Organisation Européenne et Méditerranéenne pour la Protection des Plantes
European and Mediterranean Plant Protection Organization

Normes OEPP
EPPO Standards

Diagnostics
Diagnostic

PM 7/53

Organisation Européenne et Méditerranéenne pour la Protection des Plantes
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Approval

EPPO Standards are approved by EPPO Council. The date of approval appears in each individual standard. In the terms of Article II of the IPPC, EPPO Standards are Regional Standards for the members of EPPO.

Review

EPPO Standards are subject to periodic review and amendment. The next review date for this EPPO Standard is decided by the EPPO Working Party on Phytosanitary Regulations.

Amendment record

Amendments will be issued as necessary, numbered and dated. The dates of amendment appear in each individual standard (as appropriate).

Distribution

EPPO Standards are distributed by the EPPO Secretariat to all EPPO member governments. Copies are available to any interested person under particular conditions upon request to the EPPO Secretariat.

Scope

EPPO Standards on Diagnostics are intended to be used by NPPOs in their capacity as bodies responsible for the application of phytosanitary measures. Standards on diagnostic protocols are concerned with the diagnosis of individual pests and describe different methods which can be used to detect and identify pests of phytosanitary concern for the EPPO region. General Standards on diagnostics are in preparation on: (1) the purpose of diagnostic protocols (which may differ according to the circumstances of their use); and (2) reporting and documentation of diagnoses.

In 1998, EPPO started a new programme to prepare diagnostic protocols for the regulated pests of the EPPO region (including the EU). The work is conducted by the EPPO Panel on Diagnostics and other specialist Panels. The objective of the programme is to develop an internationally agreed diagnostic protocol for each regulated pest. The protocols are based on the many years of experience of EPPO experts. The first drafts are prepared by an assigned expert author(s). They are written according to a 'common format and content of a diagnostic protocol' agreed by the Panel on Diagnostics, modified as necessary to fit individual pests. As a general rule, the protocol recommends a particular means of detection or identification which is considered to have advantages (of reliability, ease of use etc.) over other methods. Other methods may also be mentioned, giving their advantages/disadvantages. If a method not mentioned in the protocol is used, it should be justified.

The following general provisions apply to all EPPO Standards on Diagnostics:

- laboratory tests may involve the use of chemicals or apparatus which present a certain hazard. In all cases, local safety procedures should be strictly followed
- use of names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable
- laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated or that proper positive and negative controls are included.

References

EPPO/CABI (1996) *Quarantine Pests for Europe*, 2nd edn. CAB International, Wallingford (GB).

EU (2000) Council Directive 2000/29/EC of 8 May 2000 on protective measures against the introduction into the Community of organisms harmful to plants or plant products and against their spread within the Community. *Official Journal of the European Communities* L169, 1–112.

FAO (1997) *International Plant Protection Convention* (new revised text). FAO, Rome (IT).

IPPC (1993) *Principles of plant quarantine as related to international trade*. ISPM no. 1. IPPC Secretariat, FAO, Rome (IT).

IPPC (2002) *Glossary of phytosanitary terms*. ISPM no. 5. IPPC Secretariat, FAO, Rome (IT).

OEPP/EPPO (2003) EPPO Standards PM 1/2(12): EPPO A1 and A2 lists of quarantine pests. *EPPO Standards PM1 General phytosanitary measures*, 5–17. OEPP/EPPO, Paris (FR).

Definitions

Regulated pest: a quarantine pest or regulated non-quarantine pest.
Quarantine pest: a pest of potential economic importance to the area endangered thereby and not yet present there, or present but not widely distributed and being officially controlled.

Outline of requirements

EPPO Standards on Diagnostics provide all the information necessary for a named pest to be detected and positively identified by an expert (i.e. a specialist in entomologist, mycology, virology, bacteriology, etc.). Each protocol begins with some short general information on the pest (its appearance, relationship with other organisms, host range, effects on host, geographical distribution and its identity) and then gives details on the detection, identification, comparison with similar species, requirements for a positive diagnosis, list of institutes or individuals where further information on that organism can be obtained, references (on the diagnosis, detection/extraction method, test methods).

