Diagnostics
Diagnostic

Liriomyza spp.

Specific scope
This standard describes a diagnostic protocol for *Liriomyza bryoniae*, *Liriomyza huidobrensis*, *Liriomyza sativae* and *Liriomyza trifolii*.

Specific approval and amendment
This Standard was developed under the EU DIAGPRO Project (SMT 4-CT98-2252) by partnership of contractor laboratories and intercomparison laboratories in European countries. Approved as an EPPO Standard in 2004-09.

Introduction
There are 376 species currently recognized in the genus *Liriomyza* (David Henshaw, pers. comm., 2000), of which 136 are found naturally in Europe (Seymour, 1994). The adult flies of all these species look very similar. They are all small (1–3 mm in length) and, from above, are seen to be mostly black, with in most species a bright yellow scutellum. As a result, separating these species can be difficult. Close examination reveals small external differences that can be used to separate the species, such as the relative length of sections along particular wing veins, the presence, position and size of certain setae or the colour of the cuticle at the point where particular head setae arise. However, considerable variation in these character states is seen in the polyphagous pest species. As a consequence, for the pest species concerned, the ranges of variation of these characters often overlap, limiting their diagnostic value.

Four species, *Liriomyza bryoniae*, *Liriomyza huidobrensis*, *Liriomyza sativae* and *Liriomyza trifolii* are listed in EU Plant Health Directive 2000/29 (EU, 2000). *L. bryoniae* is indigenous to Europe, while the other three all originated in the New World. All are polyphagous pests of ornamental and vegetable crops. Because of the different phytosanitary measures applied when the various *Liriomyza* spp. are detected on plant material, precise species identification is required.

To identify these species, the diagnostician has not only to distinguish between them, but also to distinguish them from the background fauna of indigenous *Liriomyza* spp. (which are mostly not pests). This composition of this background fauna varies across Europe and no one morphological dichotomous key has been produced that will separate each of the four species both from each other and from the European fauna.

This protocol presents methodologies by which the identity of these four species can be confirmed, whether the material available for examination consists of larvae or pupae (Fig. 1), or adult flies (Fig. 2). Since larvae and puparia possess few distinguishing morphological characters, isozyme analysis (Appendix I) and PCR-RFLP analysis (Appendix III) are recommended as additional methods. PCR-RFLP may also be useful to confirm morphological analysis of adults or to identify damaged specimens. A further species, *L. strigata* (Meigen, 1830), is a common, polyphagous species, indigenous to Europe. Because it is sometimes a minor pest itself and because it can be found in close proximity with the four listed species, the species is included in this protocol. *L. cocculi* (Frick, 1953) is a species from Hawaii whose close relationship to *L. huidobrensis* is indicated by the structure of the male genitalia (Spencer, 1990). However, it has a dark scutellum, is unlikely to be encountered in Europe or in association with imported commodities and is not discussed further here.

Identity
Name: *Liriomyza bryoniae* (Kaltenbach, 1858)

Synonyms: *Agromyza bryoniae* (Kaltenbach, 1858); *Liriomyza solani* (Hering, 1927); *Liriomyza citrulli* (Rohdendorf, 1950)

Taxonomic position: Insecta: Diptera: Agromyzidae

EPPO computer code: LIRIBO

Phytosanitary categorization: EU Annex designation I/B
Name: *Liriomyza huidobrensis* (Blanchard, 1926)

Synonyms: Agromyza huidobrensis (Blanchard, 1926); *Liriomyza cucumifoliae* (Blanchard, 1938); *Liriomyza langei* (Frick, 1951); *Liriomyza dianthi* (Frick, 1958)

Taxonomic position: *Insecta: Diptera: Agromyzidae*

EPPO computer code: LIRIHU

Phytosanitary categorization: EPPO A2 list no. 152, EU Annex designation II/A2

Name: *Liriomyza sativae* (Blanchard, 1938)

