DOI: 10.1111/epp.12832

EPPO STANDARD ON DIAGNOSTICS

PM 7/53 (2) *Liriomyza* spp.

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

This Standard should be used in conjunction with PM 7/76 Use of EPPO diagnostic protocols.[1]

Specific approval and amendment: This Standard was initially developed under the EU DIAGPRO Project (SMT 4-CT98-2252) by a partnership of contractor laboratories and intercomparison laboratories in European countries. This revision was prepared on the basis of the IPPC Diagnostic Protocol adopted in 2016 (Appendix 16 to ISPM 27 Genus *Liriomyza*, IPPC (2016)). Molecular tests are described according to the EPPO format.

First approved in 2004–09.

Revision approved in 2021–10.

Authors and contributors are given in the Acknowledgements section.

1 | INTRODUCTION

Agromyzidae is a family of small flies whose larvae feed on the internal tissue of plants, often as leafminers and stem miners. The majority of agromyzid species are either host specific or restricted to a small group of plants that are related to each other (Benavent-Corai et al., 2005). However, a few highly polyphagous species have become agricultural and horticultural pests in many parts of the world. These include four species of *Liriomyza* that are listed in plant quarantine legislation in various countries: *L. bryoniae*, *L. huidobrensis*, *L. sativae* and *L. trifolii*. These are all polyphagous pests of both ornamental and vegetable crops. The species-level identification in this protocol is restricted to these four species.

Liriomyza is predominantly found in the north temperate zone but species are also found in the Afrotropical, Neotropical and Oriental regions. The adult flies of the 300-plus species of *Liriomyza* (including 130-plus in Europe) look very similar: they are all small (1–3 in length) and, from above, appear largely black with, in most species, a yellow frons and scutellum (e.g. Figure 1). As a result, separating the species of the genus can be difficult. Furthermore, in order to identify the four species, a diagnostician should not only recognize these four species, but also be able to identify them against the background fauna of indigenous *Liriomyza* species.

Liriomyza bryoniae is essentially a Palaearctic species with records from across Europe and Asia, and from Egypt and Morocco in North Africa (EPPO, 2020). It is highly polyphagous and has been recorded from 16 plant families (Spencer, 1990). It is a pest of tomatoes, cucurbits (particularly melons, watermelon and cucumber) and glasshouse-grown lettuce, beans and lupins (Spencer, 1989, 1990).

Liriomyza huidobrensis is thought to have originated in South America and has now spread throughout much of the world, including parts of North America, Europe, Africa, Asia and the Pacific (Lonsdale, 2011; EPPO, 2020). However, the species as formerly taxonomically defined was recently split into two morphocryptic species – *L. huidobrensis* and *L. langei* – and there is some uncertainty about the precise delineation of their relative distribution. Currently, *L. langei* has been confirmed only from the USA and it is highly likely that all invasive populations outside the USA are *L. huidobrensis* as now taxonomically defined (Scheffer & Lewis, 2001; Scheffer et al., 2001; Takano et al., 2008; Lonsdale, 2011). *Liriomyza huidobrensis* is highly polyphagous and has been recorded from 15 plant families (CABI, 2021).

FIGURE 1 Adult *Liriomyza bryoniae*. Courtesy of Department of Environment, Food and Rural Affairs, GB

[1]Use of brand names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.

The major host families and species listed by EFSA (2012) are: Apiaceae (*Apium graveolens*), Asteraceae (*Aster* spp., *Chrysanthemum* spp., *Gerbera* spp., *Dahlia* spp., *Lactuca sativa*, *Lactuca* spp.), Brassicaceae (*Brassica* spp.), Caryophyllaceae (*Gypsophila* spp.), Chenopodiaceae (*Spinacia oleracea*, *Beta vulgaris*), Cucurbitaceae (*Cucumis* spp., *Cucurbita* spp.), Fabaceae (*Medicago sativa*, *Phaseolus vulgaris*, *Pisum sativum*, *Pisum* spp., *Trifolium* spp., *Vicia faba*), Liliaceae (*Allium cepa*, *Allium sativum*) and Solanaceae (*Capsicum annuum*, *Capsicum frutescens*, *Petunia* spp., *Solanum lycopersicum*, *Solanum* spp.).

Liriomyza sativae originated in North, Central and South America and has now spread to many parts of Asia, Africa, Australia and the Pacific, but not to Europe (Lonsdale, 2011; EPPO, 2020). However, distributional notes on *L. sativae* are likely to be incomplete as there is evidence to indicate that the species is continuing to expand its range rapidly. It is another highly polyphagous pest of many vegetable and flower crops (Spencer, 1973, 1990). It has been recorded from nine plant families, although it is mainly found on hosts in the Cucurbitaceae, Fabaceae and Solanaceae (Spencer, 1973, 1990).

Liriomyza trifolii, also originally from North, Central and South America, has been spread to large parts of Europe, Africa, Asia and the Pacific, most likely as the result of trade in chrysanthemum cuttings (Martinez & Etienne, 2002; Lonsdale, 2011; EPPO, 2020). It is highly polyphagous and has been recorded from 25 plant families (Spencer, 1990). The most economically important crops it attacks are beans, celery, chrysanthemums, cucumbers, gerberas, *Gypsophila*, lettuce, onions, potatoes and tomatoes (Spencer, 1989).

A further (fifth) species, *L. strigata*, is included in the diagnostic protocol because it is closely related to both *L. bryoniae* and *L. huidobrensis*, and as such, a

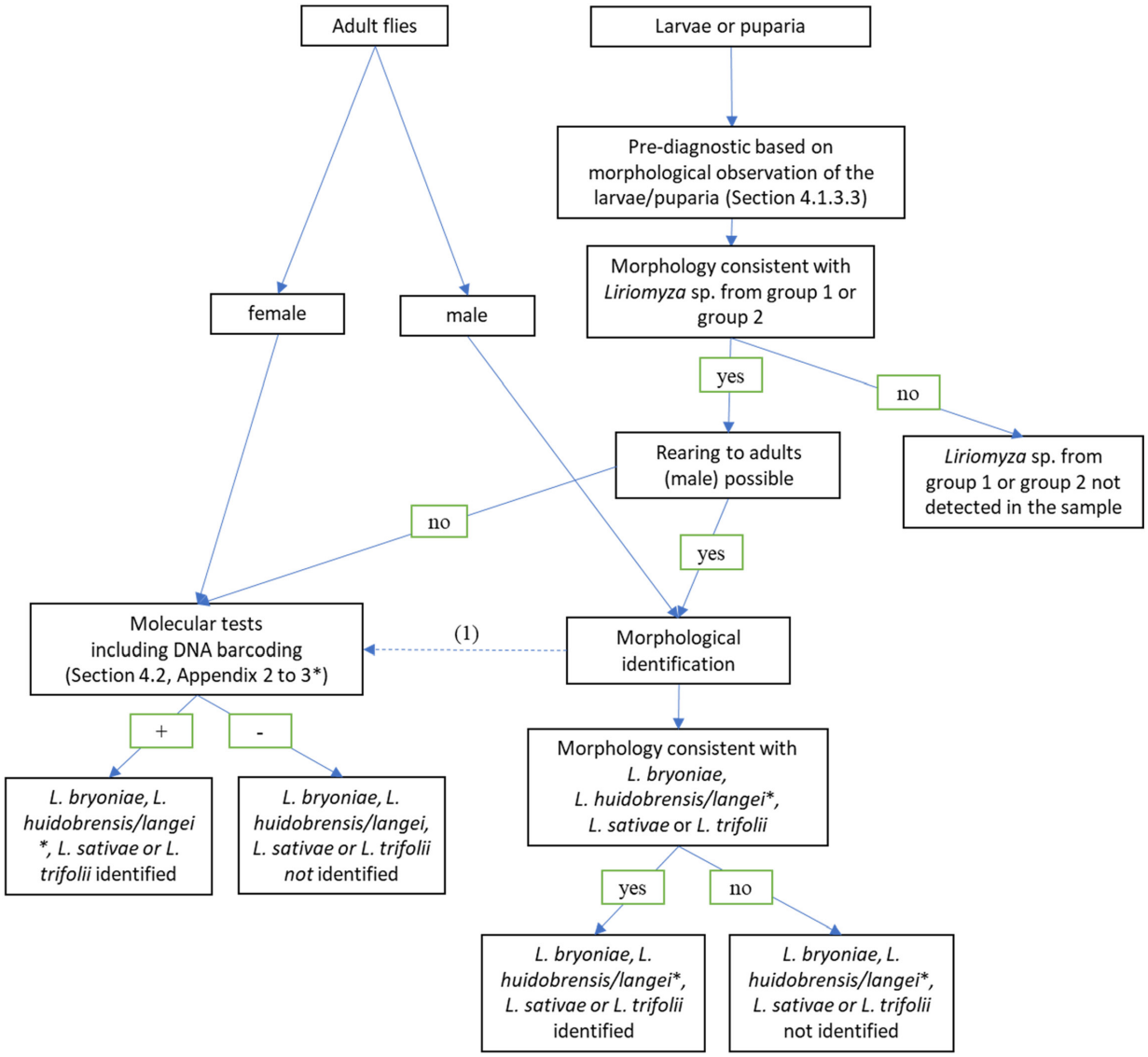

FIGURE 2 Flow diagram describing the diagnostic procedure for the detection and identification of *Liriomyza* spp.
*Tests described in Appendices 2 and 3 and morphological identification cannot be used to distinguish *L. huidobrensis* and *L. langei*
(1) In cases where adult specimens are atypical or damaged

diagnostician must be able to eliminate this species when seeking to positively identify *L. bryoniae*, *L. huidobrensis*, *L. sativae* or *L. trifolii*. *Liriomyza strigata* is an Eurasian species (Pitkin et al. (n.d.) quoting Spencer (1976), Dempewolf (2001), Ellis (n.d.) and Martinez (2013)). The eastern borders of its distribution are not clearly defined, but the range extends beyond the Ural Mountains (Spencer, 1976) and it has been doubtfully recorded in Southeast Asia (Dempewolf, 2004). It is highly polyphagous, having been recorded from 29 plant families worldwide (Spencer, 1990).

