

National Diagnostic Protocol

Summer Fruit Tortrix

Adoxophyes orana [Lepidoptera: --
Tortricidae]



NDP 30 V1

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- 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.

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

Process

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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

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Further information

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1 INTRODUCTION

Adoxophyes orana (Fischer von Röslerstamm 1834) is a polyphagous leafroller which feeds on pome and stone fruit and is a serious pest in apple orchards as it grazes on the surface of fruit. Unlike the codling moth, however, the larva of *A. orana* does not bore deeply into the fruit. The biology in central Europe as described by Dickler (1991) is here briefly summarised.

Adoxophyes orana is usually bivoltine and rarely produces a third generation. In central Europe, the overwintering larva of the second generation complete their development by feeding on the opening buds in spring, and the resulting adults lay their eggs in May. The summer generation then develops very quickly. The larvae feed on shoots, leaves and fruit and they cause major economic damage. The resulting adults appear in late summer, lay their eggs, and the larvae of the second generation hatch in autumn. Their feeding produces shallow depressions in the fruit before the larvae enter winter diapause in the 2nd or 3rd instar, in a loosely woven cocoon under bark or some other sheltered niche.

Adoxophyes is a large genus with many often very similar-looking species characterised by strong sexual dimorphism in wing shape and pattern. Superficial identification is further impeded by the generalised, typically tortricine wing pattern of most *Adoxophyes* species and the fact that there are closely related pest genera of similar appearance in both Eurasia and Australia. However, though this has never been mentioned in any keys or fact sheets, *Adoxophyes* has a unique wing venation on both fore and hind wings, which allows unambiguous recognition of the adults of the genus *Adoxophyes*. Morphology-based identification of larva and pupa of *Adoxophyes*, on the other hand, is difficult and tenuous. Hence, if at all possible, identification should be based either on adult moths or on molecular information.

2 TAXONOMIC INFORMATION

Kingdom: Animalia, **Phylum:** Arthropoda, **Class:** Insecta, **Order:** Lepidoptera, **Family:** Tortricidae, **Subfamily:** Tortricinae, **Genus:** *Adoxophyes*. *Adoxophyes orana* (Fischer von Röslerstamm 1834)

Common name: summer fruit tortrix

Brown (2005) lists six junior synonyms for *A. orana* in his World Catalogue of the Tortricidae, namely *Adoxophyes beijingensis* Zhou, Qiu & Fu 1997 (China), *Adoxophyes fasciata* Walsingham 1900 (Japan), *Archips minor* Shiraki 1913 (Taiwan), *Tortrix reticulana* Hübner [1818-1819] (Europe), *Capua sutschana* Caradja 1926 (China), and *Tortrix tripsiana* Eversmann 1844 (Russia), with *A. orana beijingensis* and *A. orana fasciata* recognised as subspecies. Yasuda (1956) initially treated *Adoxophyes fasciata* as a synonym of *A. orana* but later (Yasuda 1975, 1998) resurrected it as a separate subspecies, restricted to Japan (Hokkaido and Honshu). *Adoxophyes orana beijingensis* was described as a subspecies of *A. orana*, from apple in northern China, whereas the nominate *A. orana orana* surprisingly is reported from cotton in southern China (Zhou *et al.* 1997). All three subspecies of *A. orana* are pests on apple. They are so similar, superficially and in genitalia morphology that the diagnostic characters used in this protocol will apply to all three subspecies.

Brown (2005) recognises 50 valid species of *Adoxophyes*, centred on the Oriental and Australian regions. Some species obviously extend across more than one region, but the type localities nevertheless are a good indication of the distribution of the genus: Australia 7 species, Papuan Region 12 species, Pacific Islands 7 species, Oriental Region 13 species, Indian Ocean Islands (including Madagascar) 6 species, China 1 species, Japan 2 species, Palearctic 1 species, North America 2 species. The strong sexual dimorphism makes it often difficult to associate males and females of a given species, and taxa based on type material of only one sex are a serious impediment to resolving the species-level taxonomy. Also, there doubtlessly are still many unnamed species of *Adoxophyes*, especially in the Oriental and Australian regions. For Australia, there are 12 unnamed or uninterpretable taxa, and of the seven described species, the Australian National Insect Collection (ANIC) has validated specimens of only five.

Checklist of Australian *Adoxophyes* (type locality in brackets):

A. fasciculana (Walker, 1866) (Ceram, Molucca Islands, Indonesia)

epipepla Lower (Cooktown, Qld)

A. heteroidana Meyrick, 1881 (Rosewood, Qld)

ablepta Turner, 1945 (Toowoomba, Qld)

amblychroa Turner, 1945 (Toowoomba, Qld)

A. meliochroa (Lower, 1899) (Mackay, Qld) no material associated in ANIC

A. installata Meyrick, 1922 (Herberton, Qld) no material associated in ANIC

A. panxantha (Lower, 1901) (Cooktown, Qld)

A. templana (Pagenstecher, 1900) (New Pommern, Bismarck Archipelago)

iotherma Meyrick, 1910 (Cairns, Qld)

thelcteropa Turner, 1945 (Cape York, Qld)

A. tripselia (Lower, 1908) (Mackay, Qld)

3 DETECTION

3.1 Symptoms

The life history of *A. orana* means that as surface feeders any immatures associated with fruit should be externally visible, albeit possibly hidden either beneath a leaf webbed to the fruit or a loose silken cocoon covering the diapausing larvae. Immatures feeding in rolled leaves (Fig a) and webbed shoots will be more difficult to detect. Egg rafts may be observed on leaves (Fig b).

Damage to fruit and leaves occurs from larval feeding (Figs c, d, e). These and other damage images can be found on the *Summer fruit tortrix moth* page of the *Encyclopédie des ravageurs européens* (<http://www7.inra.fr/hyppz/RAVAGEUR/6adoora.htm#deg>) (2 Feb 2016) or the *Apple Best Practice Guide* of the UK Agricultural and Horticultural Development Board (<http://apples.ahdb.org.uk/summer-fruit-tortrix-moth.asp>) (2 Feb 2016).

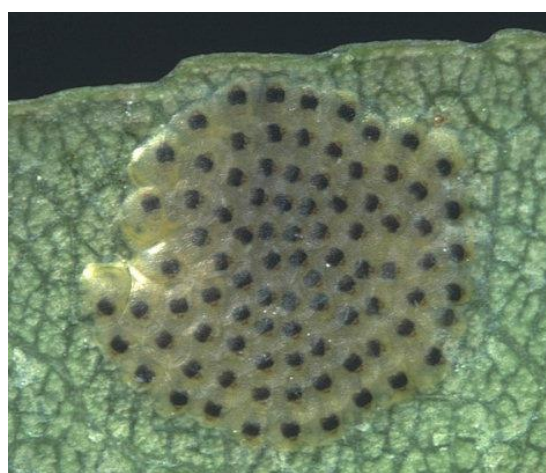


Figure a. (L). Leaf roll with *Adoxophyes orana* larvae. Photo from *Apple Best Practice Guide* of the UK Agricultural and Horticultural Development Board (<http://apples.ahdb.org.uk/summer-fruit-tortrix-moth.asp>) (2 Feb 2016).

Figure b (R). Egg rafts of *Adoxophyes orana* on apple leaf. Photo courtesy of Coutin, R *Encyclopédie des ravageurs européens* (<http://www7.inra.fr/hyppz/RAVAGEUR/6adoora.htm#deg>) (2 Feb 2016)



Figure c (L). Larvae of *Adoxophyes orana* on pear leaf. Photo courtesy of Coutin, R *Encyclopédie des ravageurs européens* (<http://www7.inra.fr/hyppz/RAVAGEUR/6adoora.htm#deg>) (2 Feb 2016)

Figure d. (R). Holes in fruit from larvae of *Adoxophyes orana*. Photo from *Apple Best Practice Guide* of the UK Agricultural and Horticultural Development Board (<http://apples.ahdb.org.uk/summer-fruit-tortrix-moth.asp>) (2 Feb 2016).



Figure e. Damage from *Adoxophyes orana* feeding. Photo courtesy of Coutin, R *Encyclopédie des ravageurs européens* (<http://www7.inra.fr/hyppz/RAVAGEUR/6adoora.htm#deg>) (2 Feb 2016)

3.2 Sampling

Given that identification of immatures is very difficult, they should either be reared to adults or preserved until the mitochondrial gene region cytochrome-c-oxidase subunit I (COI) can be sequenced for comparison with COI sequences held in the Barcode of Life Database (BOLD). For preservation techniques see 4.4 *Molecular identification through the BOLD using COI sequence data*.