Synonyms: *Liriomyza pullata* (Frick, 1952); *Liriomyza canomarginis* (Frick, 1952); *Liriomyza minutiseta* (Frick, 1952); *Liriomyza propepusilla* (Frost, 1954); *Liriomyza munda* (Frick, 1957); *Liriomyza guytoma* (Freeman, 1958)

Taxonomic position: *Insecta: Diptera: Agromyzidae*

EPPO computer code: LIRITA

Phytosanitary categorization: EPPO A1 list no. 152, EU Annex designation I/A1

Name: *Liriomyza trifolii* (Burgess, 1880)

Synonyms: *Liriomyza alliovora* (Frick, 1955)

Taxonomic position: *Insecta: Diptera: Agromyzidae*

EPPO computer code: LIRIRI

Phytosanitary categorization: EPPO A2 list no. 131, EU Annex designation II/A2

Detection

Damage symptoms

Feeding punctures and leaf mines are usually the first and most obvious sign of the presence of *Liriomyza* spp. They remain intact and relatively unchanged over a period of weeks. Mine configuration is often considered a reliable guide to the identification of agromyzid species of no economic importance (as in many such cases the species are host-specific). However, with the polyphagous pest species, mine configuration is affected by the host, by the physical and physiological condition of each leaf and by the number of larvae mining the same leaf. This wider range of variation means that identification from mine patterns alone should be treated with caution.

Feeding punctures

Feeding punctures of *Liriomyza* spp. are rounded and usually about 0.2 mm in diameter. They appear as white speckles on the upper leaf surface. The appearance of the punctures does not differ between species, nor can the pattern of their distribution on the leaf be used to separate species.

Leaf mines

The larvae feed mostly in the upper part of the leaf, mining through the green palisade tissue. Mines are usually off-white, with trails of frass appearing as broken black strips along their length. Repeated convolutions in the same small part of the leaf will often result in discoloration of the mine with dampened black and dried brown areas appearing, usually as the result of plant-induced reactions to the leaf miner. The typical appearances of mines (Web Figs 9 and 10) of these species are:

- a tightly coiled, almost blotch-like mine – *L. trifolii*
- a looser, irregular serpentine mine – *L. bryoniae* and *L. sativae*
- an irregular serpentine mine tending to be restricted by veins within segments of the leaf and undulating between upper and lower leaf surface – *L. huidobrensis*
- a mine closely following the main vein toward (and occasionally into) the petiole – *L. strigata*.

Larvae exit the fully developed mines in order to pupariate (usually in the soil, sometimes on the surface of the leaf). The exit hole characteristically takes the form of a semicircular slit.

The mines of other species of agromyzids may look similar to those described above. Nevertheless, the feeding punctures and mines of *Chromatomyia syngenesiae* can usually be separated from those described above (Web Fig. 11). The feeding punctures of *C. syngenesiae* are larger (up to 1.0 mm in diameter) and distinctly oval in shape. The mines appear cleaner, uniformly white, with less convolutions and the frass appearing as distinctly separated black dots. As with *L. huidobrensis*, the mines can undulate between the upper and lower leaf surfaces. The larvae of *C. syngenesiae*, and of *C. horticola*, pupariate within the mine with the anterior spiracles usually projecting out from the lower surface of the leaf.

Identification of family and genus

Morphological terminology used in this protocol is based on that of McAlpine *et al.* (1981).

Family: *Agromyzidae*

Agromyzids are small flies whose larvae are leaf miners, stem borers or gall-makers.
Formal description (of the adult)
The following combination of characters (Web Fig. 3), which define the family Agromyzidae, follows Hennig (1958) (as quoted in Spencer, 1987). Vibrissae present; 1–7 frontal bristles present; costal break present at the apex of Sc; cell cup small; A1 not reaching wing margin; pregenital sclerites of male with a simple (fused) tergal complex (tergites 6–8) with only two spiracles between tergite 5 and the genital segment; and anterior part of abdominal segment 7 in female forming an oviscape.