Existing EPPO Standards in this series

Forty-one EPPO standards on diagnostic protocols have already been approved and published. Each standard is

numbered in the style PM 7/4 (1), meaning an EPPO Standard on Phytosanitary Measures (PM), in series no. 7 (Diagnostic Protocols), in this case standard no. 4, first version. The existing standards are:

PM 7/1 (1) *Ceratocystis fagacearum*. *Bulletin OEPP/EPPO Bulletin* **31**, 41–44

PM 7/2 (1) *Tobacco ringspot nepovirus*. *Bulletin OEPP/EPPO Bulletin* **31**, 45–51

PM 7/3 (1) *Thrips palmi*. *Bulletin OEPP/EPPO Bulletin* **31**, 53–60

PM 7/4 (1) *Bursaphelenchus xylophilus*. *Bulletin OEPP/EPPO Bulletin* **31**, 61–69

PM 7/5 (1) *Nacobbus aberrans*. *Bulletin OEPP/EPPO Bulletin* **31**, 71–77

PM 7/6 (1) *Chrysanthemum stunt pospiviroid*. *Bulletin OEPP/EPPO Bulletin* **32**, 245–253

PM 7/7 (1) *Aleurocanthus spiniferus*. *Bulletin OEPP/EPPO Bulletin* **32**, 255–259

PM 7/8 (1) *Aleurocanthus woglumi*. *Bulletin OEPP/EPPO Bulletin* **32**, 261–265

PM 7/9 (1) *Cacoecimorpha pronubana*. *Bulletin OEPP/EPPO Bulletin* **32**, 267–275

PM 7/10 (1) *Cacyreus marshalli*. *Bulletin OEPP/EPPO Bulletin* **32**, 277–279

PM 7/11 (1) *Frankliniella occidentalis*. *Bulletin OEPP/EPPO Bulletin* **32**, 281–292

PM 7/12 (1) *Parasaissetia nigra*. *Bulletin OEPP/EPPO Bulletin* **32**, 293–298

PM 7/13 (1) *Trogoderma granarium*. *Bulletin OEPP/EPPO Bulletin* **32**, 299–310

PM 7/14 (1) *Ceratocystis fimbriata* f. sp. *platani*. *Bulletin OEPP/EPPO Bulletin* **33**, 249–256

PM 7/15 (1) *Ciborinia camelliae*. *Bulletin OEPP/EPPO Bulletin* **33**, 257–264

PM 7/16 (1) *Fusarium oxysporum* f. sp. *albedinis*. *Bulletin OEPP/EPPO Bulletin* **33**, 265–270

PM 7/17 (1) *Guignardia citricarpa*. *Bulletin OEPP/EPPO Bulletin* **33**, 271–280

PM 7/18 (1) *Monilinia fructicola*. *Bulletin OEPP/EPPO Bulletin* **33**, 281–288

PM 7/19 (1) *Helicoverpa armigera*. *Bulletin OEPP/EPPO Bulletin* **33**, 289–296

PM 7/20 (1) *Erwinia amylovora*. *Bulletin OEPP/EPPO Bulletin* **34**, 159–172

PM 7/21 (1) *Ralstonia solanacearum*. *Bulletin OEPP/EPPO Bulletin* **34**, 173–178

PM 7/22 (1) *Xanthomonas arboricola* pv. *corylina*. *Bulletin OEPP/EPPO Bulletin* **34**, 179–182

PM 7/23 (1) *Xanthomonas axonopodis* pv. *dieffenbachiae*. *Bulletin OEPP/EPPO Bulletin* **34**, 183–186

PM 7/24 (1) *Xylella fastidiosa*. *Bulletin OEPP/EPPO Bulletin* **34**, 187–192

PM 7/25 (1) *Glomerella acutata*. *Bulletin OEPP/EPPO Bulletin* **34**, 193–200

PM 7/26 (1) *Phytophthora cinnamomi*. *Bulletin OEPP/EPPO Bulletin* **34**, 201–208