As with nearly all Lepidoptera, *Adoxophyes* adults will come to light and are collected in light traps.

3.3 Australian tortricines with wing pattern similar to *A. orana*

The following species or some of their colour variants could be mistaken for *A. orana* on the basis of their brownish forewings with an outwardly oblique transverse pattern of paler and darker bands. However, except for the *Adoxophyes* species, they all have a fore- and hindwing venation as shown in Fig. 9, not with CuA1 from far below the angle of the cell as in Fig. 8. Differentiation of *A. orana* from other Australian *Adoxophyes* is addressed in 4.2, with photos in Appendix 8.1.

'Cacoecia' mnemosynana Meyrick

Australian *Adoxophyes* species (see Figs 33-55)

Epiphyas caryotis (Meyrick)

Epiphyas dotatana (Walker)

Epiphyas fabricata (Meyrick)

Epiphyas liadelpa (Meyrick)

Epiphyas postvittana (Walker)

Epiphyas pulla (Turner)

Epiphyas xylodes (Meyrick)

Merophyas divulsana (Walker)

Merophyas petrochroa (Lower)

Merophyas therina (Meyrick)

Procalyptis parooptera (Turner)

4 IDENTIFICATION

To assist with identification of material caught in Australia, photos of adult Australian *Adoxophyes* spp. and *Procalyptis parooptera* (Turner 1925) with *A. orana* have been included in Appendix 1, and genitalia photos in Appendix 2. A glossary of morphological terms is available in the Morphology Chapter of Horak (1991). For effective identification, the tortricids should first be examined morphologically, with confirmation of suspect material by sequencing.

4.1 Preparation of Material

Instructions for the preparation of genitalia slides are described by Robinson (1976) and can be found in *Moths of Australia* (Common 1990). Embedding of the genitalia may not be necessary for identification, however if a species of relevance to quarantine is identified a slide should be made and kept as a voucher. Wing venation can often be seen on the underside with tangential light as the wings are slightly raised. If this is not possible, removal of the wings and immersion in 70% ethanol will make the venation easily visible. Instructions for preparation of permanent wing slides can be found in *Moths of Australia* (Common 1990).

4.2 Identification of adult *Adoxophyes orana*

4.2.1 Identification of adult *Adoxophyes* as a tortricid

(Use this if you do NOT know the family. The costal fold is present only in some tortricids, but it is present in *Adoxophyes* and is sufficient to refer a male to the Tortricidae.)

- Female: ovipositor lobes leaf-like, dorso-ventrally flattened (Figure 1-2): treat as a tortricid, go to 4.2.2.
- Male: costal fold present on forewing (Figure 3): treat as a tortricid, go to 4.2.2.

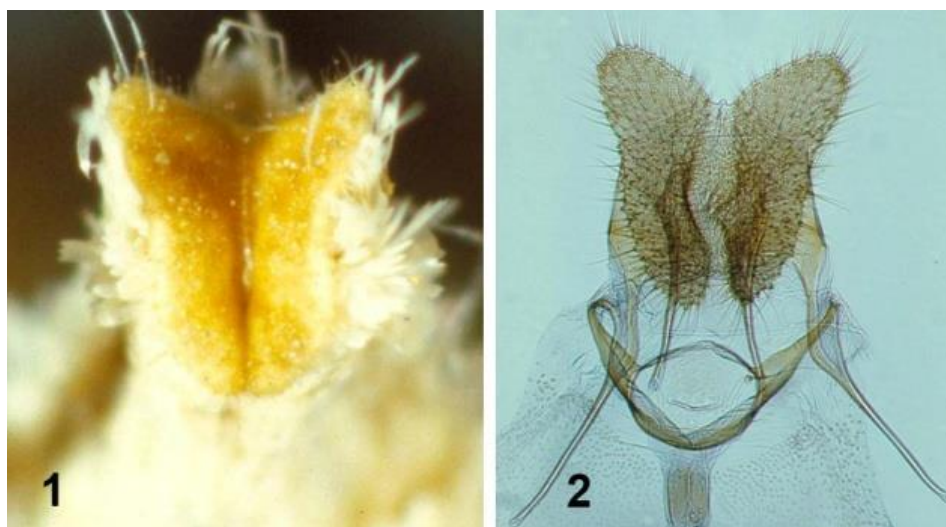


Figure 1-2 Tortricid ovipositor lobes.



Figure 3 *Adoxophyes* male.

If above two characters cannot be established (specimen damaged), the following character combination applies to nearly all tortricids, certainly to *Adoxophyes*:

1. No tympanal organ (check abdomen and thorax)
2. Proboscis (tongue, haustellum) unscaled also at base
3. Presence of ocellus AND chaetosema (Figure 4-7)
4. Scales on lower frons are orientated upwards (Figure 4-5)
5. Labial palpi S-shaped and porrect or subscending, i.e. horizontally extended or at most obliquely rising AND apical segment short, stout or tapering (Figure 4-5)
6. Maxillary palpi minute

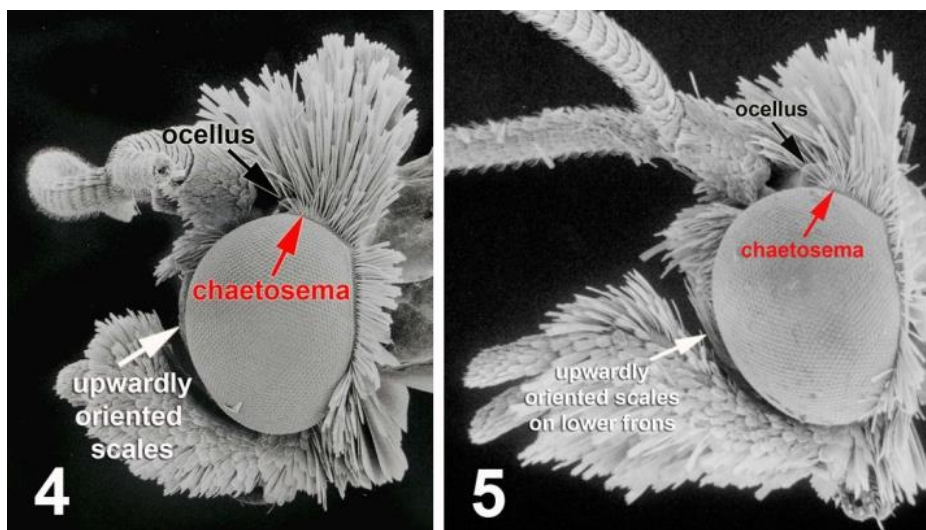


Figure 4-5 Tortricid heads.

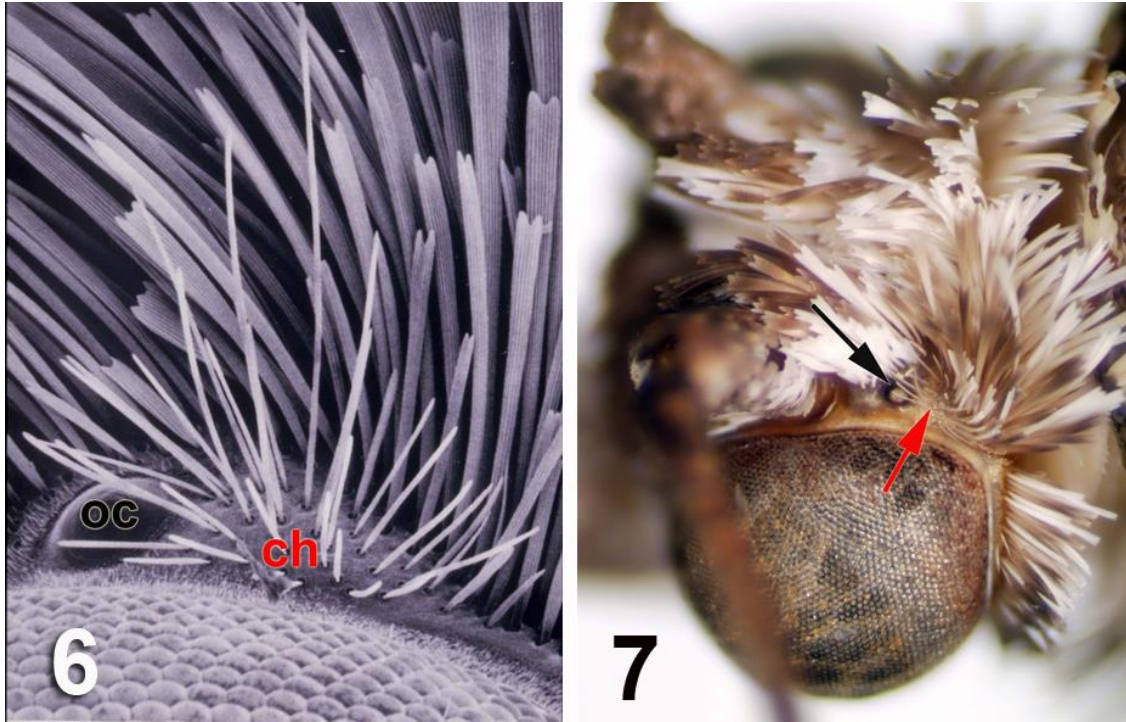


Figure 6-7 Ocellus (oc) and chaetosema (ch) on tortricid head.