Practical diagnosis (based on the larval stages)
In practice, agromyzids are recognizable because their larvae feed in the living tissue of plants (three-quarters of them are leaf miners). There are leaf miners in other Dipteran families. Typically, agromyzid larvae are cylindrical in shape, tapering anteriorly; with projections bearing the anterior and posterior spiracles, the former positioned on the dorsal surface of the prothorax, the latter backwardly directed at the rear; prominent, strongly sclerotized mouthparts, the mandibles with its longitudinal axis at oblique or right angles to the rest.
of the cephalopharyngeal skeleton and usually bearing two or more pairs of equally sized teeth, directed anteriorly, the ventral cornua (the posteriorly directed 'arms') commonly shorter than the dorsal ones. For a summary of information on the morphology and biology of the immature stages of agromyzids, with a large bibliography and illustrations of the cephalopharyngeal skeleton and posterior spiracles for a number of species, see Ferrar (1987).

Genus: Liriomyza

*Formal description (of the adult)*

Small flies, 1–3 mm in length; fronto-orbital setulae reclinate; usually with a dark prescutellar area concolorous with the scutum, rarely yellow; scutellum yellow in most species, rarely dark; costa extends to vein M1; discal cell small; dm-cu crossvein present in most species; stridulating organ...
present in males (a ‘scraper’, a chitinized ridge on the hind-femora, and a ‘file’, a line of low chitinized scales on the connecting membrane between the abdominal tergites and sternites).

**Practical diagnosis**

The economically important species discussed in this protocol are seen from above to be mostly black with a yellow frons and a bright yellow scutellum. The legs are variably yellow. They possess the typical wing venation for the genus (Web Fig. 4).

**Natural species groups**

The *Liriomyza* spp. considered here separate into two distinct natural groups, based on the structure of the male genitalia, and the colour and the structure of the posterior spiracles of the larvae. However, the external characters of the adult flies useful for identification (Table 1), particularly those based on colour, do not fall neatly into these two groupings: Group 1 (*L. bryoniae*, *L. huidobrensis*, *L. strigata*, *L. cocculi*); Group 2 (*L. sativae*, *L. trifolii*).

**Identification of the different life stages**

**Eggs**

The eggs are laid into the leaf tissue. They are white and oval, about 0.25 mm in length. Neither genus nor species identification is possible.

**Larvae and pupae**

There are three larval instars, which feed as they tunnel through the leaf tissue. The newly emerged larvae are about 0.5 mm long but reach 3.0 mm when full-grown. They are typical of agromyzids in gross form (see above, and Web Fig. 12a). Pupae are oval, about 2.0 mm in length, very slightly flattened ventrally, with projecting anterior and posterior spiracles. In practice, for larvae and pupae, the two natural groups can be distinguished from each other morphologically but not the species within the groups. Species determination requires electrophoretic analysis (see Appendix I) or PCR-RFLP (Appendix III).

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**Table 1** Morphological characters of *Liriomyza* spp., adult

|                      | Male distiphallus            | Vertical setae (see Web Fig. 3) | Anepisternum (see Web Fig. 3) | Vein Cu 1 A (see Web Fig. 4) |
|----------------------|-----------------------------|----------------------------------|-------------------------------|------------------------------|
| *L. bryoniae*        | Two distal bulbs, bulb rims circular | Both vertical setae on yellow ground | Predominantly yellow, small black mark at front lower margin | a twice length of b           |
| *L. huidobrensis*    | Two distal bulbs, meeting only at their rims | Both vertical setae on black ground | Yellow with variable black patch generally across the lower three-quarters | a 2–2.5 times length of b     |
| *L. sativae*         | One distal bulb with a slight constriction between upper and lower halves | Outer vertical seta on black ground which may just reach inner vertical seta which otherwise is on yellow | Predominantly yellow, with dark area varying in size from a small bar along the lower margin to a patch along the entire lower margin, well up the front margin and narrowly up the hind margin | a 3–4 times length of b      |
| *L. strigata*        | Two distal bulbs, meeting from their rims to their bases | At least outer vertical seta on black ground (or yellow) | Yellow, black patch variable and can extend across the lower half | a 2–2.5 times length of b     |
| *L. trifolii*        | One distal bulb with marked constriction between lower and upper halves | Both vertical setae on yellow ground | Yellow, small blackish grey mark at front lower margin | a 3–4 times length of b      |