PM 7/27 (1) *Puccinia horiana*. *Bulletin OEPP/EPPO Bulletin* **34**, 209–212

PM 7/28 (1) *Synchytrium endobioticum*. *Bulletin OEPP/EPPO Bulletin* **34**, 213–218

PM 7/29 (1) *Tilletia indica*. *Bulletin OEPP/EPPO Bulletin* **34**, 219–228

PM 7/30 (1) *Beet necrotic yellow vein benyvirus*. *Bulletin OEPP/EPPO Bulletin* **34**, 229–238

PM 7/31 (1) *Citrus tristeza closterovirus*. *Bulletin OEPP/EPPO Bulletin* **34**, 239–246

PM 7/32 (1) *Plum pox potyvirus*. *Bulletin OEPP/EPPO Bulletin* **34**, 247–256

PM 7/33 (1) *Potato spindle tuber pospiviroid*. *Bulletin OEPP/EPPO Bulletin* **34**, 257–270

PM 7/34 (1) *Tomato spotted wilt tospovirus*. *Bulletin OEPP/EPPO Bulletin* **34**, 271–280

PM 7/35 (1) *Bemisia tabaci*. *Bulletin OEPP/EPPO Bulletin* **34**, 281–288

PM 7/36 (1) *Diabrotica virgifera*. *Bulletin OEPP/EPPO Bulletin* **34**, 289–294

PM 7/37 (1) *Thaumetopoea pityocampa*. *Bulletin OEPP/EPPO Bulletin* **34**, 295–298

PM 7/38 (1) *Unaspis citri*. *Bulletin OEPP/EPPO Bulletin* **34**, 299–302

PM 7/39 (1) *Aphelenchoides besseyi*. *Bulletin OEPP/EPPO Bulletin* **34**, 303–308

PM 7/40 (1) *Globodera rostochiensis* and *Globodera pallida*. *Bulletin OEPP/EPPO Bulletin* **34**, 309–314

PM 7/41 (1) *Meloidogyne chitwoodi* and *Meloidogyne fallax*. *Bulletin OEPP/EPPO Bulletin* **34**, 315–320

Some of the Standards of the present set result from a different drafting and consultation procedure. They are the output of the DIAGPRO Project of the Commission of the European Union (no. SMT 4-CT98-2252). This project involved four 'contractor' diagnostic laboratories (in England, Netherlands, Scotland, Spain) and 50 'inter-comparison' laboratories in many European countries (within and outside the European Union), which were involved in ring-testing the draft protocols. The DIAGPRO project was set up in full knowledge of the parallel activity of the EPPO Working Party on Phytosanitary Regulations in drafting diagnostic protocols, and covered regulated pests which were for that reason not included in the EPPO programme. The DIAGPRO protocols have been approved by the Council of EPPO as EPPO Standards in series PM 7. They will in future be subject to review by EPPO procedures, on the same terms as other members of the series.

Diagnostics[1]
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

[1]The Figures in this Standard marked 'Web Fig.' are published on the EPPO website www.eppo.org.

Name: *Liriomyza huidobrensis* (Blanchard, 1926)[2]
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 guytona* (Freeman, 1958)
Taxonomic position: *Insecta*: *Diptera*: *Agromyzidae*
EPPO computer code: LIRISA
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: LIRITR
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.

[2]Note: it has recently been proposed that *L. huidobrensis* is in fact a complex of two cryptic species. This follows a study of specific sequences in mitochondrial and nuclear genomes (Scheffer, 2000; Scheffer & Lewis, 2001). The name *Liriomyza langei* has been applied to North American populations, and the name *L. huidobrensis* to Central and South American populations. All invasive populations were found to belong to *L. huidobrensis* as so defined. *L. langei* and *L. huidobrensis* could not be separated morphologically, but a PCR-RFLP protocol for separating them has been published (Scheffer *et al.*, 2001). This can only distinguish between these two taxa, and *L. bryoniae* would produce as false a result as *L. huidobrensis*. The authors do not comment on other species such as *L. strigata*. They also note that, potentially, the primers used would also amplify parasitoid DNA and therefore recommend restricting use of the protocol to adult material. For the purposes of this Standard, the name *L. huidobrensis* will continue to be applied to all the specimens originating from the trans-American populations that cannot currently be separated by morphological means.