For more detailed information on the distribution and hosts plants of the-above mentioned *Liriomyza* species, see EPPO Global Database (EPPO, 2020). A flow diagram describing the diagnostic procedure for the detection and identification of *Liriomyza* spp. is presented in Figure 2.

2 | IDENTITY

Name: *Liriomyza bryoniae* (Kaltenbach, 1858)
Other scientific name: *Agromyza bryoniae* (Kaltenbach, 1858); *Liriomyza solani* (Hering, 1927); *Liriomyza hydrocotylae* Hering, 1930; *Liriomyza mercurialis* Hering, 1932; *Liriomyza triton* Frey, 1945; *Liriomyza citrulli* (Rohdendorf, 1950); *Liriomyza nipponallia* Sasakawa, 1961
Taxonomic position: *Insecta*: *Diptera*: *Agromyzidae*
EPPO Code: LIRIBO
Phytosanitary categorization: EU Protected Zone Quarantine Pest (Annex III)

Name: *Liriomyza huidobrensis* (Blanchard, 1926)
Other scientific name: *Agromyza huidobrensis* (Blanchard, 1926); *Liriomyza cucumifoliae* (Blanchard, 1938); *Liriomyza dianthi* (Frick, 1958)
Taxonomic position: *Insecta*: *Diptera*: *Agromyzidae*
EPPO Code: LIRIHU
Phytosanitary categorization: EPPO A2 list no. 283, EU Protected Zone Quarantine Pest (Annex III).

The taxonomic relationship between *L. huidobrensis* (Blanchard) and *L. langei* (Frick) is complex. *Liriomyza huidobrensis* was originally described from specimens taken from *Cineraria* in Argentina by Blanchard (1926). Frick (1951) described *L. langei* from California as a species that he noted was primarily a pest of peas, although it had also damaged *Aster*. In 1973, Spencer then synonymized the two species as they were (and de facto remain) morphologically indistinguishable. Following a study of their mitochondrial and nuclear DNA sequences (Scheffer, 2000; Scheffer & Lewis, 2001), and supported by later rearing experiments (Takano et al., 2008), the two species were formally separated as two cryptic species (Lonsdale, 2011). The name *L. langei* Frick was resurrected and applied to the cryptic species from California, and the name *L. huidobrensis* (Blanchard) was applied to the cryptic species from South and Central America.

Lonsdale (2011) attempted to delineate diagnostic morphological characters that could differentiate "most" specimens of the two species, but found the characters "subtle and sometimes overlapping" so he recommended the use of molecular data to support identification whenever possible. Scheffer and her collaborators consider that the ranges of the two species do not overlap (although Lonsdale (2011) recorded *L. huidobrensis* from California, once in 1968 and once in 2008, he states that it is unknown if the populations established), and that all of the invasive populations that they had studied were *L. huidobrensis* as so defined (Scheffer & Lewis, 2001; Scheffer et al., 2001). This means that reports from California in the literature predating Scheffer's papers should be considered as applying to *L. langei* (with low uncertainty). *Liriomyza langei* is predominantly a Californian species although it has apparently been introduced into Hawaii, Oregon and Washington; populations found in Florida, Utah and Virginia in the mid-1990s did not establish (Lonsdale, 2011). Only *L. huidobrensis* has been confirmed in Mexico (Lonsdale, 2011), but Takano et al. (2005) reported that specimens of *L. langei* (described as the Californian clade) were intercepted at a Japanese inspection site on fresh vegetables originating from Mexico.

Name: *Liriomyza sativae* (Blanchard, 1938)
Other scientific name: *Liriomyza verbenicola* (Hering, 1951); *Lemurimyza lycopersicae* (Pla and de la Cruz, 1981); *Liriomyza pullata* (Frick, 1952); *Liriomyza canomarginis* (Frick, 1952); *Liriomyza minutiseta* (Frick, 1952); *Liriomyza propepusilla* (Frost, 1954); *Liriomyza munda* (Frick, 1957); *Liriomyza guytona* (Freeman, 1958): *Liriomyza subpusilla* (Frost)
Taxonomic position: *Insecta*: *Diptera*: *Agromyzidae*
EPPO Code: LIRISA
Phytosanitary categorization: EPPO A2 list no. 282, EU A1 Quarantine pest (Annex II A)

Name: *Liriomyza trifolii* (Burgess, 1880)
Other scientific name: *Liriomyza alliovora* (Frick, 1955); *Liriomyza phaseolunata* (Frost)
Taxonomic position: *Insecta*: *Diptera*: *Agromyzidae*
EPPO Code: LIRITR
Phytosanitary categorization: EPPO A2 list no. 131, EU Protected Zone Quarantine Pest (Annex III)

3 | DETECTION

3.1 | General information

In practice, agromyzids are recognizable because their larvae feed in the living tissue of plants (three-quarters of them are leafminers). However, there are leafminers in other Dipteran families such as Anthomyiidae and Drosophilidae. Leafminer species are also present in Lepidoptera families; however, excrements of

Agromyzidae larvae are located in the margin of the mines whereas they are located in the center for Lepidoptera larvae. Feeding punctures and leaf mines are usually the first and most obvious signs of the presence of *Liriomyza*. While fully formed mines should be readily visible to quarantine officials, early signs of infestation are much less obvious and are easily overlooked (Spencer, 1989). Mines remain intact and relatively unchanged over a period of weeks. Mine configuration may be used to guide to the identification of agromyzid species (as in many such cases the species are host specific). However, considering the broad host range, the pattern of mining may be 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 variability means that identification from mine pattern alone is not recommended. Examples of mine pattern for the four quarantine species and *L. strigata* are provided in Figures 3–5.

3.1.1 | Feeding and oviposition punctures

Female flies use their ovipositor to puncture the leaves of the host plants, causing wounds that serve as sites for feeding (by both female and male flies) or for oviposition. Feeding punctures of *Liriomyza* species are rounded, usually about 0.2 mm in diameter, and appear as white speckles on the upper surface of the leaf. Oviposition punctures are usually smaller (0.05 mm) and more uniformly round. Feeding punctures made by the polyphagous agromyzid pest species *Chromatomyia horticola* and *Chromatomyia syngenesiae* are distinctly larger and more oval than those made by *Liriomyza* flies. The appearance of feeding and oviposition punctures does not differ among *Liriomyza* species, and the pattern of their distribution on the leaf cannot be used to identify species. Feeding punctures cause the destruction of a large number of cells and are clearly visible to the naked eye.

3.1.2 | Leaf mines

The larvae feed mostly just below the upper surface of the leaf, mining through the green palisade tissue. Mines are usually off-white, with trails of frass appearing as broken black lines along the length of the leaf. Repeated convolutions in the same small area of

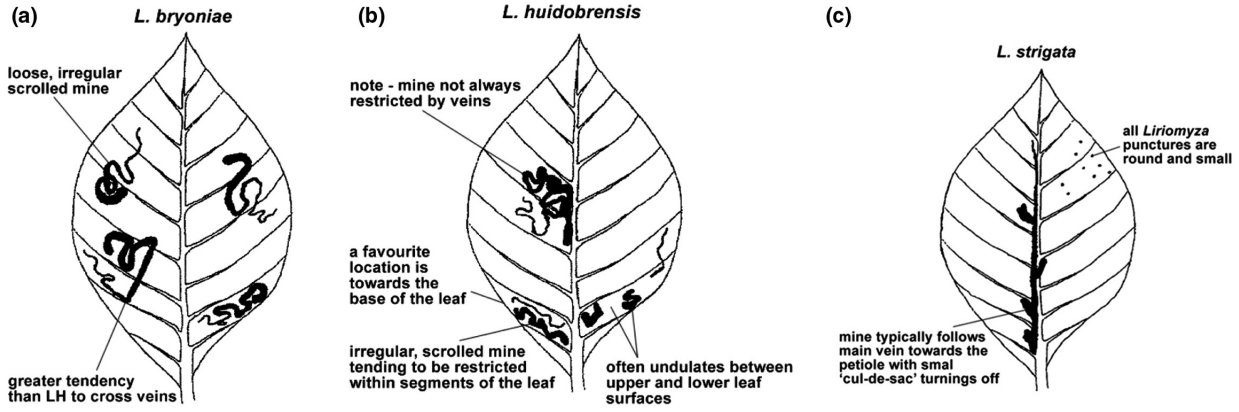

FIGURE 3 Typical characteristics of mines of (a) *Liriomyza bryoniae*, (b) *Liriomyza huidobrensis* and (c) *Liriomyza strigata*