| Third antennal segment | Frons & orbits | Femur | Mesonotum | Wing length |
|------------------------|----------------|-------|-----------|-------------|
| *L. bryoniae*          | Small, yellow | Bright yellow with some brownish striations | Black, largely shining but with distinct matt undertone | 1.75–2.1 mm |
| *L. huidobrensis*      | Slightly enlarged, usually darkened | Frons yellow, generally more orange than pale lemon-yellow; upper orbits slightly darkened at least to upper ors | Yellow, variably darkened with black striations | 1.7–2.25 mm |
| *L. sativae*           | Small, yellow | Frons and orbits bright yellow | Bright yellow | 1.3–1.7 mm |
| *L. strigata*          | Small, yellow | Frons and orbits yellow | Yellow with some brownish striations | 1.8–2.1 mm |
| *L. trifolii*          | Small, yellow | Frons and orbits yellow | Yellow, occasional | 1.3–1.7 mm |

Information, except with respect to the distiphallus, compiled from Spencer (1973, 1976).

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Group 1
Larvae are cream-coloured but in the final instar additionally develop a yellow-orange patch dorsally at the anterior end, which can extend right around to the ventral surface. Each posterior spiracle consists of an ellipse with pores along the margin (Web Fig. 12b). It can be difficult to make out the number of pores, which according to Spencer (1973), are: L. bryoniae 7–12 pores; L. huidobrensis about 6–9 pores and L. strigata 10–12 pores. Puparia are variable in coloration, from yellow-orange to dark brown. In L. bryoniae and L. strigata, they are mostly, but not exclusively, at the lighter end of the colour range. Mostly the colour of L. huidobrensis puparia tends to anthracite. The form of the larval spiracles is retained in the puparium although the pores are less clearly discernible.

Group 2
Larvae are translucent when newly emerged, yellow-orange later. Each posterior spiracle is tricorn-shaped with three pores, each on a distinct projection, the outer two elongate (Web Fig. 12c). Puparia are yellowish-orange, sometimes a darker golden-brown. Again the form of the larval spiracles is retained but the detail is less obvious.

Adults
External characters
Important morphological characters are shown in Table 1. For morphological keys, descriptions of species and illustrations of the male aedeagus of a number of European species of Liriomyza (and other agromyzids), see Spencer (1972, 1976). For species descriptions and illustrations of species worldwide, including economically important species, see Spencer (1973, 1990).

Identification based on distiphallic structure
The distiphallus is the terminal part of the aedeagus (the intromittent organ, part of the male genitalia) (Web Fig. 14a,d; Web Fig. 18 (Plate 1)) and its complex three-dimensional structure is here of considerable diagnostic value. Indeed, the distiphallus provides a single character by which all five species can be reliably identified. In other words, all other species of Liriomyza, including those not discussed here, can be eliminated.

The distiphallus is a very small, fragile structure enclosed by membranes and requires careful dissection and subsequent examination under a high power microscope. The basic structure of the distiphallus differs in the two natural species groups: in Group 1, there are two distal bulbs side by side (Web Fig. 14b), while in Group 2 there is only one distal bulb with a medial constriction dividing distinct lower and upper sections (Web Fig. 14c). Separation of the five species using the distiphallus is described in Appendix II. Brief summary descriptions of the five species are provided below.

Group 1 – distiphallus with two distal bulbs
L. bryoniae: bulb rims of distiphallus circular; relatively yellow, medium-size fly with both vertical setae on yellow.
L. huidobrensis: bulbs of distiphallus meet only at their rims; a larger and darker fly with both vertical setae on black and the black extending forward along the upper orbits; third antennal segment usually darkened.
L. strigata: bulbs of distiphallus meet along their length; medium to large, moderately dark fly with at least the outer vertical seta on black.