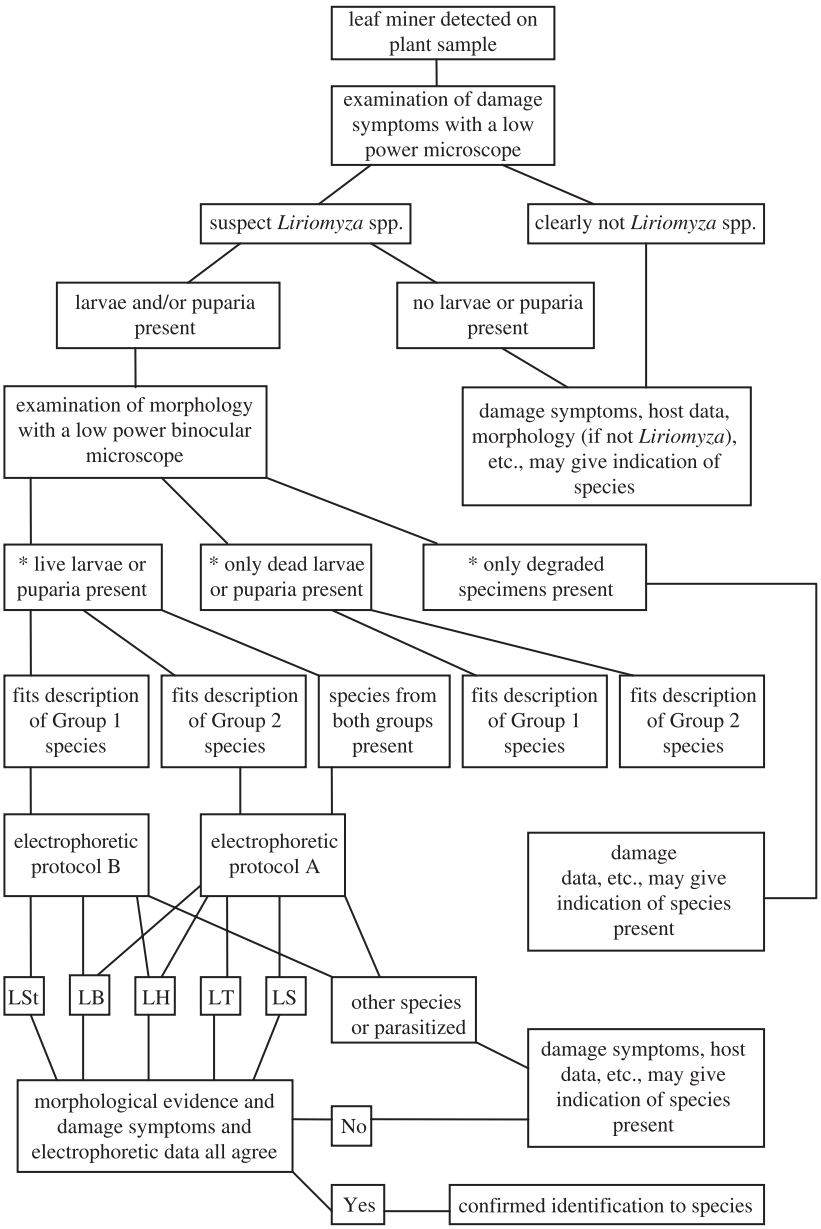


Fig. 1 Application of the protocol for larvae and puparia.