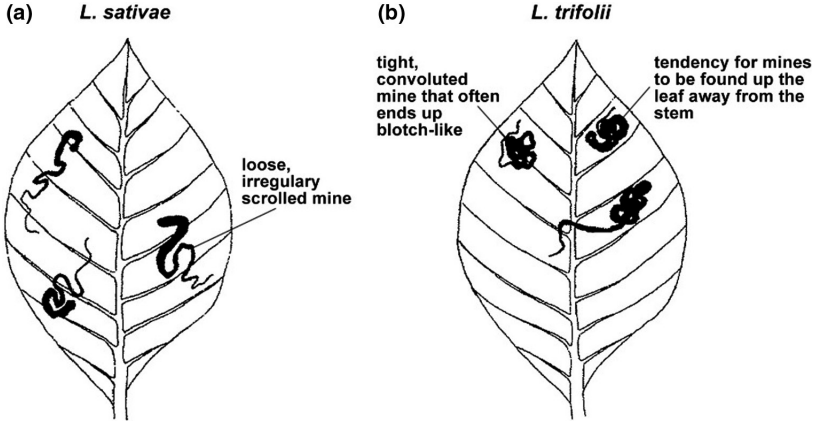

FIGURE 4 Typical characteristics of mines of (a) *Liriomyza sativae* and (b) *Liriomyza trifolii*

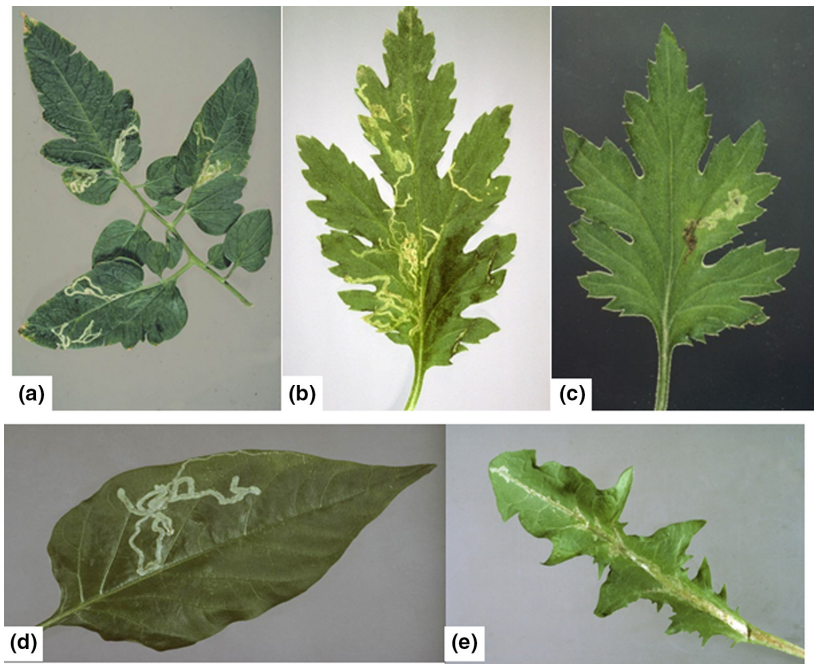

FIGURE 5 Typical mines of *Liriomyza* spp.: (a) *L. bryoniae* on tomato; (b) *L. huidobrensis* on chrysanthemum; (c) *L. trifolii* on *chrysanthemum*; (d) *L. sativae* on pepper; and (e) *L. strigata* on an unidentified host. Courtesy of Department of Environment, Food and Rural Affairs, GB

the leaf will often result in discolouration of the mine, with dampened black and dried brown areas appearing, usually as the result of plant-induced reactions to the leafminer.

There are three larval stages, all of which feed within the leaves. The larvae predominantly feed on the plant in which the eggs are laid. The larvae of *Liriomyza* spp. leave the leaf when ready to pupariate (Parrella & Bethke, 1984), and their exit hole characteristically takes the form of a semi-circular slit; in contrast, the larvae of *C. horticola* and *C. syngenesiae* pupate inside the leaf at the end of the larval mine, with the anterior spiracles usually projecting from the lower surface of the leaf. *Liriomyza* puparia, therefore, may be found in crop debris, in the soil or sometimes on the leaf surface.

Specimens may be found in different locations of the plant and surrounds depending on the life stages present, as follows:

- Eggs – inserted just below the leaf surface;
- Larvae – inside mines on leaves;
- Pupae – in crop debris, in the soil or sometimes on the leaf surface;
- Adult – free-flying, or on leaf surfaces while producing feeding and oviposition punctures.

3.2 | Collection and preservation of specimens

Liriomyza flies can be collected as immature life stages in association with mined leaf samples or as adults. Because the morphological characters used to diagnose species are based on male genitalia, adult males are needed in order to confirm species identification (see Section 4). Adult females are often identifiable with certainty only to genus level. Collecting multiple specimens (ideally 20) from a plant or a location will increase the likelihood of obtaining male flies, which is important unless molecular tests are to be used for diagnosis.

3.2.1 | Collecting adults

Adult flies are normally found on the foliage and can be collected by hand or with a sweep-net into glass vials or collected with an aspirator. Alternatively, they can be collected using yellow sticky traps, particularly in glasshouses. However, the most practical and reliable method for collecting leafminer flies such as *Liriomyza* species is to collect mined leaves containing living larvae. These can be placed in a large jar for rearing to adult flies in the laboratory. Techniques for rearing agromyzids are described in Griffiths (1962).

For morphological identification adults and larvae can be boiled in water for a few minutes, transferred in 70% ethanol and stored indefinitely, although their colour fades over time. Dry storage of adults, for example as pinned specimens, is also possible.

For molecular testing, specimens should be killed and preserved in 96–100% ethanol, stored frozen (at about −20 or −4.0°C) or preserved on FTA cards (Whatman) (Blacket et al., 2015).

3.2.2 | Collecting immature life stages

If the intention is to collect and preserve plant samples, leaves with suspect feeding punctures or mines should be picked and placed between sheets of newspaper to permit slow drying.

Leaves with occupied mines from which it is intended to rear individuals can be safely removed for identification. They need to be packed in slightly damp, but not overly wet, laboratory tissue, and mailed in padded and sealed bags. In the laboratory, the mined leaves with living larvae can be placed in sealed Petri dishes with damp filter paper inserts and stored in an incubator at about 23°C (checking every two or three days to remove leaves that began to decay).

4 | IDENTIFICATION

Identification of leafminer species by morphological examination is restricted to adult male specimens because there are no adequate keys for the species-level identification of adult females or for eggs, larvae or pupae. Identification of adult material is possible by examination of morphological characters, in particular the genitalia of the male fly. The morphological characters of the male genitalia are examined under a high-power microscope (recommended at 400× magnification) able to perform biometric measurements and regularly calibrated using a micrometer. Using this protocol with good quality preparations should allow adults of the four quarantine species of *Liriomyza* to be identified with certainty by morphological examination alone (with the exception of *L. huidobrensis* and *L. langei* for the reasons discussed in Section 2).

Molecular tests can be applied to all life stages, including the immature stages for which morphological identification to species level is not possible. Additionally, in cases where adult specimens are atypical or damaged, molecular tests may provide further relevant information about identity. However, the analytical specificity of molecular tests may be limited as they were developed for a purpose and evaluated against a restricted number of species. Therefore, the results from molecular tests need to be carefully interpreted.

4.1 | Morphological identification

Examination of the male genitalia (in particular, the distiphallus; Figure 6) is necessary in order to obtain a positive identification for any of the four target species of *Liriomyza*. Determination of flies' sex and preparation of male distiphallus for examination (based on Malipatil & Ridland, 2008) are presented in Appendix 1. Evidence of distiphallic structure should be compared with characters of external morphology (Table 1) in order to confirm the species identification.

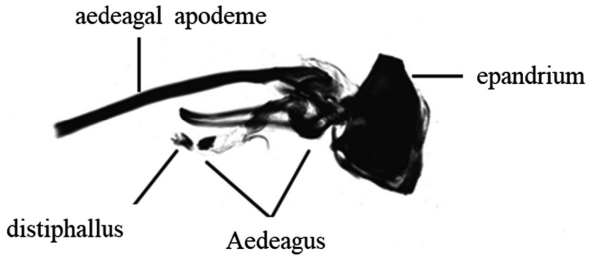

FIGURE 6 Male genitalia of *Liriomyza huidobrensis* (lateral view). Courtesy of Department of Environment, Food and Rural Affairs, GB

4.1.1 | Identification of the family Agromyzidae

Worldwide, the family Agromyzidae comprises about 2,500 species (Spencer, 1989, 1990). Detailed descriptions of agromyzid morphology are given by Spencer (1972, 1973, 1987), Dempewolf (2004) and Boucher (2010).

Morphological nomenclature in this Standard follows Yeates et al. (2004). This online resource can also be consulted for clear illustrations of the anatomy of a typical acalyptrate fly (such as Agromyzidae).

4.1.1.1 | Adults

The following combination of characters defines the family Agromyzidae (Hennig, 1958; Spencer, 1987; Boucher, 2010):

- Small in size, up to 1–6 mm, but usually 1–3 mm;
- Vibrissae present;
- One to seven frontal setae present;
- Wing with costal break present at the apex of Sc (Figures 7 and 8);
- Wing cell cup small – wing veins A_1 + CuA_2 not reaching wing margin (Figure 8);
- Male with pregenital sclerites with a fused tergal complex of tergites 6–8, with only two spiracles between tergite 5 and the genital segment;
- Female with the anterior part of abdominal segment 7 forming an oviscape (Figure 7 Q – ovipositor sheath).