4.2.2 Identification of a tortricid as *Adoxophyes*

(Use this if you know you have a tortricid or/and you suspect it could be *Adoxophyes*)

1. Antennal segments each with 2 rings of scales (Figure 11) AND hindwing without cubital pecten (NOT as Figure 13) (= not Olethreutinae)
and
2. Forewing with CuA1 distinctly from below posterior angle of discal cell; distance between bases of CuA1 and M3 rarely less than half the distance between bases of CuA1 and CuA2, usually more (Figure 8 and 10)

Adoxophyes

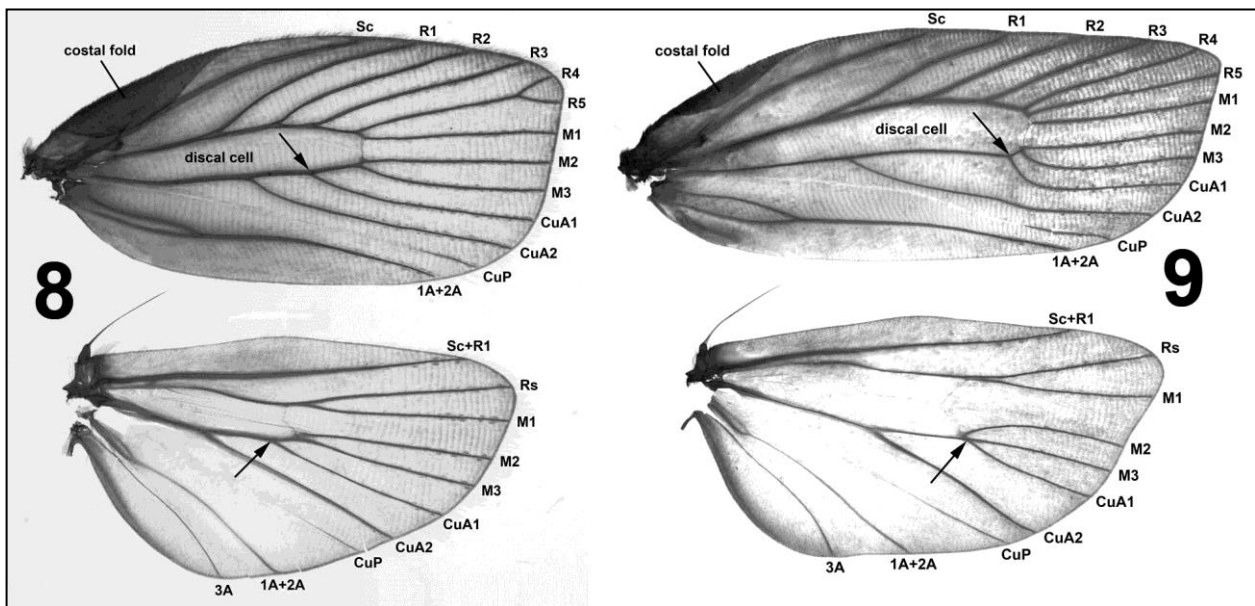
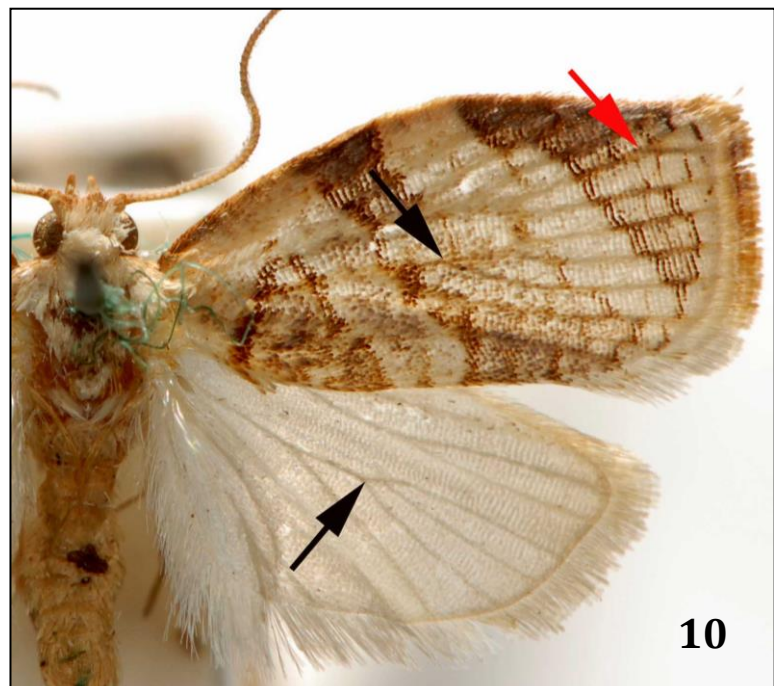


Figure 8 Wing venation of *Adoxophyes* sp.

Figure 9 Wing venation of *Epiphyas postvittana*

Figure 10 Wing venation of Australian *Adoxophyes* sp. (black arrows: base of CuA1; red arrow: stalked R4+R5)



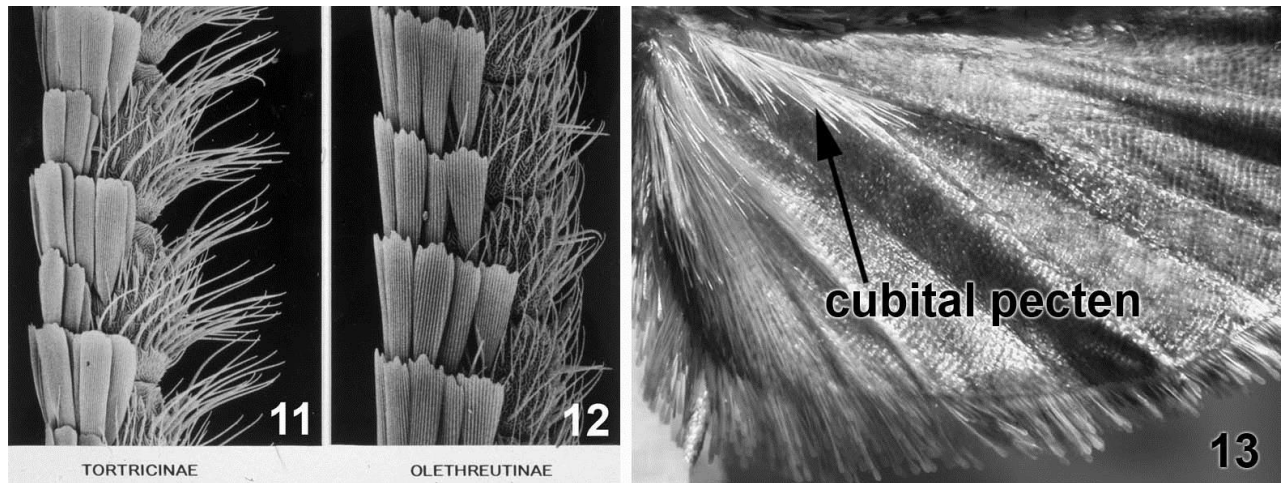


Figure 11-12 Scales on antennal segment: 2 rings per segment in Tortricinae (11); 1 ring per segment in Olethreutinae (12)

Figure 13 Cubital pecten in olethreutine hindwing (absent in *Adoxophyes* and most other Tortricinae).