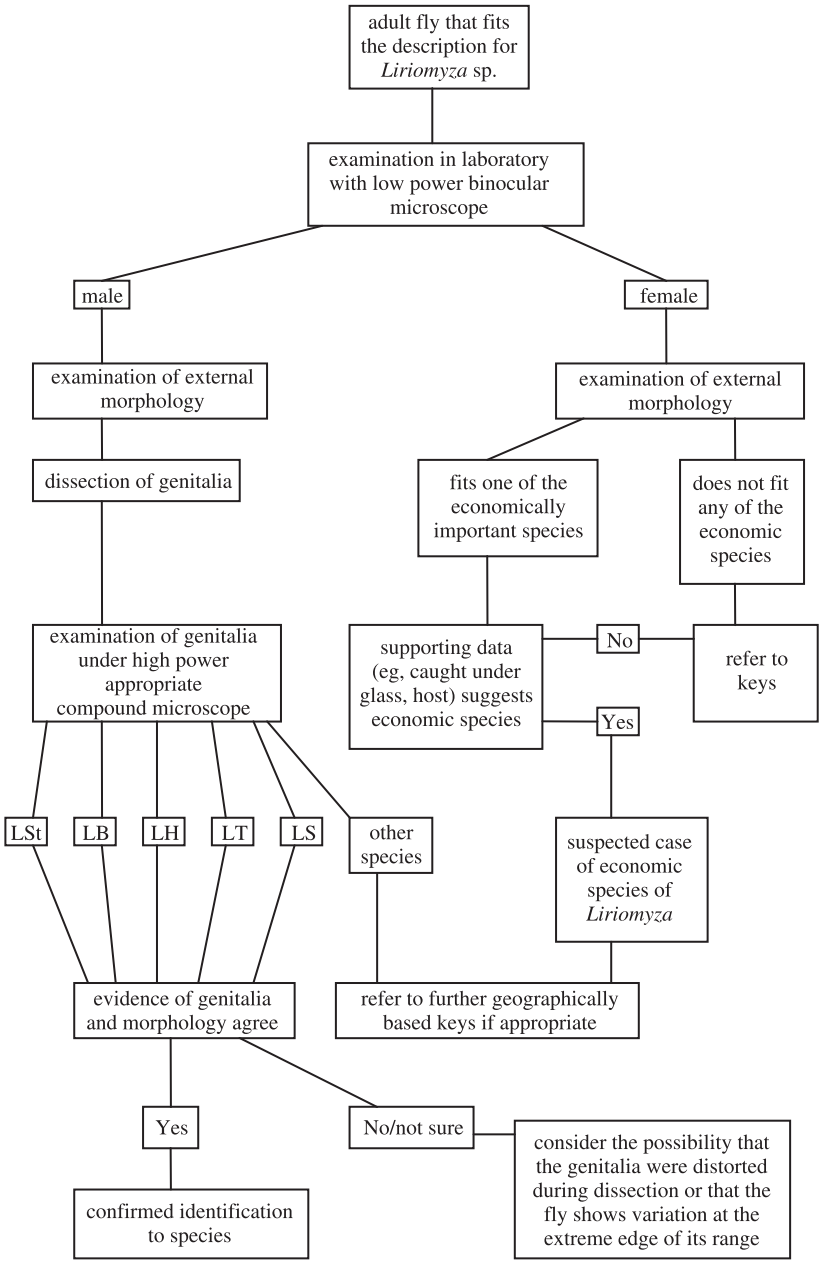
(LB = *L. bryoniae*; LH = *L. huidobrensis*; LT = *L. trifolii*; LS = *L. sativae*; LSt = *L. strigata*)
* PCR-RFLP analysis is an alternative method, which may be used regardless the condition of the specimen. It can also be used as a confirmation of morphological analysis of adults.

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



(LB = *L. bryoniae*; LH = *L. huidobrensis*; LT = *L. trifolii*; LS = *L. sativa*e; LSt = *L. strigata*)

Fig. 2 Application of the protocol for adult flies.

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

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	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	Frons bright yellow, orbits slightly paler	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	Black, matt	1.7–2.25 mm
L. sativae	Small, yellow	Frons and orbits bright yellow	Bright yellow	Black, shining	1.3–1.7 mm
L. strigata	Small, yellow	Frons and orbits yellow	Yellow with some brownish striations	Black, shining but slightly matt	1.8–2.1 mm
L. trifolii	Small, yellow	Frons and orbits yellow slight brownish striations	Yellow, occasional	Matt black with grey undertone	1.3–1.7 mm

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

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).