Group 2 – distiphallus with one distal bulb
L. sativa: slight medial constriction on the distiphallus bulb; smaller, moderately dark fly with at least the outer vertical seta on black; section a of wing vein Cu1A much longer relative to section b than in Group 1 species.
L. trifoli: marked medial constriction on the distiphallus bulb; relatively yellow, smaller fly with both vertical setae on yellow; section a of wing vein Cu1A much longer relative to section b than in Group 1 species.

Reporting and documentation
Guidance on reporting and documentation is given in EPPO Standard PM7– (in preparation).

Further information
Further information on this organism can be obtained from:
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Acknowledgements
This protocol was originally drafted by D. W. Collins, Central Science Laboratory, York (GB) E-mail: dom.collins@csl.gov.uk. Many of the line drawings found in this protocol are based on original versions by Paul Seymour, formerly of the Central Science Laboratory, UK. Paul Seymour also took all the photographs of Liriomyza genitalia. The PCR-RFLP protocol was developed by Linda Kox, Plant Protection Service, Wageningen (NL).

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Appendix I

Electrophoretic identification of larvae and puparia to species

The use of allozyme electrophoresis to identify the immature stages of selected Liriomyza spp. was developed by Menken & Ulenberg (1983, 1986), the methodology technologically improved by Oudman (1992) and the protocols refined by Oudman et al. (1995) and Collins (1996). The protocols given here are those of Oudman et al. (1995), Protocol A, and Collins (1996), Protocol B, and one should be selected according to the identification question being asked. A diagrammatic representation of the successive steps undertaken in this procedure is presented as Fig. 1.

Protocol A uses three isoenzymes to distinguish between the four listed species, L. bryoniae, L. huidobrensis, L. sativae and L. trifolii. Protocol B separates the three species in natural group 1, L. bryoniae, L. huidobrensis and L. striigata, and explicitly both eliminates Chromatomyia horticola and C. syngenesia and provides warning against potentially misleading results caused by the presence of the endoparasiotid Dacnusa sibirica Telenga, 1934.

Interpretation of the band patterns from unknown samples requires direct comparison with a known standard, usually taken from a laboratory culture of L. bryoniae.

Equipment

The apparatus used for sample preparation and the electrophoretic run is manufactured by Helena Laboratories (Beaumont, US). The basic components required are an electrophoretic tank (cat. no. 1283), paper wicks (cat. no. 5081) and an applicator kit (cat. no. 4093), the latter made up of the applicator itself with 12 microtips, a sample well plate and an aligning base for the gels. Electrophoresis is carried out on pre-manufactured Titan III cellulose acetate plates (catalogue no. 3024 or 3033).

Sample storage

Isozyme electrophoresis requires biochemically active enzymes. Samples should either still be live or stored in the freezer until removal immediately before use. Samples may be stored for several weeks within plastic microtubes at −20°C. Longer-term storage should be at −80°C.

Gel preparation

The cellulose acetate plates are pre-soaked for 20–30 min in 800 mL 25 mM Tris Glycine, pH 8.5 buffer solution to which NADP (70 mg L−1) and MgCl₂ (70 mg L−1) have been added. Three gels are required for protocol A, two gels for protocol B. Gel/electrode buffer: 3.03 g Tris, 14.41 g glycine, make up to 1000 mL with distilled water, add NADP (70 mg L−1) and MgCl₂ (70 mg L−1). Stain buffers: 1.21 g Tris, 100 mL distilled water, titrate to pH 8.0 with 1 M HCl.