Confirmation that it is *Adoxophyes*:

- Wing venation: R4+R5 stalked in forewing AND CuA1 from below angle of cell in hindwing (Figure 8 and 10)
- Male: costal fold present (Figure 3) AND valva elongate ovate (Figure 16)
- Female: signum at junction of corpus and ductus bursae AND never with a capitulum (Figure 17).

4.2.3 Identification of adult *Adoxophyes orana*

1. Hindwing grey with ochreous tinge, overall about as dark as forewings but more greyish (Figure 14-15) (Australian *Adoxophyes* all with hindwings pale, paler than forewings (Figure 10) except male of *A. panxantha* complex sp. 4, Figure 58 Appendix 8.1).

AND

2. Genitalia: Male (Figure 16)
Female (Figure 17)



Figure 14-15 Male (14) and female (15) of *Adoxophyes orana*.

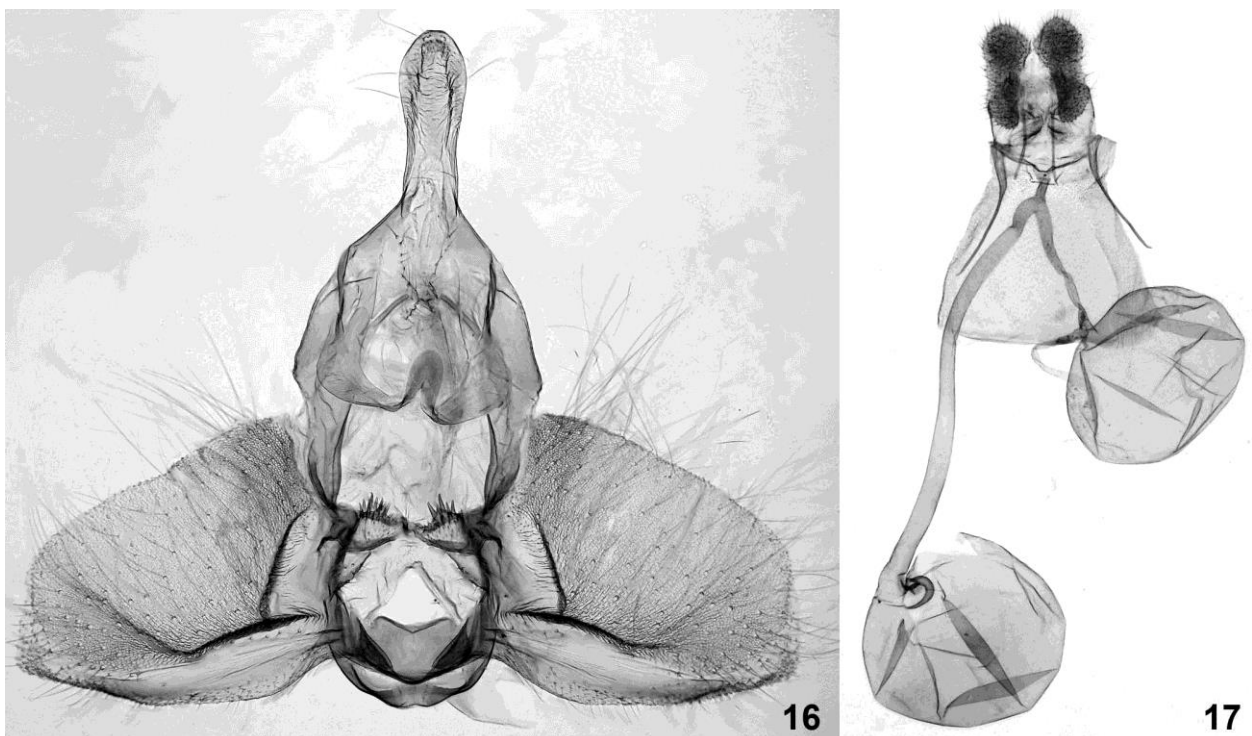


Figure 16-17 Genitalia of *Adoxophyes orana*: male (16), female (17).

4.3 Identification of late instar larva of *Adoxophyes*

4.3.1 Identification of *Lepidopteron* larva as a tortricid

(This does not comprise ALL tortricid larvae, but will identify an *Adoxophyes* larvae)

- Anal comb present AND with parallel not strongly curved prongs (Figure 18 NOT Figure 19): tortricid larva, go to 4.3.2

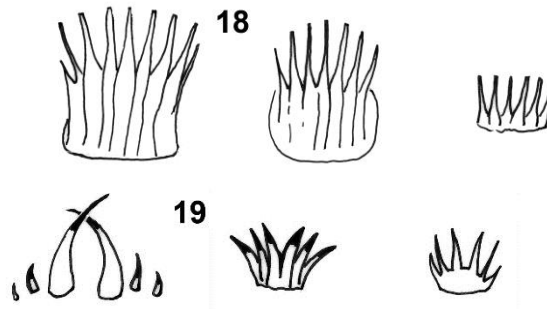


Figure 18-19 Anal combs: tortricids (18), gelechiids (19).

- If anal area damaged and comb possibly lost, the following combination identifies *Adoxophyes* and some other tortricid larvae (Fig. 20):
 1. Larva not in case
 2. Prolegs stout
 3. On T1 setal group L with three setae, all arranged horizontally on same pinaculum
 4. On A1-7 setal group L with L1 and L2 on common pinaculum ventral to spiracle
 5. On A9 seta SD1 normal, setiform, no thinner than on other segments
 6. On A9 setae D1 wider apart than setae D2, and closer to seta SD1
 7. On A8 SD pinaculum usually anterior to spiracle 8, if dorsal to spiracle 8 then contiguous to it
 8. On anal shield setae D1 usually anterior to level of SD1, or directly dorsal to SD1.

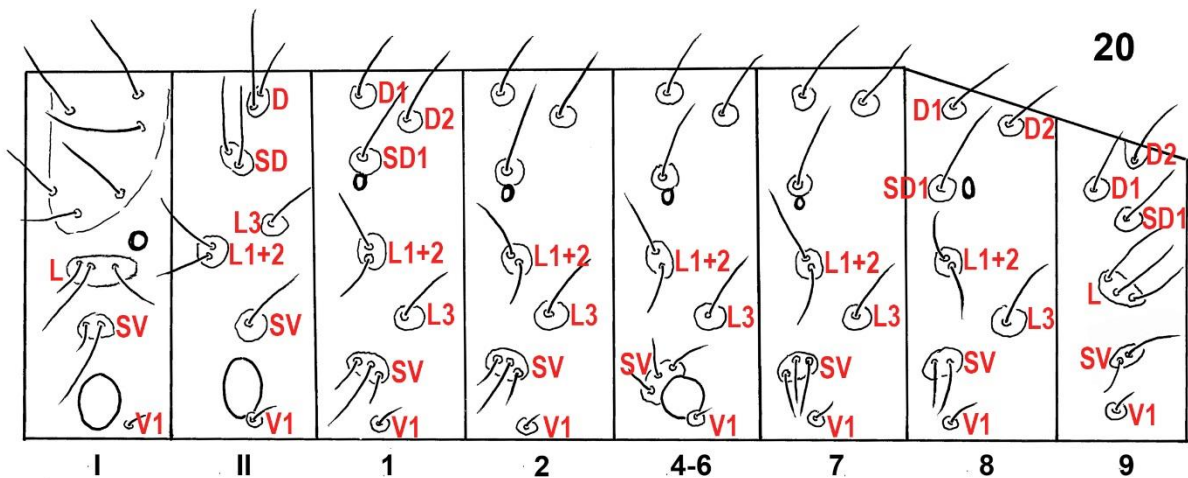


Figure 20 Setal map of *Adoxophyes orana*.

4.3.2 Identification of a tortricid larva as *Adoxophyes*

The following character combination should identify larvae of *Adoxophyes* (chaetotaxy of *A. orana*: Figure 20), but any identification has to be regarded as tentative only, to be confirmed with reared adults or molecular methods.