4.1.1.2 | Larvae

Generally the larvae (Figure 9a) are cylindrical in shape, tapering anteriorly, with projections bearing the anterior and posterior spiracles (Figure 9b and d), the former located on the dorsal surface of the prothorax, the latter posteriorly directed at the rear. The larvae also possess strongly sclerotized mouthparts; the mandibles with their longitudinal axis are at about right angles to the rest of the cephalopharyngeal skeleton (Figure 9c) and usually bear two or more pairs of equally sized anteriorly directed teeth, with the ventral cornua (the posteriorly directed paired "arms") commonly shorter than the dorsal ones.

TABLE 1 Adult morphological characters of selected *Liriomyza* species[a]

	L. bryoniae	*L. huidobrensis*[b]	*L. sativae*	*L. strigata*	*L. trifolii*
Vertical setae	Both vertical setae on yellow ground but black at hind margin of eye sometimes reaching base of outer verticle	Both vertical setae on black (dark) ground	Outer vertical setae on black ground. Inner vertical normally on dark ground or at margin with yellow	Black colouration behind the eyes extending to at least the outer vertical setae	Both vertical setae on yellow ground
Frons and orbits	Frons bright yellow, orbits slightly paler	Frons yellow, generally more orange than pale lemon-yellow; upper orbits slightly darkened to upper orbital setae	Frons and orbits bright yellow	Frons bright yellow, orbits slightly paler	Frons and *orbits* yellow
Third antennal segment	Small, bright yellow	Normally brownish yellow. In palest specimens only faintly darkened (infuscated)	Small, yellow	Small, bright yellow	Small, bright yellow
Anepisternum[c]	Predominantly yellow, small black mark at front lower margin	Generally black on lower three-quarters but variable	Predominantly yellow, with dark area, which in the palest specimens is limited to a small grey bar along the lower margin and in the darkest extends along the entire lower margin	Yellow with black patch along lower margin. In darkest specimens entire lower half is black	Yellow, small blackish grey grey on lower margin
Mesonotum	Black, largely shiny but with distinct matt undertone	Semi shiny black	Black, shiny	Black, shiny *but slightly matt*	Blackish grey, matt
Femur	Bright yellow with some brownish striations	Yellow, variably darkened with black striations In darkened specimens appearing almost completely black but yellow always detectable on inner sides	Bright yellow	Yellow *with some brownish striations*	Yellow with slight, variable brownish striation
Wing length	1.75–2.1 mm	1.7–2.25 mm	1.3–1.7 mm	*1.8–2.1 mm*	1.3–1.7 mm
Vein Cu 1A	*a* twice length of *b*	*a* 2–2.5 times length of *b*	*a* 3–4 times length of *b*	*a* 2–3 times length of *b*	*a* 3–4 times length of *b*
Male abdominal tergites	Second and third visible tergites divided by a yellow medial furrow	Only the second visible tergite divided by a yellow medial furrow	Only the second visible tergite divided by a yellow medial furrow	–	Second to fifth visible tergites divided by a yellow medial furrow
Male distiphallus	Two distal bulbs; bulb rims circular	Two distal bulbs, meeting only at their rims; bulb rims drawn out antero-ventrally	One distal bulb with a slight constriction between upper and lower halves in dorso-ventral view; bulb appears more strongly sclerotized with a shorter basal stem	Two distal bulbs, meeting from their rims to their bases; bulb rims drawn out antero-ventrally	One distal bulb with marked constriction between lower and upper halves in dorso-ventral view; bulb appears less distinctly sclerotized with a longer basal stem
Epiphallus[d]	Apex of epiphallus is rounded	Apex of epiphallus is rounded	Apex of epiphallus is pointed	Apex of epiphallus is rounded	Apex of epiphallus is pointed

Source: Mostly compiled from Spencer (1973, 1976), with information on the male abdominal tergites from Shiao (2004) (who did not include *L. strigata* in his analysis). Text in italics is for characters not found in the references cited before but mentioned in Appendix 16 to ISPM 27 Genus *Liriomyza,* IPPC (2016).

[a]See also Figures 7, 8 and 10–14.

[b]*L. langei* is morphologically indistinguishable from *L. huidobrensis.*

[c]Spencer refers to the mesopleura (nearly equivalent to Anepisternum)

[d]Additional character compared with IPPC protocol.

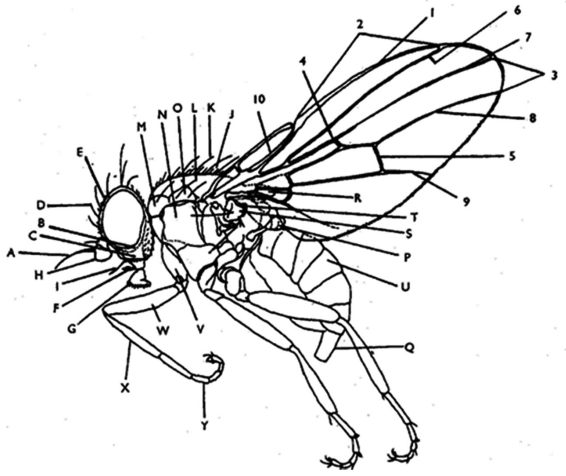

FIGURE 7 Adult female morphology of Agromyzidae. Source: Spencer (1973). Side view of typical *Agromyza* sp. (after Sasakawa): A, arista; B, cheek; C, jowl; D, orbital bristles; E, orbital setulae; F, palp; G, proboscis; H, third antennal segment; I, vibrissa; J, acrostichals; K, dorso-central bristles; L, mesonotum; M, humerus; N, mesopleural area; O, notopleural area; P, haltere; Q, ovipositor sheath; R, scutellum; S, squama; T, squamal fringe; U, tergites; V, coxa; W, femur; X, tibia; Y, tarsi. 1, costa; 2, second costal section; 3, fourth costal section; 4, first cross-vein; 5, second cross-vein; 6, R_1; 7, R_{4+5}; 8, M_{1+2}; 9, $M_{3+4;}$ 10, sub-costa (Sc)

For a summary of information on the morphology and biology of the immature stages of agromyzids, with an extensive bibliography and illustrations of the cephalopharyngeal skeleton and posterior spiracles for a number of species, see Ferrar (1987).

4.1.2 | Identification of the genus *Liriomyza*

Adult flies of the genus *Liriomyza* have the following morphological characters (Spencer, 1976):

- Fronto-orbital setulae reclinate (backward pointing);
- Dark pre-scutellar area concolorous with the scutum in most species, rarely yellow;
- Scutellum yellow in most species, rarely dark;
- Subcosta becomes a fold distally and ends in costa separately (Figure 8);
- Costa extends to vein M_{1+2}[2](Figure 8);
- Discal cell (dm) small (Figure 8);
- Second (outer) crossvein (dm–cu) present in most species (Figure 8);
- 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).

[2]In most of the *Liriomyza* species the costa vein ends at the point where M_{1+2} reaches the wing margin as noted in Spencer (1976). However, there are a number of other *Liriomyza* species, including *L. sativae* and *L. trifolii*, that have slightly varied length costa, and for which the costa are fractionally extending beyond the point where M_{1+2} reaches the wing margin (Figure 9).

In practice, most species of *Liriomyza* (including the four target species included in this diagnostic protocol) are seen from above to be mostly black with a yellow frons and a bright yellow scutellum. The legs are variably yellow. The target species possess the typical wing venation (Figure 9) and the generalized male genitalia of the genus.

There are several genera that may be confused with *Liriomyza*. The closely related genera *Phytomyza*, *Chromatomyia* and *Phytoliriomyza* can generally be separated from *Liriomyza* by their proclinate (forward pointing) fronto-orbital setulae (always reclinate or occasionally upright or missing in *Liriomyza*), and by the scutellum, which is generally grey or black but occasionally slightly yellowish centrally (entirely yellow in most *Liriomyza*). In *Phytomyza* and *Chromatomyia*, the costa extends only to R_{4+5}, whereas in *Phytoliriomyza* and *Liriomyza* it extends to vein M_{1+2}[2] (Spencer, 1977). *Phytoliriomyza* species are gall-forming (on a stem or leaf) internal feeders, whereas *Chromatomyia*, *Phytomyza* and *Liriomyza* species are typically leafminers.

4.1.3 | Identification of *Liriomyza* species

4.1.3.1 | Morphological characters of adult Liriomyza *spp.*

A simplified summary of the main diagnostic characters for *L. bryoniae*, *L. huidobrensis*, *L. sativae* and *L. trifolii* (as well as for *L. strigata* for the purposes of elimination) is given in Table 1. This is accompanied by illustrative images (photomicrographs) of the distiphallus in Figures 10–12.

More detailed descriptions and illustrations of the morphology of these species are provided by Spencer (1965, 1973), Dempewolf (2004), Malipatil et al. (2004) and Shiao (2004). Key diagnostic features are shown in the Pest and Disease Image Library (Malipatil, 2007a, 2007b, 2007c).