Liriomyza individuals are subject to attack by parasitoid wasps and the host electrophoretic band pattern may be replaced by that of the parasitoid. The replacement process is not instantaneous and a range of intermediate patterns incorporating elements from both host and parasitoid may be seen (Collins, 1996). Atypical band patterns should therefore be treated with caution. Ideally, at least 2–3 individuals should be run from a sample so as to eliminate the possibility of a single individual producing an atypical or (very rarely) a misleading band pattern.
Table 2  Staining solutions of G6PDH, IDH, ME and PEP

| Chemical (stock solution) | G6PDH | IDH | ME | PEP |
|---------------------------|-------|-----|----|-----|
| Tris-HCl, 0.1 m, pH 8.0   | 0.6   | 0.6 | 0.6| 0.6 |
| NADP (2 mg mL⁻¹)          | 1.5   | 1.5 | 1.5| mL  |
| O-Dianisidine (4 mg mL⁻¹) | –     | –   | –  | 8.0 |
| MgCl₂ (20 mg mL⁻¹)        | 5.0   | 5.0 | 2.0| 2.0 |
| n-glucose-6-phosphate (20 mg mL⁻¹)| 12.0 | –   | –  | drops |
| DL-isocitric acid (100 mg mL⁻¹) | –  | 15.0 | – | – |
| DL-malic acid (70 mg mL⁻¹) | –     | 12.0| –  | drops |
| Leu-Gly (dry)             | –     | –   | 10.0| mg  |
| MTT (10 mg mL⁻¹)          | 5.0   | 5.0 | 5.0| – drops |
| PMS (10 mg mL⁻¹)          | 1.0   | 1.0 | 1.0| – drops |
| Peroxidase (10 mg mL⁻¹)   | –     | –   | 5.0| drops |
| t-aminooxyde oxidase (10 mg mL⁻¹) | – | –  | 5.0| drops |
| Agar (16 mg mL⁻¹)         | 2.0   | 2.0 | 2.0| 2.0 mL|

MTT = methyl thiazolyl blue; PMS = phenazine methosulphate.

Sample preparation

Individual larvae or puparia are homogenized in either 10 µL of NADP solution in a microtube using a moulded plastic crusher (with the homogenate then being transferred to the well of the sample plate) (Protocol A) or in 5 µL of NADP solution in situ in the well of the sample plate using a heat-sealed Pasteur pipette (Protocol B). Samples taken from the freezer should be kept below 4°C (e.g. in melting ice) until immediately before use.

Electrophoresis

Each of the outside chambers of the electrophoresis tank is filled with 100 mL 25 mM Tris Glycine, pH 8.5 buffer solution. Paper wicks are soaked in this solution and then attached to the inner walls of these two chambers along their length so that in each case one side drops into the solution and the other just overhangs into the next chamber. Each gel in turn is removed from the buffer solution, blotted between sheets of filter paper, in order to remove excess liquid, and placed onto the aligning base. The homogenates are then applied from the sample plate to the gel using the applicator. Three to four applications per gel may be required to ensure sufficient homogenate on the gel. The gel is then placed across the middle two chambers of the electrophoretic tank with the cellulose side down so that good contact is made between the cellulose and the wicks.

Protocol A: the gels are run simultaneously for 18 min at 200 V (1 mA per gel). Protocol B: the gels are initially run simultaneously for 18 min at 200 V (1 mA per gel). Electrophoresis is then interrupted and the first plate removed (to be stained for glucose-6-phosphate dehydrogenase). The second plate is then run for a further 20 min, still at 200 V.

Staining

Staining schedules essentially follow those outlined by Hebert & Beaton (1989). Staining solutions are prepared fresh from stock solutions while the electrophoresis is in progress. Note that PMS and t-aminooxyde oxidase are light-sensitive and should only be added to the relevant staining solutions (Table 2) immediately before they are used. The gels are removed from the electrophoresis tank and placed on a plexiglass plate. The staining solution is mixed with approximately 2 mL molten agar and gently and evenly poured over the gel. Bands are usually visible within a minute or two but, if this proves not to be the case, the staining reactions may be incubated in the dark for up to 45 min at 37°C. The staining reaction may be brought to a halt at any time by placing the agar-overlain gel plate in a 7% (v/v) solution of acetic acid.