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. sativae*:** 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. trifolii*:** 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:
D. W. Collins, Central Science Laboratory, Sand Hutton, York YO41 1LZ (UK) E-mail: dom.collins@csl.gov.uk.

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. strigata*, and explicitly both eliminates *Chromatomyia horticola* and *C. syngenesiae* and provides warning against potentially misleading results caused by the presence of the endoparasitoid *Dacnusa sibirica* Telenga, 1934[3].

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 $MgCl_2$ (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 $MgCl_2$ (70 mg L^{-1}). Stain buffers: 1.21 g Tris, 100 mL distilled water, titrate to pH 8.0 with 1 M HCl.

[3]*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	mL
NADP (2 mg mL^{-1})	1.5	1.5	1.5	–	mL
O-Dianisidine (4 mg mL^{-1})	–	–	–	8.0	drops
$MgCl_2$ (20 mg mL^{-1})	5.0	5.0	2.0	2.0	drops
D-glucose-6-phosphate (20 mg mL^{-1})	12.0	–	–	–	drops
DL-isocitric acid (100 mg mL^{-1})	–	15.0	–	–	drops
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
L-amino acid 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.

Table 3 Key for separation of *Liriomyza* spp. by allozyme electrophoresis: *Protocol A*. The most common phenotype of *L. bryoniae* found on the gel is used as a standard: G6PDH 25, IDH 18, ME 31/38 (Fig. 5)

1	G6PDH band faster than *L. bryoniae* standard	*L. huidobrensis*
	G6PDH the same or slower than the *L. bryoniae* standard	2
2	IDH band faster than the *L. bryoniae* standard*	*L. sativae*
	IDH band same as or slower than the *L. bryoniae* standard	3
3	ME band slower than the *L. bryoniae* standard	*L. trifolii*
	ME band the same as the *L. bryoniae* standard (heterozygote) or only one of the *L. bryoniae* homozygote bands present	*L. bryoniae*

**L. bryoniae* also has one rare allele, which is faster than the standard. This is still marginally slower than the *L. sativae* band.

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 L-amino acid 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 dehyrogenase (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 distiphallic 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*
	G6PDH band the same or slightly slower than the *L. bryoniae* standard	2
2	PEP-1 band present (band within 15 mm of origin; occasionally travels towards cathode)	3
	PEP-1 band displaced, absent or heavy streaking associated with it	4
3	PEP-1 band the same or slower than the *L. bryoniae* standard	*L. bryoniae*
	PEP-1 band faster than the *L. bryoniae* standard (between 10 and 15 mm)	*L. strigata*
4	PEP-1 band displaced to become a poorly resolved band located between 20 and 30 mm	*L. trifolii*; *L. sativae*, *C. syngenesiae*, *C. horticola*
	PEP-1 band absent or heavy streaking associated	Parasitism by *D. sibirica*

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

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 mounting medium (or a similar semiviscous mounting fluid such as Berlese solution or Hoyer's solution) on a cavity slide. Under a binocular stereoscopic microscope and using fine mounted needles, carefully dissect out the genital complex from the cuticle and the immediate, surrounding membranes (see Web Fig. 18 [Plate 1]). Using fine mounted needles, position the genital complex for lateral viewing under a compound light microscope (recommended at 400 × magnification). 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 raction (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′-GTT(A/T)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 µM each primer, 200 µM dNTPs (Promega), 1 Unit HotStarTaq DNA polymerase (Qiagen), 5 µL 10 × reaction buffer [with 15 mM $MgCl_2$] , 1 µL DNA. The PCR is performed in a 96-well thermocycler

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*
*Dde*I	790	790	567	790	790	619
			223			171
*Hinf*I	421	421	421	421	421	421
	369	369	283	310	342	310
			27	59	27	59
			59			
*Ssp*I	392	399	399	717	399	391
	326	391	391	73	391	326
	72					73
*Taq*I	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)[a]
		30	30	30	67	21
		21				

[a]*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.