Identification of the adults can also be carried out with keys. Malipatil and Ridland (2008) provide a key to 17 species of economic importance, including a few species endemic to Australia. In addition, an identification system for pest species from around the world based on photomicrographs is available in Dempewolf (2004). With particular reference to keys for *Liriomyza* species, there are some extensive regional back catalogues and keys available through the works of Spencer. These cover the regional background fauna, which obviously differs from region to region, and by doing so differentially affects the positive process of eliminating non-target taxa. A full list of these works is listed in Spencer (1973). In addition, considering the host plant on which the suspected quarantine *Liriomyza* species has been detected can help by narrowing down the other potential agromyzid species that may occur in the same biological context and

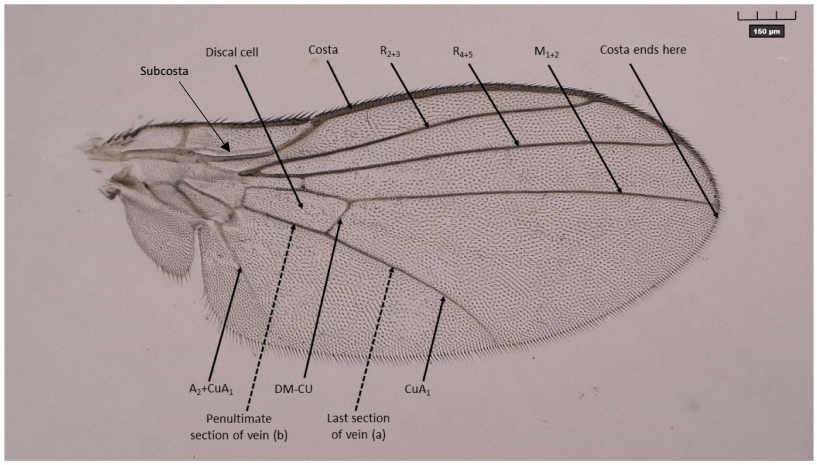

FIGURE 8 Wing venation of *Liriomyza* spp. Courtesy L. Gidron, PPIS, IL

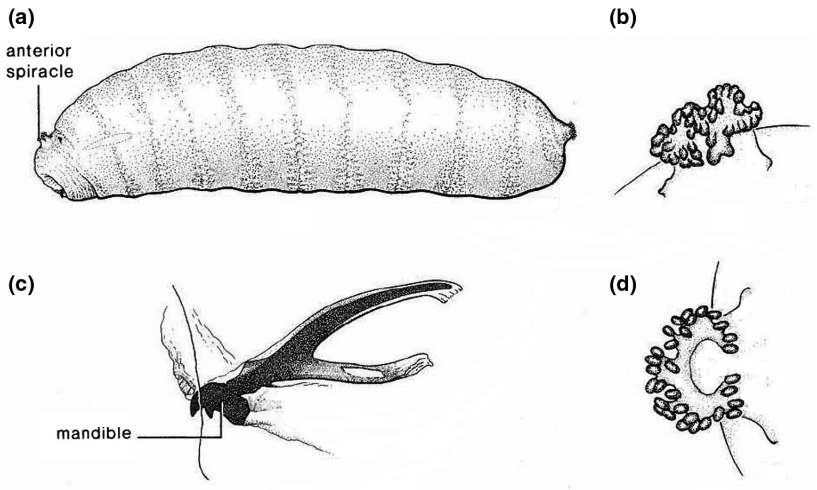

FIGURE 9 Larval morphology of Agromyzidae (*Phytomyza chelonei*): (a) lateral view; (b) anterior spiracle; (c) cephalopharyngeal skeleton; and (d) posterior spiracle. Source: Stehr (1991)

which may need to be eliminated from consideration (e.g. for Europe, see Ellis (n.d.)).

4.1.3.2 | Distiphallic structure of adult male Liriomyza *spp.*

The *Liriomyza* species considered here separate into two distinct natural groups based on the structure of the male genitalia (particularly the distiphallus) as well as the body colour and the structure of the posterior spiracles of the larvae:

- Group 1 – *L. bryoniae*, *L. huidobrensis* and *L. strigata*;
- Group 2 – *L. sativae* and *L. trifolii*.

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.

The distiphallus is a very small, fragile structure enclosed by membranes. It is the terminal part of the aedeagus (the intromittent organ, part of the male genitalia; Figure 6) and its complex three-dimensional structure is of considerable diagnostic value. Indeed, the distiphallus provides a differential character by which all four target species can be identified reliably. The basic structure of the distiphallus differs in the two natural species groups: in group 1, there are two distal bulbs side by side (Figure 10), while in group 2, there is only one distal bulb, which has a medial constriction dividing it into distinct lower and upper sections (Figure 11). A key that facilitates identification of the four target species using the distiphallus is provided below. For convenience, the key also includes *L. strigata*, which is closely related to *L. bryoniae* and *L. huidobrensis* and is also polyphagous and therefore to be found on similar host plants.

However, the differences between some of the species pairs are subtle and the evidence of the distiphallic structure should be cross-checked with the evidence of external morphology (Table 1) in order to ensure that the distiphallic structure has not been misinterpreted. If all of the evidence correlates, all other species of *Liriomyza*, including those not discussed here, can be eliminated.

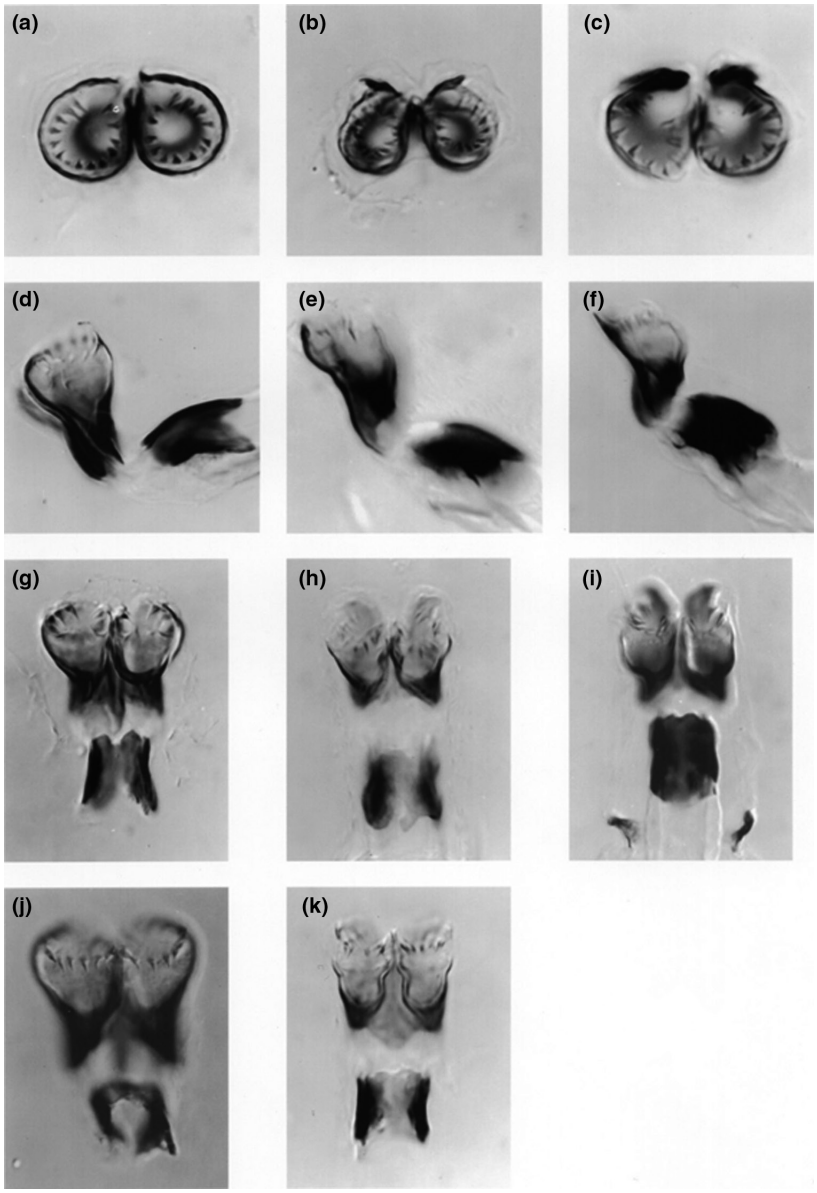

FIGURE 10 Distiphallus of *L. bryoniae*, *L. huidobrensis* and *L. strigata*. (×400 magnification): (a) *L. bryoniae*, anterior view; (b) *L. huidobrensis*, anterior view; (c) *L. strigata*, anterior view; (d) *L. bryoniae*, lateral view; (e) *L. huidobrensis*, lateral view; (f) *L. strigata*, lateral view; (g) *L. bryoniae*, dorso-ventral view; (h) *L. huidobrensis*, dorso-ventral view; (i) *L. strigata*, dorso-ventral view; (j) *L. bryoniae*, dorso-ventral view (in a different plane from (g)); and (k) *L. huidobrensis*, dorso-ventral view (in a different plane from (h)). Courtesy of Department of Environment, Food and Rural Affairs, GB

Diagnostic key for identification of *Liriomyza* spp. using the male distiphallus

This key is to be used in conjunction with Figures 10 and 11.

1.	With one distal bulb (Figure 11e,f)	**2**
	With a pair of distal bulbs (Figure 10a–c,g–k)	**3**
2.	With marked constriction between the apical and basal parts of the bulb: basal section strongly curved (Figure 11f)	***L. trifolii***
	With slight constriction only between the apical and basal parts of the bulb: basal section not strongly curved (Figure 11e)	***L. sativae***
3.	With bulb rims circular (not drawn out antero-ventrally); evenly sclerotized (Figure 10a)	***L. bryoniae***
	With bulb rims spiralled (drawn out antero-ventrally) (Figure 10b,c)	**4**
4.	With bulbs meeting in the midline only at their rims (Figure 10h)	***L. huidobrensis****
	With bulbs meeting in the midline from their rims to their bases (Figure 10i)	***L. strigata***

* *L. langei* is morphologically indistinguishable from *L. huidobrensis*.

