (Eurogentec)	10×	2.5	1×	10×	2.5	1×
$MgCl_2$ (Eurogentec)	50 mM	2.5	5 mM	50 mM	2.5	5 mM
dNTPs (Eurogentec)	5 mM	1.0	0.2 mM	5 mM	1.0	0.2 mM
S. litt_S. litu-fw	10 µM	0.75	0.3 µM	10 µM	0.75	0.3 µM
S. litt_S. litu-rv	10 µM	0.75	0.3 µM	10 µM	0.75	0.3 µM
S. littoralis-T	10 µM	0.25	0.1 µM	–	–	–
S. litura-T	–	–	–	10 µM	0.25	0.1 µM
Hot Goldstar Taq (Eurogentec)	5 U µL^{-1}	0.25	1.25 U	5 U µL^{-1}	0.25	1.25 U
Subtotal		24.0			24.0	
DNA dilution: 10^0		1.0			1.0	
Total		25.0			25.0	

*Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.45 µm filtered) and nuclease-free.

- NIC and NAC should be negative (C_t equal or above 40).

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve, and a C_t value below 40;
- A test will be considered negative, if it does not produce an exponential amplification curve or if it gives a C_t value equal to or above 40.

4 Performance criteria available

4.1 Analytical sensitivity data: *S. littoralis* 2.0 pg DNA and *S. litura* 1.0 pg DNA.

4.2 Analytical specificity data: analytical specificity was determined by testing both target and non-target species in the four specific *Spodoptera* tests, i.e., 114 *Spodoptera* specimens covering 18 of the 31 *Spodoptera* species and 12 non-*Spodoptera* specimens (4 *S. albula,* 1 *S. androgea* (Cramer), 1 *S. cilium,* 1 *S. cosmioides* (Walker), 10 *S. dolichos,* 7 *S. eridania,* 1 *S. exempta,* 15 *S. exigua,* 12 *S. frugiperda,* 6 *S. latifascia,* 18 *S. littoralis,* 10 *S. litura,* 5 *S. mauritia,* 2 *S. ochrea,* 1 *S. ornithogalli* (Guenée), 2 *S. pecten,* 4 *S. pulchella,* 2 *S. triturata,* 2 *Apospasta fuscirufa,* 5 *Copitarsia corruda* Pogue & Simmons, 1 *Elaphria agrotina* (Guenée), 3 *E. nucicolora,* 1 *Periodroma saucia*). Target organisms yielded the following *Ct* values: *S. littoralis* 19.0 ± 2.3, and *S. litura* 25.3 ± 2.5.

4.3 Data on Repeatability: In total, 11 samples (2 *S. eridania*, 6 *S. frugiperda*, 1 *S. littoralis* and 2 *S. litura*) were divided in three equal parts from which 22 samples were tested as biological duplicates. All 44 results (11 samples × 2 biological duplicates × 2 technical duplicates) obtained a positive signal with the corresponding specific real-time PCR assay, and no signal with the other tests. The real-time PCR tests for the identification of *S. eridania*, *S. frugiperda*, *S. littoralis* and *S. litura* were found to be 100% repeatable.

4.4 Data on Reproducibility: In total, 11 samples (2 *S. eridania*, 6 *S. frugiperda*, 1 *S. littoralis* and 2 *S. litura*) divided in three equal parts were tested in technical duplicate. All 66 results (11 samples × 3 parts × 2 technical duplicates) obtained a positive signal with the corresponding specific real-time PCR assay, and no signal with the other tests. The real-time PCR tests for the identification of *S. eridania*, *S. frugiperda*, *S. littoralis* and *S. litura* were found to be 100% reproducible.