1. Setal group SV on A7-9 with 3, 2, and 2 setae respectively
2. Head capsule with P and MD setal series axes forming a straight line or an obtuse angle close to 180° (**a** in Figure 22-23) (rather than an angle between 100° and 140°).
3. On A9 setae D2 on common pinaculum separate from D1 and SD1 pinacula AND D2 setae closer to each other than either is to seta D1
4. Anal shield setae D1 originating at, or slightly posterior to, half shield length
5. Body without strongly pigmented pattern, at most faint D and SD stripes of darker scobination
6. V1 further apart on A9 than on A7 and A8 (unlike *Epiphyas*, *Merophyas*, *Isotenes*, *Acropolitis*, NZ archipines, all with V1 equally far apart on A7-9)
7. Prolegs uniordinal on anterior margin, otherwise biordinal (Figure 21) (diagnostic for *Adoxophyes* in Europe but somewhat similar in *Isotenes*)
8. On A2-7 spiracle only slightly larger than base of SD1 (much larger in *Epiphyas*)
9. AF2 setae level with or posterior to hindmargin of frontoclypeus (Figure 22) (unlike *Epiphyas* (Figure 23), *Merophyas*, *Isotenes*, *Acropolitis*)
10. Second ocellus closer to third than to first
11. On T2 and T3 pinaculum of seta V1 separate from coxa but on a plate contiguous with coxa (in contrast to *Archips* etc.)

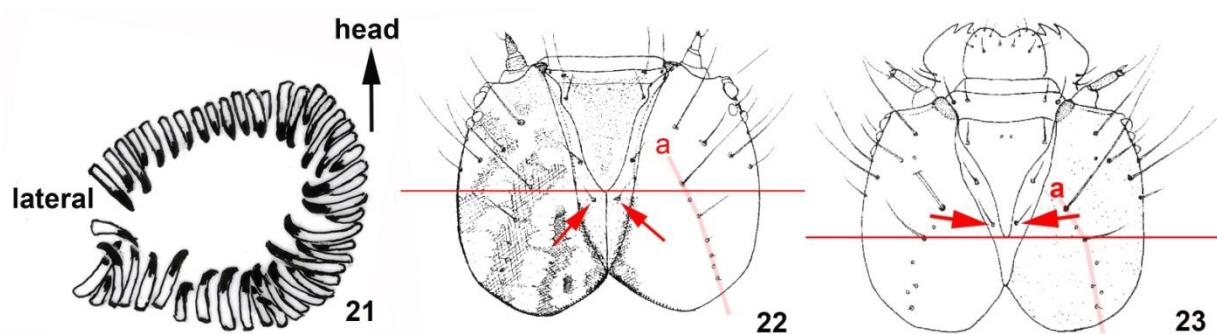


Figure 21-23 Crochets of abdominal proleg (21) and head capsule of *Adoxophyes orana* (22) and head capsule of *Epiphyas postvittana* (23). **a**: Axis of P and MD setal series. (Figure 22 and 23 modified from A Diagnostic Guide to Tortricidae encountered in field surveys in New Zealand, 2008, MAF).

4.4 Identification of *Adoxophyes orana* pupa

Pupa of Australian Archipini all have the second row of dorsal spines much smaller, not really different from Australian *Adoxophyes*; pupa of *Homona*, *Procalyptis* and *Dichelopa* have R4 and R5 stalked, like *Adoxophyes*, unlike *Epiphyas*, *Merophyas* and *Isotenes* which have R4 and R5 separate.

4.4.1 Characters of tortricid pupa

After Patocka and Turcani (2005).

- No functional mandibles
- Wings and extremities discernible
- Pupa not obtecta: dorsal side of abdomen with transverse rows of spines and pupa protrudes from pupation place before emergence
- Proboscis present (lateral to medial labium), its lobes long, parallel and distally adjacent
- Antenna not longer, but not much shorter than forewing
- Pupa longer than 5 mm
- Prothoracic femora visible
- Vertex not longer, frequently shorter than prothorax
- Tips of forewings not further than to base or middle of 4th abdominal segment
- Labium with labial palpi present
- Mesothoracic coxae conspicuous and adjacent to each other
- Clypeus without transverse projection

4.4.2 Characters of *Adoxophyes* pupa

After Patocka and Turcani (2005).

This is based on *A. orana*, not all these characters apply to Australian *Adoxophyes* which do not have the dorsal spines in the distal row as small as *A. orana*.

- Cremaster more or less long and slender in ventral view
- Four pairs of caudal setae, with the two central pairs (hooks) arising close to each other in joint depression (Figures 27, 28)
- Transverse furrow at base of 2nd and 3rd abdominal segments not interrupted in middle
- 10th abdominal segment without spines dorsally
- Spines of basal row on 2nd and 3rd abdominal segment normal spines or tubercles not longitudinal ridges
- The 4th and 5th abdominal segment bear in their distal rows more than 30 very tiny spines between setae D2 (Figure 26) (other European genera [and Australian *Adoxophyes*]: no more than 20 bigger spines between setae D2)
- Proboscis exceeds prothoracic femora
- Forewing with bifurcation of the two radial veins R4 and R5 (Figure 24) (also in *Homona*, *Procalyptis* and *Dichelopa*, but unlike *Epiphyas*, *Merophyas* and *Isotenes* which have R4 and R5 separate).

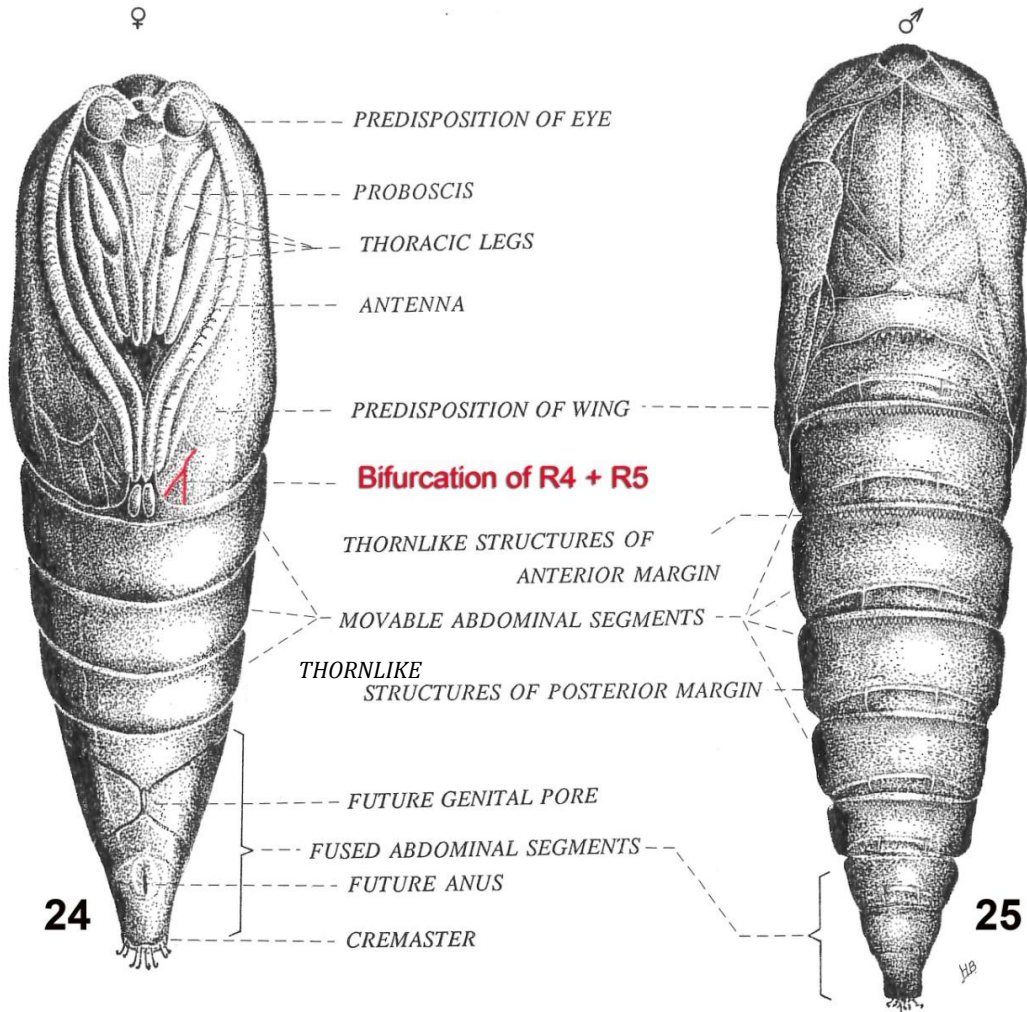


Figure 24-25 Semi-schematic representation of pupa of *Adoxophyes orana*: female, ventral (24); male, dorsal (25) (From Beeke and de Jong, 1991)