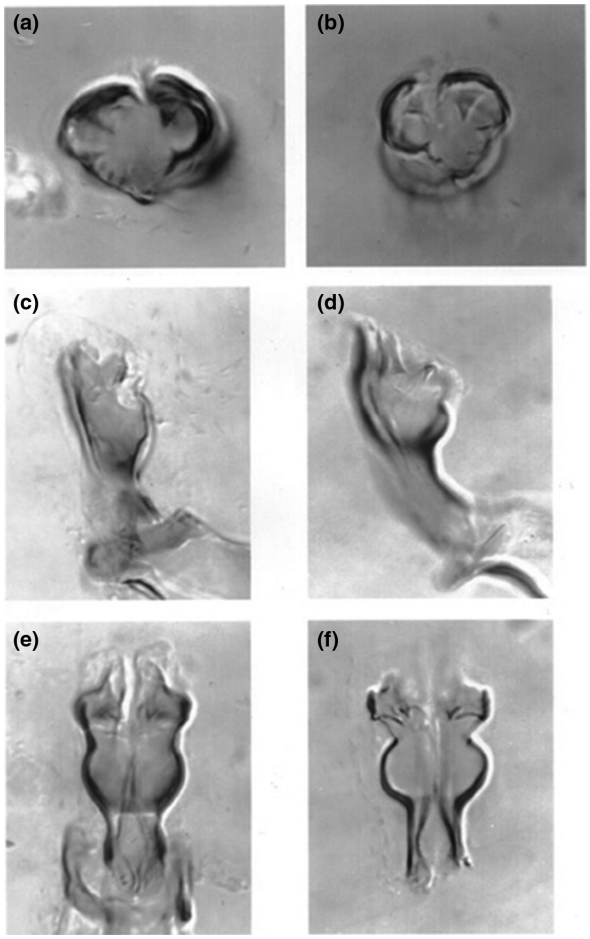

FIGURE 11 Distiphallus of *L. sativae* and *L. trifollii* (×400 magnification): (a) *L. sativae*, anterior view; (b) *L. trifolii*, anterior view; (c) *L. sativae*, lateral view; (d) *L. trifolii*, lateral view; (e) *L. sativae*, dorso-ventral view; and (f) *L. trifolii*, dorso-ventral view. Courtesy of Department of Environment, Food and Rural Affairs (GB)

4.1.3.3 | Morphological characteristics of the immature stages of the four target species of Liriomyza

Of the four life stages (egg, larva, pupa and adult) only the adult male flies can be positively identified to species level using morphological features (the shape of the male genitalia). The morphological characteristics of larvae and pupae can be used to distinguish between the members of the two natural species groups described in section 4.1.3.2. This information can contribute towards species identification but is insufficient by itself to allow species identification. To complement morphological identification, molecular tests can be used to distinguish between the species included in the protocol (section 4.2).

4.1.3.3.1 | 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.

4.1.3.3.2 | 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 fully grown. The larvae are typical agromyzids in their gross form (see section 4.1.1). Pupae (Figure 15) are oval cylinders in shape, 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 as follows (but not the species within the groups).

4.1.3.3.3 | Group 1 larvae. Larvae of *L. bryoniae*, *L. huidobrensis* and *L. strigata* are cream-coloured but in the final instar develop a yellow–orange patch dorsally at the anterior end, which can extend around to the ventral surface (Figure 16). Each posterior spiracle consists of an ellipse with pores along the margin (Figure 17a). It can be difficult to observe the numbers 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 colour, from yellow–orange to dark brown. In *L. bryoniae* and *L. strigata*, puparia are mostly, but not exclusively, at the lighter end of the colour range. The colour of *L. huidobrensis* puparia mostly tends to anthracite. The form of the larval spiracles is retained in the puparium although the pores are less clearly discernible.

4.1.3.3.4 | Group 2 larvae. Larvae of *L. sativae* and *L. trifolii* are translucent when newly emerged and yellow–orange over the entire body later. Each posterior spiracle is tricorn shaped with three pores, each on a distinct projection, the outer two elongate (Figure 17b). Puparia are yellowish orange, sometimes a darker golden brown. The form of the larval spiracles is retained in the puparium but the detail is less obvious.

4.2 | Molecular methods

Two molecular tests that can be used to support *Liriomyza* species identification are included in the present Standard and were developed by Kox et al. (2005) and Nakamura et al. (2013). These tests are described in full in Appendices 2 and 3 and are also part of the IPPC Protocol.

A PCR-RFLP test (Scheffer et al., 2001) included in the IPPC Protocol and a multiplex PCR (Scheffer et al., 2014) have been developed to distinguish *L. huidobrensis* and *L. langei*. In addition, an on-site test to confirm the identity of *L. huidobrensis*, *L. sativae* and *L. trifolii* has been developed by OptiGene (LAMP) in collaboration with Fera Science Ltd and the optimized LAMP tests have been validated according to EPPO Standard PM 7/98. A multiplex real-time PCR test for the detection of *L. huidobrensis*, *L. sativa* and *L. trifolii* has also been developed by Sooda et al. (2017). There is little experience in the region with these tests and they are consequently not described in this Standard.

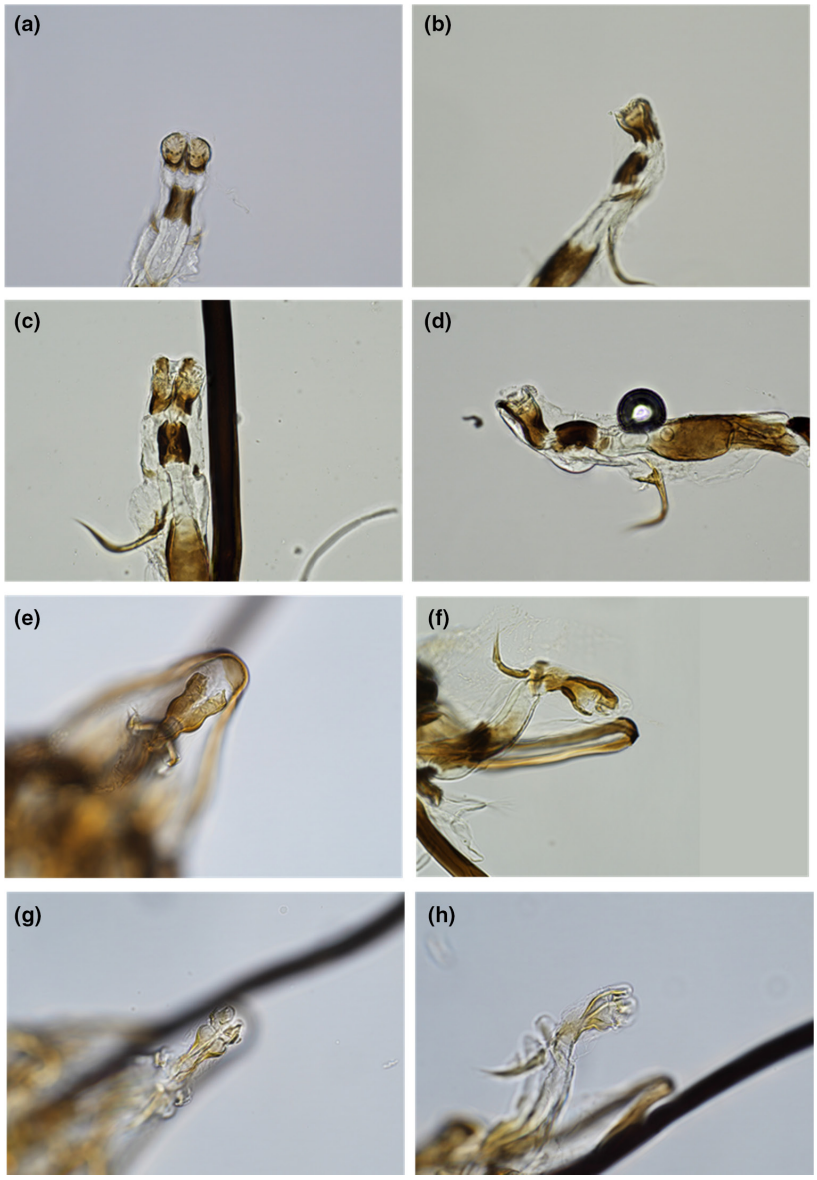

FIGURE 12 Distiphallus of *L. bryoniae* (a, b), *L. strigata* (c, d), *L. sativae* (e, f), *L. trifolii* (g, h) (×400 magnification). Courtesy of A. Gluhovs, National Phytosanitary Laboratory (LV)

A protocol for DNA barcoding based on COI is described in Appendix 1 of PM 7/129 *DNA barcoding as an identification tool for a number of regulated pests: DNA barcoding Arthropods* (EPPO, 2020) and can also support the identification of *Liriomyza* spp. Sequences are available in different databases; those in the EPPO-Q-bank (https://qbank.eppo.int/arthropods/) are curated. DNA barcoding identification results should be interpreted carefully for possible issues such as: (1) potential preferential PCR amplification of parasitoids or nuclear mitochondrial copies of the COI gene (e.g. nuclear mitochondrial pseudogenes (numt)); (2) the possibility of misidentification with closely related sister species (i.e. species complexes); and (3) a different scope of geographic coverage of the reference specimens in the sequence databases.