Protocol A: the three gels are, respectively, stained for glucose-6-phosphate dehydrogenase (G6PDH), isocitrate dehydrogenase (IDH) and malic enzyme (ME). Protocol B: the first gel to be removed from the electrophoresis tank is stained for G6PDH, the second for leucine-glycine peptidase (PEP).

Interpretation of band patterns

Interpretation of the band patterns is achieved using the biochemical keys presented in Tables 3 and 4.

Appendix II

Identification to species using the male distiphallus

A diagrammatic representation of the successive steps undertaken in this procedure is presented as Fig. 2. The distiphallus of male *Liriomyza* spp. is a very small, fragile structure enclosed by membranes and requires careful dissection before examination under a high power microscope. Evidence of distiphallicular structure should be correlated with evidence of external morphology (Table 1) in order to confirm the identification.
### Table 4 Key for separation of *Liriomyza* spp. by allozyme electrophoresis: Protocol B. See Web Figs 6–8

| 1 | G6PDH band faster than the *L. bryoniae* standard | *L. huidobrensis* |
|---|-------------------------------------------------|-----------------|
| 2 | G6PDH band the same or slightly slower than the *L. bryoniae* standard | *L. strigata* |
| 3 | PEP-1 band present (band within 15 mm of origin; occasionally travels towards cathode) | *L. trifolii* |
|   | PEP-1 band displaced, absent or heavy streaking associated with it | *L. sativae* |
| 4 | PEP-1 band displaced to become a poorly resolved band located between 20 and 30 mm | *C. syngenesiae* |
|   | PEP-1 band absent or heavy streaking associated | *C. horticola* |

### Determining the sex of flies

In the male, the lobes of the epandrium, which are dark and pubescent and not so heavily sclerotized as the female tube, curve around and down at the rear of the abdomen, from the dorsal to the ventral sides (Web Fig. 13a). A slit-like opening is seen between the lobes, triangular when more fully open, through which the rest of the male genitalia can be viewed. The lobes hardly extend beyond the last tergite. In the female, the abdominal segments beyond segment 6 form a black, heavily sclerotized tube which extends out beyond the 6th tergite (Web Fig. 13b) with a circular opening visible in posterior view at the end of the tube. The 6th tergite covers the basal half of the tube from above, though it is visible in lateral and ventral views.

### Preparation and examination of the distiphallus

Using fine mounted needles, carefully separate the abdomen from the rest of the fly. Briefly wet in absolute ethanol, and bring to the boil in 10% KOH (or NaOH) and boil for 60–90 s. Transfer to cold glacial acetic acid and leave for 3 min. Blot off excess glacial acetic acid and transfer to a drop of Heinz lysis buffer using a micropestle. DNA is extracted using standard DNA extraction methods, e.g. the High Pure PCR Template Preparation Kit (Roche Diagnostics, Almere, NL). DNA extraction is applied to adults, puparia or larvae ground in DNA extraction mixture is composed as follows: 0.6 µL each primer, 200 µM dNTPs (Promega), 1 Unit HotStarTaq DNA polymerase (Qiagen), 5 µL 10 × reaction buffer [with 15 mM MgCl₂], 1 µL DNA. The PCR is performed in a 96-well thermocycler.

### Table 5 Diagnostic key for identification of *Liriomyza* spp. using the male distiphallus (to be used in conjunction with Web Fig. 15 and Figs. Plates 2 and 3)

| 1 | With one distal bulb | 2 |
|---|---------------------|---|
| 2 | With a pair of distal bulbs | 3 |
| 3 | With bulb rims circular (not drawn out antero-ventrally); evenly sclerotized | 4 |
|   | With bulb rims spiralled (i.e. drawn out antero-ventrally); strongly sclerotized antero-ventrally | 4 |

Re-position the genital complex for ventral viewing of the distiphallus (again at 400 × magnification). Use the key in Table 5 for diagnostic determination of the species.