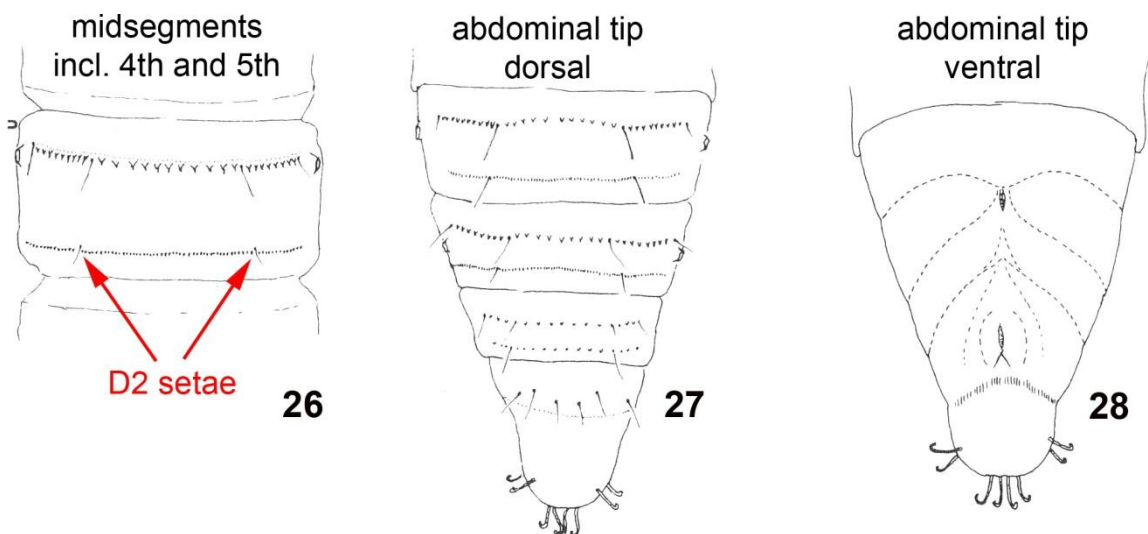


Figure 26-28 Details of pupa of *Adoxophyes orana* (From Beeke and de Jong, 1991)

4.5 Identification through the BOLD using COI sequence data

For DNA barcoding, a single leg (approximately 2–3 mm long) is sufficient for DNA extraction. Other parts such as the antennae, wing veins/membranes can be used but legs are preferred since they are paired appendices. The posterior end of the abdomen and the genitalia can be recovered after DNA extraction. Eggs, caterpillars and pupae can also be used for DNA barcoding as long as the specimens are preserved as vouchers. The head capsule, a transversal section of a segment, a thoracic leg of a caterpillar and any epidermis tissue from a pupa can be used. All tissue can be collected fresh and allowed to dry rapidly and/or preserved in 95% ethanol. Preserved specimens can be stored at -20°C until required. More details on specimen collection and preservation can be found at <http://lepbarcoding.org/protocols.php>. (Accessed 2/2/2016). Barcoding should only be used as a definitive identification of specimens already morphologically identified.

4.5.1 Material and equipment requirements

- Sterile 1.5 and 2 mL microcentrifuge tube
- Beakers
- Scalpel blades
- Vortexer
- Benchtop centrifuge
- Microscope
- 0.5–10 µL and 200–1000 µL pipettes
- 0.5–10 µL and 200–1000 µL sterile filtered tips
- Latex (powder free) or nitrile gloves
- Shaking waterbath or incubator
- Tube rack
- Measuring cylinder
- Micropestles
- 100% ethanol
- Polymerase Chain Reaction machine
- Gel electrophoresis apparatus
- UV transilluminator
- Qiagen DNeasy Blood and Tissue Kit or ZyGEM *prepGem* Insect
- Qiagen QIAquick PCR Purification Kit
- DNA marker ladders (any brand is suitable, but needs to be in 100 bp increments)
- 1 X TAE buffer
- Agarose
- Ethidium bromide or SYBR® Safe DNA Gel Stain

4.5.2 DNA extractions

Using the commercial kit DNeasy Blood and Tissue Kit (Qiagen, Australia). Instructions below are an excerpt from the manufacturer's protocol supplied with the kit.

Purified DNA extracts can last for many years stored at -20°C.

1. If samples are preserved in ethanol, transfer specimen to a fresh sterile 2 mL microcentrifuge and rinse several times with sterile distilled water.

2. For larvae, add 180 μ L of buffer ATL and 20 μ L proteinase K (600 mAU/mL), using a sharp sterile needle, pierce a hole in the caterpillar.
3. For tissue (i.e. adult leg), add 100 μ L of buffer ATL and grind material using a sterile micropestle (an abrasive such as silicon carborundum can be used). Once material is fully ground, add the remaining 80 μ L buffer ATL and 20 μ L proteinase K.
4. Incubate tube at 56°C for at least 2 hours to overnight with slight agitation to mix solution and enhance cell lysis. It is recommended to lay tubes on a microcentrifuge rack, secure tubes using masking tape. Fix rack onto shaking tray such as an incubator shaker, shaking waterbath or rocking platform.
5. After incubation, the caterpillar (if used) can be removed and preserved in absolute ethanol.
6. Vortex tubes for 15 seconds.
7. Add 200 μ L of buffer AL, vortex for 5–10 seconds and add 200 μ L of absolute ethanol and vortex for 5–10 seconds.
8. Transfer the mixture to the labelled DNeasy mini spin column placed in a collection tube.
9. Centrifuge the columns at $\geq 6,000$ g for 1 minute. Discard the flow through and the collection tube.
10. Transfer the DNeasy spin column into a new 2 mL collection tube and add 500 μ L of buffer AW1 and centrifuge at $\geq 6,000$ g for 1 minute. Discard the flow through and the collection tube.
11. Transfer the DNeasy spin column into a new 2 mL collection tube and add 500 μ L of buffer AW2 and centrifuge at 20,000 g for 3 minutes. Discard the flow through and the collection tube. It is important to dry the membrane.
12. Transfer the column to a new sterile 2 mL microcentrifuge and add 200 of buffer AE directly to the membrane without touching it. Allow to sit at room temperature for 1 minute. Centrifuge the columns at $\geq 6,000$ g for 1 minute to elute DNA.
13. Add a further 200 μ L of buffer AE directly to the membrane without touching it. Allow to sit at room temperature for 1 minute. Centrifuge the columns at $\geq 6,000$ g for 1 minute to elute DNA.
14. Store the purified genomic DNA extract at -20°C until required.

DNA extractions can also be conducted using *prepGem* Insect as per manufacturer's instructions (ZyGem, New Zealand), a fast, one step and closed tube process. This kit is useful for a rapid result, however it is unclear how long the DNA can be stored.

1. Finely chop the specimen into minute pieces (preferably as small as possible) under a microscope.
2. Transfer the material to a PCR tube, add 35 μ L of PCR grade water, 4 μ L of 10 x Buffer followed by 1 μ L *prepGem*.
3. Incubate the specimens in a PCR thermocycler for 15 min at 75 °C followed by 5 min at 95 °C.
4. Transfer the supernatant to a new sterile 1.5 mL tube.
5. Store the DNA solution at -20°C until required.

4.5.3 Polymerase chain reaction

DNA barcoding targets a ~650 bp section on the 5' region of the COI gene and is used to identify species in the animal kingdom (Hebert *et al.* 2003). It is a standardised method using a mitochondrial gene which is easily transferable to different laboratories and the data can be compared across a large number of taxa (Hanner 2005, Armstrong 2010). Primers commonly used for DNA barcoding of Lepidoptera are listed in Table 1.