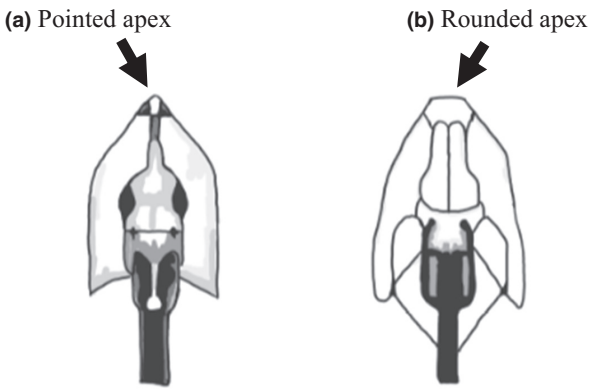

FIGURE 13 Schematic representation of the epiphallus of *Liriomyza* spp. (a) *L. sativae* and *L. trifolii*; (b) *L. bryoniae*, *L. huidobrensis*, *L. strigata*. Courtesy of ANSES (FR)

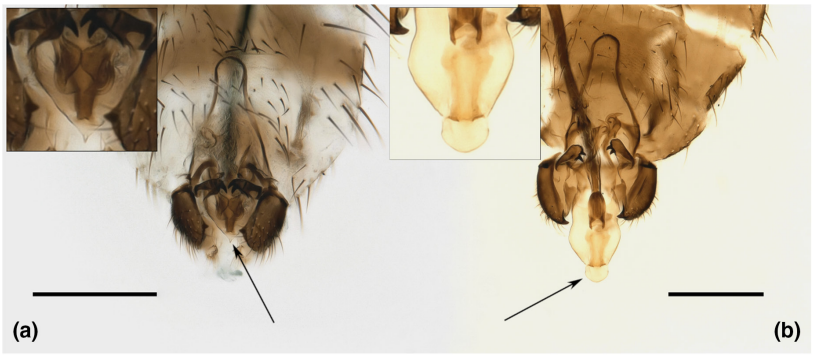

FIGURE 14 Abdomen terminalia of *Liriomyza* spp., ventral view (after clearing in KOH). The arrows identify the apex of epiphallus, magnified in the respective boxes at top left: (a) *L. trifolii*, pointed apex of epiphallus; (b) *L. huidobrensis*, rounded apex of epiphallus (extracted from epandrium). Scalebar of 0.2 mm. Photos courtesy A. Taddei, ANSES LSV, Entomology and Invasive Plant Unit, FR

FIGURE 15 Pupa of *Liriomyza* sp. Courtesy of Victorian State Government Department of Environment, Land, Water and Planning, AU

FIGURE 16 Third larval instar of *L. bryoniae*. Courtesy of Department of Environment, Food and Rural Affairs, GB

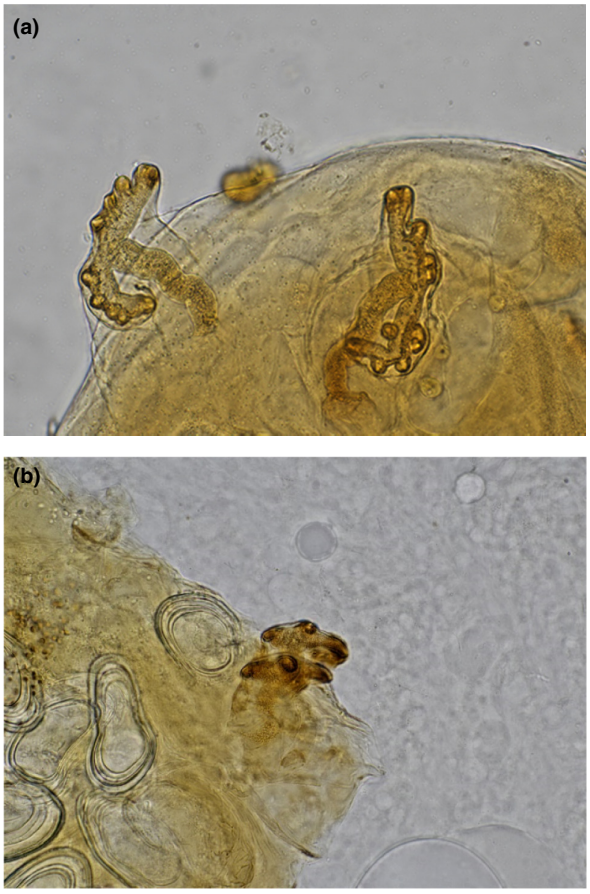

FIGURE 17 Typical shape of posterior spiracles in group 1 (a) and group 2 (b) larvae (magnification ×400). Courtesy of A. Gluhovs, National Phytosanitary Laboratory, LV

5 | REFERENCE MATERIAL

Slides may be available for loan from Fera Science Limited, GB.

6 | REPORTING AND DOCUMENTATION

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

7 | PERFORMANCE CHARACTERISTICS

When performance characteristics are available, these are provided with the description of the test. Validation data is 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).

8 | FURTHER INFORMATION

Countries were asked to suggest experts who could provide further information but no nominations were received.

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 validation 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.

ACKNOWLEDGEMENTS

This protocol was originally drafted by D. W. Collins, formerly FERA Sciences Ltd, York (GB). The first revision was prepared by E. Wulff, Danish Veterinary and Food Administration, Division of Plant Diagnostics, Ringsted (Denmark). Many of the line drawings found in this protocol are based on original versions by Paul Seymour, formerly of the Central Science Laboratory, GB. Paul Seymour also took all of the photographs of *Liriomyza* genitalia. The second revision was prepared to align the EPPO Protocol to Appendix 16 to ISPM 27 Genus Liriomyza, IPPC. L Gidron (PPIS, IL) prepared the revision of Table 1. The Standard was reviewed by the Panel on Diagnostics in Entomology.

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APPENDIX 1 - DETERMINATION OF FLIES' SEX AND PREPARATION OF MALE DISTIPHALLUS FOR EXAMINATION

1. Determining the sex of flies

In the male fly, the lobes of the epandrium, which are dark and pubescent and not as heavily sclerotized as the female tube, curve around and down at the rear of the abdomen, from the dorsal to the ventral sides (Figure 18a). 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 barely extend beyond the last tergite. In the female fly, the abdominal segments beyond segment 6 form a black, heavily sclerotized tube that extends beyond the sixth tergite (Figure 18b), with a circular opening visible in posterior view at the end of the tube. The sixth tergite covers the basal half of the tube from above, although it is visible in lateral and ventral views.

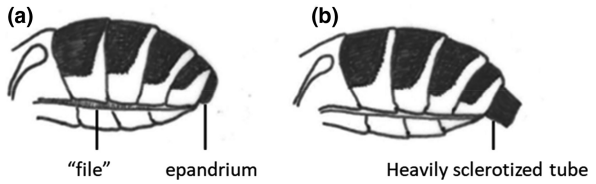

FIGURE 18 Abdomen in (a) male and (b) female *Liriomyza*. Photo courtesy of Department of Environment, Food and Rural Affairs, GB

2. Preparation of the male distiphallus for examination

The abdomen should be removed from the body to enable clearing of tissues and observation. This can be accomplished by using fine dissecting needles (which can be made by gluing the blunt end of a pointed micro pin into the end of a wooden matchstick, first making a shallow hole with a normal pin) to carefully separate the abdomen from the rest of the fly. The abdomen can be boiled in 10% potassium hydroxide (KOH) or sodium hydroxide (NaOH) for 2–4 min or, alternatively, left in cold 10% KOH or NaOH overnight to clear the tissues. Transferring the treated abdomen to a bath of distilled water will neutralize the KOH or NaOH. The abdomen is then ready for transfer to a drop of glycerol on a cavity slide.

Under a binocular stereomicroscope and using the fine dissecting needles, the genital complex is carefully dissected out from the surrounding membranes, cuticle and associated musculature. Using the fine dissecting needles, the genital complex is positioned for lateral viewing under a compound microscope at up to 400× magnification. The genital complex is repositioned for ventral viewing of the distiphallus at 400× magnification, without the addition of a cover. The distiphallus needs to be viewed in different orientations (e.g. lateral, dorsal, ventral), which requires repositioning under a lower magnification.

To make semi-permanent slides (e.g. for routine identification), the genital complex should be transferred to a drop of glycerol on a clean flat slide. The genitalia are immersed gently in the mountant, and a round coverslip is lowered carefully over it to evenly spread the mountant.

If permanent slide mounts are required, the abdomen should be cleared in KOH (as described above) and neutralized in cold glacial acetic acid. Then, the abdomen can be transferred to 70% ethanol and, using the fine dissecting needles under a binocular stereomicroscope, the genital complex carefully dissected from the surrounding membranes, cuticle and associated musculature. The dissected genitalia should be transferred first to absolute ethanol for 2–4 min, then to clove oil (in which, if necessary, they can be stored for any length of time). The genitalia are transferred to 70% ethanol (for approximately 10 min), then to 95% ethanol (for approximately 10 min) and finally to clove oil (for at least 5 min). The genitalia can then be permanently mounted on a slide in a drop of Canada balsam under a coverslip. All slide mounts must be labelled with adequate data detailing locality, host, date of collection, name of collector (if known), species name, name of identifier and a code label to cross-reference to the remaining specimen.

The remainder of the fly specimen should be mounted on a card point with an appropriate label cross-referenced to its genitalia mounted on the slide. More details on or variations to the method are provided by Spencer (1981, 1992), Spencer and Steyskal (1986).

APPENDIX 2 - PCR-RFLP (KOX ET AL., 2005)

The test below is described as carried out to generate the validation data provided in section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1. This PCR RFLP developed by Kox et al. (2005) is suitable for the identification of the four target *Liriomyza* species (*L. bryoniae*, *L. huidobrensis*/*L. langei*, *L. sativae* and *L. trifolii*).
1.2. The target sequence is the mitochondrial cytochrome oxidase II (COII) gene.
1.3. The amplicon size is 790 bp.
1.4. Oligonucleotides

Forward primer: TL2-J-3037	5′-ATGGCAGATTA GTGCAATGG-3′	Simon et al. (1994)
Reverse primer: TK-N-3785Lir	5′-GTTWAAGAGAC CATTRCTTG-3′	Kox et al. (2005)

These primers are annealing in the leucine tRNA and lysine tRNA genes, respectively, spanning the 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*.