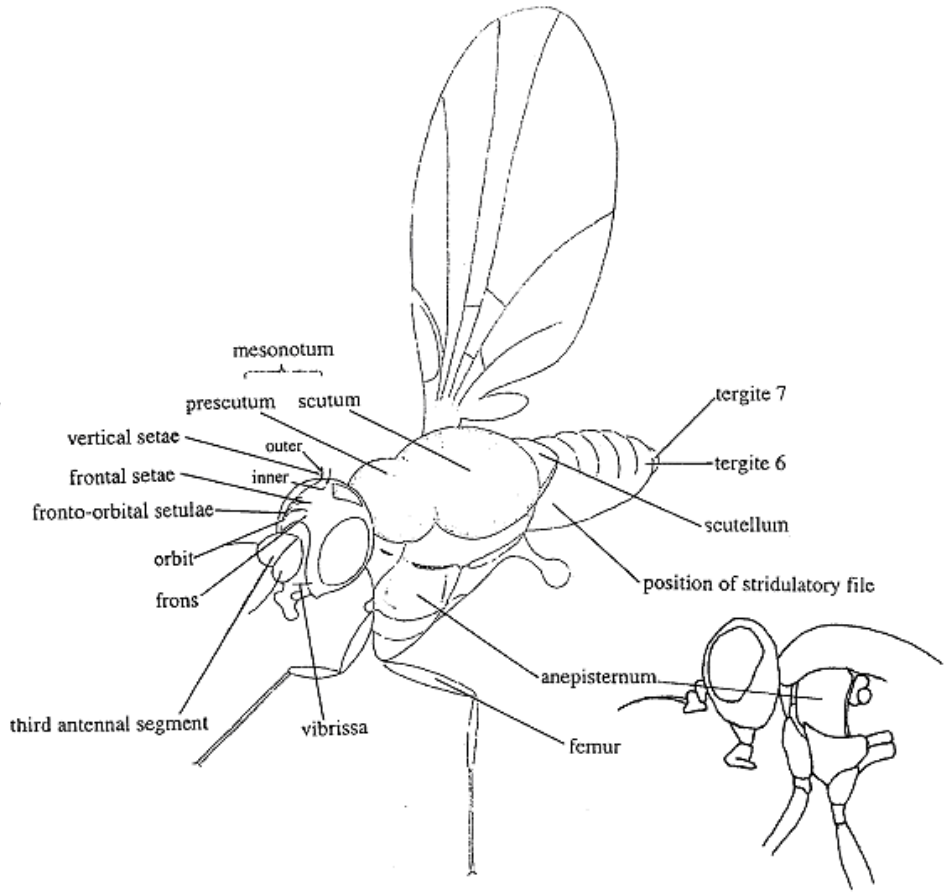
RFLP analysis

5 µL 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. 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.