Table 1 Primers commonly used for DNA barcoding

Primer name	Primer sequence (5' - 3')	Target gene	Reference
LCO1490	GGTCAACAAATCATAAAGATATTG	COI	Folmer <i>et al.</i> 1994
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	COI	Folmer <i>et al.</i> 1994
BC1-Fm	GTAAAACGACGGCCAGTTCWACWAAYCAYAARGAYATYGG	COI	Cho <i>et al.</i> 2008
Scar3RDm	CAGGAAACAGCTATGACAAAATRTAWACTTCDGGRTGNCC	COI	Mitchell and Maddox 2010
Enh_LepR1	CTCCWCCAGCAGGATCAAAA	COI	Hajibabaei <i>et al.</i> 2006
MF1	GCTTTCCCACGAAATAAATAATA	COI	Hajibabaei <i>et al.</i> 2006
MH-MRI	CCTGTTCCAGCTCCATTTTC	COI	Hajibabaei <i>et al.</i> 2006
LepF1	ATTCAACCAATCATAAAGATAT	COI	Hebert <i>et al.</i> 2004
LepR1	TAAACTTCTGGATGTCCAAAAA	COI	Hebert <i>et al.</i> 2004

Note:

- LCO1490/HCO2198 combination will give 700 bp product
- BC1-Fm/Scar3RDm combination will give 667 bp when using the flanking M13 primer for sequencing
- LepF1/LepR1 combination will give 648 bp
- LepF1/Enh_LepR1 combination will give 618 bp
- For specimens >10 years MF1/ LepR1 combination will give 407 bp
- LepF1/MH-MRI combination will give 311 bp PCR product.

In the PCR clean cabinet:

1. Make up the PCR mastermix with additional 10% more than required i.e. for 8 samples, make a mastermix for 9 reactions (Table 2). The PCR components may differ depending on each laboratory's preference, therefore the test may need to be optimised in the first instance.
2. Thaw components and spin briefly to collect the liquid before pipetting.
3. Pipette everything into the 1.5 mL microcentrifuge.
4. Mix components by gently inverting the tube a few times and then spin briefly in a benchtop centrifuge to collect the liquid.
5. Add 45 µL of PCR cocktail to each PCR tube and add 5 µL DNA template.

Table 2 PCR mastermix components

Stock	Amount required for 1 reaction
Bioline 5X MyFi reaction buffer	10
LCO (10 µM) (forward primer)	2
HCO (10 µM) (reverse primer)	2
Bioline MyFi Taq	2
Sterile Distilled Water	29
Total volume	45 µL

Each PCR run requires two controls, one positive control (known insect DNA specimen that will be amplified using this PCR protocol) and one negative control (PCR master mix only, no DNA Template). Recommended PCR thermal cycle conditions using the above PCR mix is

Step	Temp. (°C)	Time	
Initial denaturing	95	1m	
Denaturing	95	15s	35 cycles
Annealing	50	15s	
Extension	72	15s	

4.5.4 Agarose gel electrophoresis

While the PCR is running, prepare the 1% agarose gel. The time and voltage specified below is for the common electrophoresis gel system. This may differ if a high speed gel system is used.

NB: This gel recipe uses Ethidium Bromide (EtBr). EtBr is a carcinogen used to bind to DNA and fluoresces under UV light. If using EtBr, all safety precautions must be heeded. Double gloves must be used at all times to handle EtBr, the inner gloves may be the normal lab gloves but the outer glove must be nitrile. Lab coats or gowns must be worn at all times. Make sure the outer gloves are removed before exiting the dark room. All equipment and benches inside the dark room must be considered to be contaminated with EtBr.

SYBR® Safe DNA Gel Stain can be used instead.

Preparation

1. Gels can be either small (30 mL) for less than 14 samples or large (70 mL) for over 14 samples.
2. For small gels, weigh 0.3 g of agarose or large, weigh 0.7 g and pour into the dedicated 250 mL gel bottle.
3. Add 30 mL or 70 mL 1 x TAE buffer to the bottle.
4. Prepare the gel trays, depending on the number of samples, the small gels have the 12 or 15 well combs, the large gels have the 15, 20 or 30 well combs. Always leave 1 lane free for the DNA reference marker of choice (NB. Recommend a 1 KB DNA ladder).
5. Heat the agarose in the microwave (dark room) until agarose is completely melted. Cool the solution in the water bath in the sink until cool enough to hold in your gloved hands (~50°C).
6. Add EtBr solution (5 mg/mL) to the molten agarose, 5 µL (small gel) and 9 µL (large gel) and swirl gently to mix. NB. SYBR® Safe DNA Gel Stain can also be used according to manufacturer's instructions.
7. Carefully pour molten agarose into the gel trays prepared earlier.
8. Always leave gels to set for at least 1.5 to 2 hours. Once the gel is ready for use, transfer to the gel bath containing 1 X TAE buffer. Make sure the buffer fully covers the gel.

Gel electrophoresis

1. Add 4 µL 1kb DNA ladder to the first lane of the gel, then proceed to add 4 µL samples.

2. Cover the tank with the supplied lid and run the gel for 30–40 minutes at 80 or 90V.
3. Once complete transfer the gel to the UV transilluminator and take a photo.

4.5.5 PCR purification

PCR purification can be achieved using QIAquick PCR purification kit (Qiagen, Australia). Instructions below are an excerpt from the manufacturer's protocol.

1. Transfer PCR products to a 1.5 mL microcentrifuge and estimate the volume using the micropipette.
2. Add 5 volumes of Buffer PB1 to 1 volume of the PCR sample and mix thoroughly.
3. Transfer the solution to QIAquick spin column (purple) in the provided collection tube.
4. Centrifuge the column for 1 minute at 13,000 rpm (17,900 g).
5. Discard the flow through. Add 750 μ L Buffer PE and centrifuge for 1 minute at 13,000 rpm (17,900 g).
6. Discard the flow through and centrifuge again for a further 1 minute.
7. Place the column in a new 1.5 mL microcentrifuge.
8. Add 30 μ L Sterile Distilled Water (pH 7.3 – 7.5) directly to the centre of the membrane. DO NOT TOUCH THE MEMBRANE. Water is recommended, since the elution buffer supplied in the kit may interfere with the sequencing reaction.
9. Wait for 1 minute and then centrifuge for 1 minute at 13,000 rpm (17,900 g).
10. Prepare a 1% agarose gel as described earlier.
11. Add 3 μ L of purified PCR product to 1 μ L of loading dye. Also dilute 4 μ L of low DNA mass ladder with 1 μ L loading dye. Load the mass ladder to the first lane and proceed with remaining samples.
12. Run gel for 40 minutes at 80V.
13. Estimate the DNA concentration based upon the intensity of the band compared to the ladder and not the position of the band.

4.5.6 Sequencing and Bioinformatics

The purified COI PCR product can be sent to any sequencing facility for sequencing such as Australian Genome Research Facility, Micromon or Macrogen (list is not exhaustive). There are range of software available that can be used to edit sequences, these may include Geneious and/or Bioedit (list is not exhaustive). Once edited and checked, the sequence can be entered into the barcode of life system (BOLD)(<http://www.boldsystems.org/>) – identification 'species level' field. The BOLD will indicate whether a species match was identified based upon at least 500 bp of the COI gene. NB. If no species match is available, the next option is to repeat the same search and select the 'all barcode records'. The comparison with a barcode reference library allows species identification from a taxonomically unknown specimen (Wilson 2010). The BOLD uses the barcode index number (BIN) system, a species-level taxonomic registry for the animal kingdom based upon the COI divergences for the animal kingdom to be less than 2% (Ratnasingham and Hebert 2013). Confidence in species identification would be based upon 100% sequence match of the DNA barcode region. Within the 'species level' match in the BOLD system, the unknown specimen in question will be matched to the closest matched species and with the BIN provided. If the algorithm of the program cannot reliably assign a species, the system will alert the user. Additional phylogenetic analyses can be conducted using Geneious, MEGA or any other equivalent software.

5 CONTACTS FOR FURTHER INFORMATION

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7.1 Other useful references

Interactive Agricultural Ecological Atlas of Russia and neighbouring countries (AgroAtlas):

http://www.agroatlas.ru/en/content/pests/Adoxophyes_orana/

Mini Risk Assessment: Summer Fruit Tortrix Moth, *Adoxophyes orana* (Fischer von Röslerstamm, 1834) [Lepidoptera: Tortricidae]:

http://www.aphis.usda.gov/plant_health/plant_pest_info/pest_detection/downloads/pr/aoranapra.pdf

INRA *Adoxophyes orana* Fact Sheet: <http://www.inra.fr/hyppz/RAVAGEUR/6adoora.htm#cyc>

8. Appendix

8.1 Photos of adult Australian *Adoxophyes* spp., *A. orana* and *Procalyptis parooptera*

Male left, female right, all same magnification. The labelling of unidentified Australian species follows the tentative labelling in the Australian National Insect Collection as these preliminary labels are used in BOLD. As long as the identity of three named Australian species (*A. meliochroa*, *A. installata* and *A. panxantha*) has not been established the status 'sp. nov.' for any species of *Adoxophyes* is tentative and has to be confirmed.