1.5. The test was initially developed on a 96-well PTC200 thermocycler (MJ-Research).

2. Methods

2.1. Nucleic acid extraction and purification.

DNA is extracted from a single adult, pupariae or larvae. DNA is extracted using standard DNA extraction methods, e.g. the high-purity PCR template preparation kit (Roche Diagnostics, Almere, the Netherlands) according to the instructions in the mammalian tissue protocol. Before DNA extraction, each specimen is ground in 200 µL of lysis buffer using a micropestle. The DNA is eluted in 50 µL of 10 mM Tris, pH 8.5. Laboratories may find that alternative extraction techniques work equally well.

2.2. Polymerase chain reaction (PCR)

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	NA	To make up to 49 µL	NA
10× reaction buffer (with 15 mM $MgCl_2$)	—	5.0	—
dNTPs (Promega)	10 mM	1.00	0.2 mM of each dNTP
Forward Primer (TL2-J-3037)	10 µM	3.00	0.6 µM
Reverse Primer (TK-N-3785Lir)	10 µM	3.00	0.6 µM
HotStarTaq DNA polymerase (Qiagen)	5 U μL^{-1}	0.20	1 U
Subtotal		49	
DNA extract		1	
Total		50	

2.2.2. PCR cycling conditions: 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..

2.3. Restriction fragment length polymorphism (RFLP) reaction.

A 5 µL aliquot of PCR product (without further purification) is digested with the enzymes *Dde*I, *Hinf*I, *Ssp*I and *Taq*I in separate reactions according to the manufacturer's instructions.

2.4. For fragment sizes of digested PCR products, see Table 2.

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 isolation and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction – clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated – nucleic acid extraction and subsequent amplification of the target organism.
- Negative amplification control (NAC) to rule out false positives owing 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 PCR amplification and of the RFLP reaction – amplification and digestion of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product).

3.2. Interpretation of results:

Verification of the controls:

- NIC and NAC – no band is visualized
- PIC and PAC – bands of the expected size as given in Table 2 are visualized.

When these conditions are met:

- A sample will be considered positive if bands of the expected size as given in Table 2 are visualized.
- A sample will be considered negative if bands of a size different than the ones expected as given in Table 2 are visualized.
- Tests should be repeated if no band is visualized or if any contradictory or unclear results are obtained

4. Performance characteristics available

Data is according to Kox et al. (2005).

4.1. Analytical sensitivity data

TABLE 2 Fragment sizes of digested PCR products of *Liriomyza* spp.

Restriction enzyme	Fragment sizes					
	L. bryoniae	*L. huidobrensis*[a]	*L. sativae* 'USA'[b]	*L. sativae* 'Asia'[b]	*L. trifolii*	*L. strigata*
*Dde*I	790	790	567	790	619	790
			223		171	
Hinf I	421	421	421	421	421	421
	369	369	283	310	310	342
			59	59	59	27
			27			
*Ssp*I	392	399	399	717	391	399
	326	391	391	73	326	391
	72				73	
*Taq*I	486	306	306	306	306	267
	163	163	210	210	163	219
	111	159	163	163	159	141
	30	111	81	81	141 (or 111 +30)[c]	72
		30	30	30	21	67
		21				

Source: Data from Kox et al. (2005).

[a]Including cryptic species *L. langei*.

[b]USA and Asia are known alternative variants; both of these are *L. sativae*.

[c]*L. trifolii* is heterogeneous for this restriction site.

One specimen (larvae, pupae or adult) was used for DNA extraction.

4.2. Analytical specificity data

Analytical specificity was determined by testing both target and non-target species. For inclusivity, several specimens per target species were tested (*L. bryoniae* (8), *L. huidobrensis* (21), *L. sativae* (9) and *L. trifolii* (15)). The specificity of the test (exclusivity) was further investigated by analysing specimens of four additional *Liriomyza* species: *L. strigata* (2), *L. langei* (1), *L. chinensis* (2) and *L. scorzonerae* (2). The *L. langei* and *L. huidobrensis* specimens could not be distinguished with this test. The other three species were separated successfully. If a sample is suspected as *L. huidobrensis*, further testing may be needed to confirm it is not the cryptic species *L. langei*.

APPENDIX 3 - CONVENTIONAL MULTIPLEX PCR (NAKAMURA ET AL., 2013)

The test below is described as carried out to generate the validation data provided in section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1. This conventional multiplex PCR is suitable for the identification of the four target *Liriomyza* species (*L. bryoniae*, *L. huidobrensis*/, *L. langei*, *L. sativae* and *L. trifolii*). This test was developed for *Liriomyza* identification in Japan and specificity has been directed to that purpose. As a result, cross-reactivity with *L. strigata* and populations of *L. trifolii* outside Japan have not been verified..

1.2. The test was developed by Nakamura et al. (2013).

1.3. This test uses six primers that target the mitochondrial *cytochrome oxidase* I (COI) gene.

1.4. Amplicon size:

649 bp (*L. bryoniae*), 359 bp (*L. chinensis*), 107 bp (*L. huidobrensis*/*L. langei*), 207 bp (*L. sativae*) and 461 bp (*L. trifolii*).

1.5. Primers:

Lb600-F	5′-CTAGGAATGAT TTATGCAATG-3′	*Liriomyza bryoniae*
Lc920-F	5′-CATGACACTTAT TATGTTGTTGCA-3′	*Liriomyza chinensis*
Lh1150-F	5′-CAATCGGATCT TCAATTTCCCTTC-3′	*Liriomyza huidobrensis*/ *langei*
Ls1040-F	5′-TTATTGGTGTAA ATTTAACC-3′	*Liriomyza sativae*
Lt780-F	5′-TTATACACCAAC TACTTTGTGAA-3′	*Liriomyza trifolii*
L1250-R	5′-GAATWGGRWAAAT YACTTGACGTTG-3′	Common to *Liriomyza*

The sixth primer binds to a segment of the COI gene conserved in all *Liriomyza* species, and is used as a reverse primer, to complete primer pairing. The size of the PCR products can be used to discriminate among

L. bryoniae, *L. huidobrensis*, *L. sativae*, *L. trifolii* and *L. chinensis*.

1.6. The test was performed in a thermal cycler model 2720 (Applied Biosystems of Life Technologies Japan, Tokyo, Japan).

2. Methods

2.1. Nucleic acid extraction and purification

DNA is extracted from a thorax that is removed from a single adult specimen with a fine cutter and then crushed with a plastic rod in 90 µL of MTD with 10 µL of ETD (proteinase K; component of QuickGene DNA tissue kit S; Fujifilm Holdings Corporation, Tokyo, Japan). The solution is incubated at 55°C for 24 h and purified with the QuickGene DNA tissue kit S protocol using QG-Mini80 (Fujifilm Holdings Corporation). Other insect parts and stages may work as well. Laboratories may find that alternative extraction techniques work equally well.

2.2. Polymerase chain reaction (PCR)

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	NA	To make up to 9.5 µL	NA
Takara Ex Taq PCR buffer (with 20 mM $MgCl_2$)	10×	1.0	1×
dNTPs (Promega)	10 mM	0.20	0.2 mM of each dNTP
Forward primers	10 µM	0.50	0.5 µM of each primer
Reverse primer	10 µM	0.50	0.5 µM
Takara Ex Taq DNA polymerase	5 U μL^{-1}	0.20	1 U
Subtotal		9.5	
DNA extract		0.5	
Total		10	

2.2.2. PCR cycling conditions: 1 min denaturation step at 94°C followed by 32 cycles of (30 s at 94°C, 30 s at 55°C and 2 min at 72°C).

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 – clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated – nucleic acid extraction and subsequent amplification of the target organism
- 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. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. These can include amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

3.2. Interpretation of results:

Verification of the controls:

- NIC and NAC – no band is visualized;
- PIC and PAC (and if relevant IPC) – a band of the expected size (as specified above) is visualized.

When these conditions are met:

- A sample will be considered positive for one of the species if a band of the expected size as specified above is visualized.
- A sample will be considered negative, if no band or a band of a different size than expected is visualized.
- Tests should be repeated if any contradictory or unclear results are obtained

4. Performance characteristics available

Data is according to Nakamura et al., 2013.

4.1. Analytical sensitivity data

The thorax of a single adult specimen was used for DNA extraction.

4.2. Analytical specificity data

Analytical specificity was determined by testing both target and non-target species. For inclusivity, several specimens per target species were tested (*L. bryoniae* (3), *L. huidobrensis* (3), *L. sativae* (4) and *L. trifolii* (2)). The specificity of the test (exclusivity) was further investigated by analysing specimens of one additional *Liriomyza* species (*L. chinensis* (8)) and of the following species: *Chromatomyia horticola* (6), *Dacnusa nipponica* (1), *Diglyphus isaea* (1), *Hemiptarsenus varicornis* (1) and *Neochrysocharis okazakii* (1). Unlike the PCR-RFLP test of Kox et al. (2005), the specificity of this test against *L. strigata* has not been verified. This test is not able to distinguish *L. huidobrensis* from *L. langei*. If a sample is suspected as *L. huidobrensis*, further testing may be needed to confirm it is not the cryptic species *L. langei*.