### Appendix III

**Identification of Liriomyza species by PCR-RFLP analysis**

A polymerase chain reaction (PCR) method amplifying a 790 bp-fragment of the cytochrome oxidase II (COII) gene followed by restriction fragment length polymorphism (RFLP) analysis was developed by L. Kox (Plant Protection Service, Wageningen, NL).

**DNA extraction**

DNA extraction is applied to adults, puparia or larvae ground in lysis buffer using a micropestle. DNA is extracted using standard DNA extraction methods, e.g. the High Pure PCR Template Preparation Kit (Roche Diagnostics, Almere, NL) according to the instructions in the mammalian tissue protocol. The DNA is eluted with 50 µL of 10 mM Tris, pH 8.5.

**PCR**

The PCR primers are (Simon et al., 1994):

- TL2-J-3037 (5’-ATGGCAGATTAGTGCAATGG-3’)
- TK-N-3785Lir (5’-GT(TA)AAGAGACCATT(A/G)CTTG-3’)

 annealing in the leucine tRNA and lysine tRNA genes, respectively, spanning the mitochondrial cytochrome oxidase II (COII) gene. These primers are not specific for *Liriomyza*, they amplify the COII gene of several insects. Primer TK-N-3785 was optimized for *Liriomyza*, the 50 µL-reaction mixture is composed as follows: 0.6 µL each primer, 200 µM dNTPs (Promega), 1 Unit HotStarTaq DNA polymerase (Qiagen), 5 µL 10 × reaction buffer [with 15 mM MgCl₂], 1 µL DNA. The PCR is performed in a 96-well thermocycler.
RFLP analysis

5 μL of PCR product (without further purification) is digested with the enzymes DdeI, HinfI, SspI and TaqI in separate reactions according to the manufacturer’s instructions. Digested PCR products are electrophoresed on 2% agarose gel along with a 100-bp DNA ladder to size fragments and visualized and photographed under UV light.

Interpretation of band patterns

For fragment sizes of digested PCR products, see Table 6.

Table 6  Fragment sizes of digested PCR products of Liriomyza spp.

| Restriction enzyme | Fragment sizes | L. bryoniae | L. huidobrensis | L. sativae ‘USA’ | L. sativae ‘Asia’ | L. strigata | L. trifolii |
|-------------------|----------------|-------------|-----------------|-----------------|-----------------|-------------|------------|
| DdeI              | 790            | 790         | 567             | 790             | 790             | 619         |            |
|                   |                |             |                 |                 |                 |             |            |
| HinfI             | 421            | 421         | 421             | 421             | 421             | 421         |            |
|                   | 369            | 369         | 283             | 310             | 342             | 310         |            |
|                   |                |             | 27              | 59              | 27              | 59          |            |
|                   |                |             |                 |                 |                 |             |            |
| SspI              | 392            | 399         | 399             | 717             | 399             | 391         |            |
|                   | 326            | 391         |                 | 73              |                 | 391         | 326        |
|                   | 72             |             |                 |                 |                 |             |            |
| TaqI              | 486            | 306         | 306             | 306             | 267             | 306         |            |
|                   | 163            | 163         | 210             | 210             | 219             | 163         |            |
|                   | 111            | 159         | 163             | 163             | 141             | 159         |            |
|                   | 30             | 111         | 81              | 81              | 72              | 141         | (or 111 +30) |
|                   | 30             | 30          | 30              |                 | 67              | 21          |            |
|                   | 21             |             |                 |                 |                 |             |            |

*L. trifolii is heterogeneous for this restriction site.

(e.g. PTC200, MJ-Research) with the following parameters: 15 min 95°C, 35 cycles of 15 s at 94°C, 1 min at 55°C, and 45 s at 72°C, followed by a final extension for 10 min at 72°C and rapid cooling to room temperature. After amplification, 5 μL samples of the PCR products are electrophoresed on 1.5% agarose gel according to standard methods (Sambrook et al., 1989) along with a 100-bp DNA ladder (e.g. 100-bp ladder MBI Fermentas) to size fragments. PCR products are viewed and photographed under UV light.