Figs 29, 30. *Adoxophyes orana*, Switzerland



Figs 31, 32. *Adoxophyes orana*, Denmark



Figs 33, 34. *Adoxophyes fasciculana*, Australia



Figs 35, 36. *Adoxophyes templana*, Australia



Figs 37, 38. *Adoxophyes* sp. ANIC 2, Australia



Figs 39, 40. *Adoxophyes heteroidana*, Australia



Figs 41, 42. *Adoxophyes tripselia*, Australia



Figs 43, 44. *Adoxophyes* sp. nov. C, Australia



Figs 45, 46. *Adoxophyes* sp. nov. A, Australia



Figs 47, 48. *Adoxophyes* sp. nov. D, Australia



Figs 49, 50. *Adoxophyes* sp. nov. E, Australia



Figs 51, 52. *Adoxophyes* sp. 12, Australia



Figs 53, 54. *Adoxophyes meliochroa* complex sp. 7, Australia



Fig. 55. *Adoxophyes meliochroa* complex sp. 9, Australia



Figs 56, 57. *Adoxophyes panxantha* complex sp. 3, Australia



Figs 58, 59. *Adoxophyes panxantha* complex sp. 4, Australia

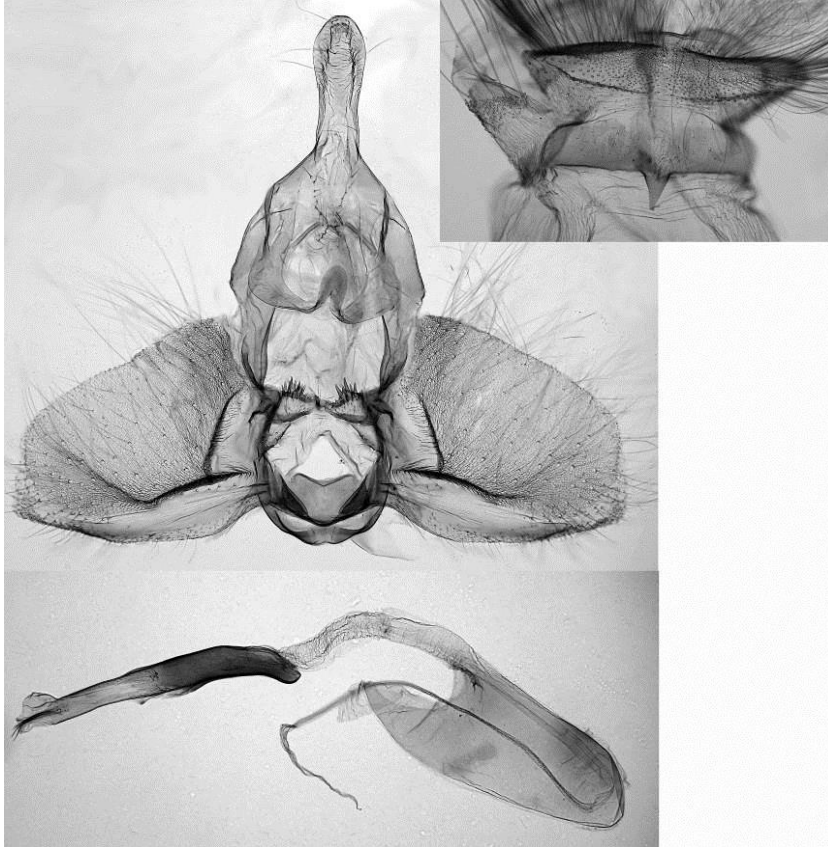


Figs 60, 61. *Adoxophyes panxantha* complex sp. 5, Australia

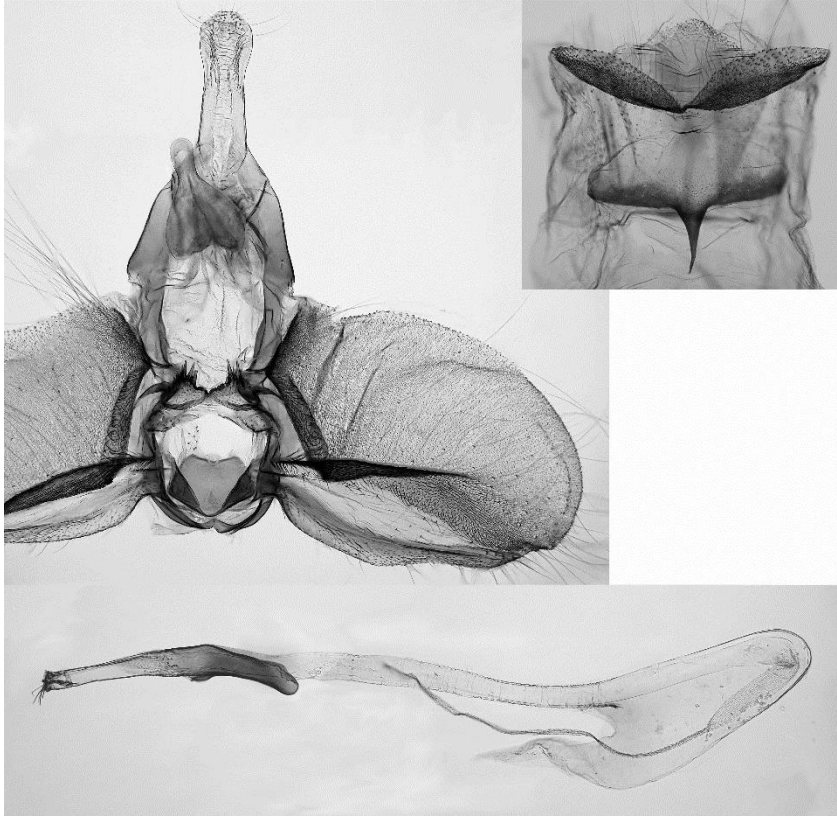


Figs 62, 63. *Procalyptis parooptera*, Australia

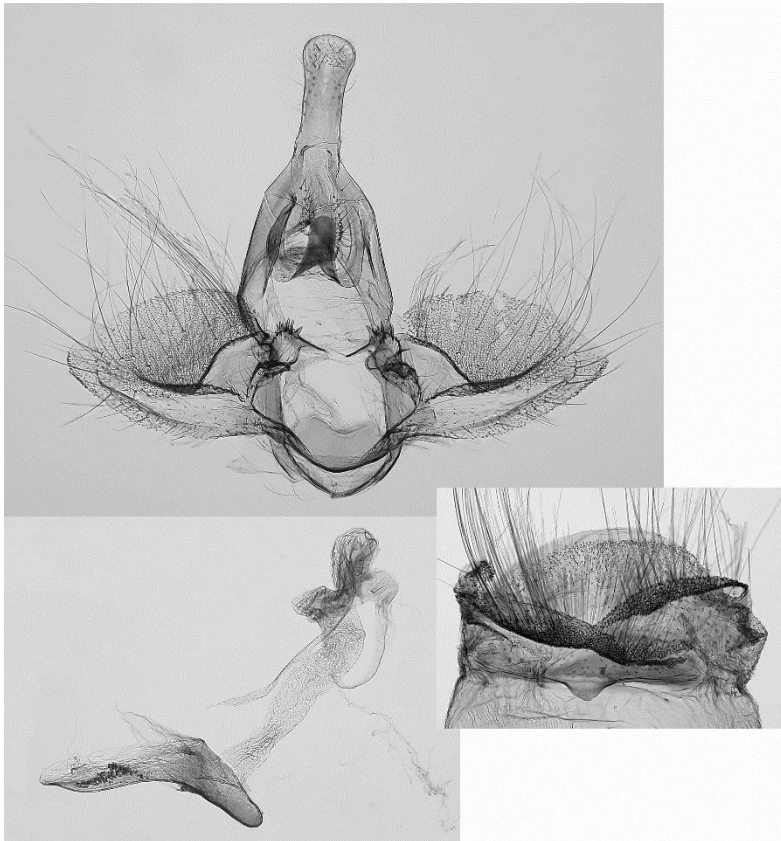
8.2 Genitalia photos of *Adoxophyes orana*, *A. fasciculana* and *A. panxantha* complex sp. 4



Figs 64-66. *Adoxophyes orana*, male genitalia



Figs 67-69. *Adoxophyes fasciculana*, male genitalia



Figs 70-72. *Adoxophyes panxantha* complex sp. 4, male genitalia