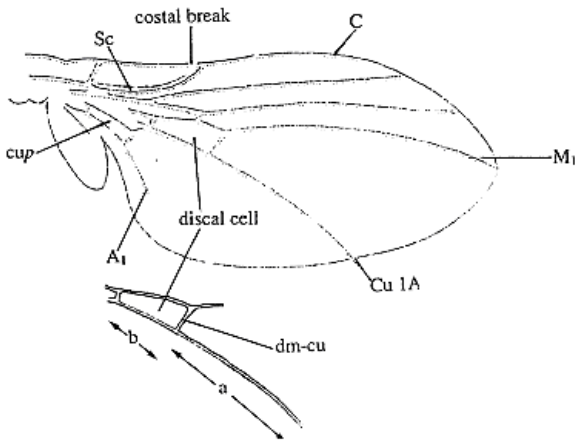
Interpretation of band patterns

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

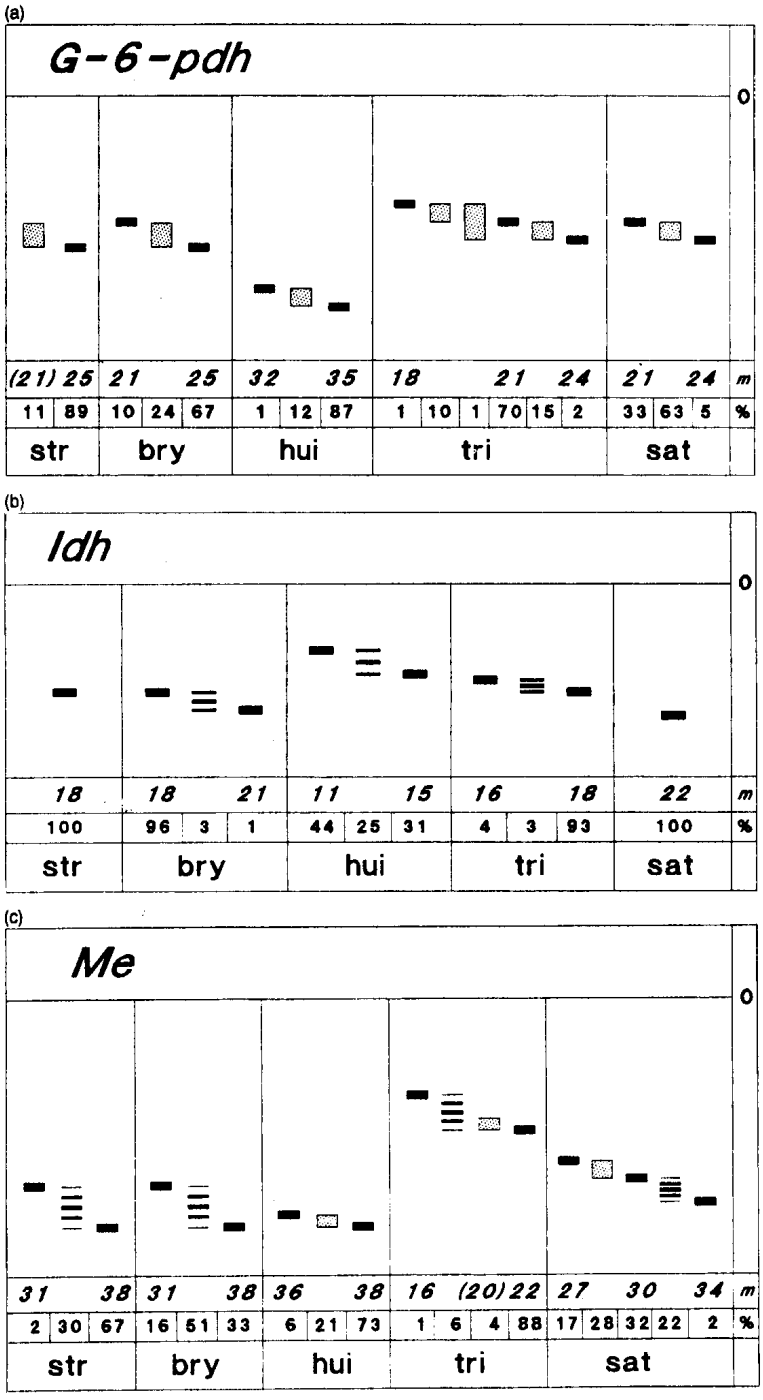
Web Fig. 3 Generalized diagrams of an adult male *Liriomyza* illustrating the morphological characters mentioned in this protocol



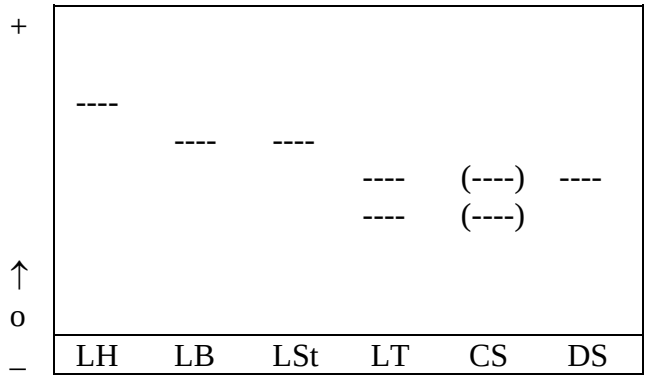
Web Fig. 4 *Liriomyza*, wing venation



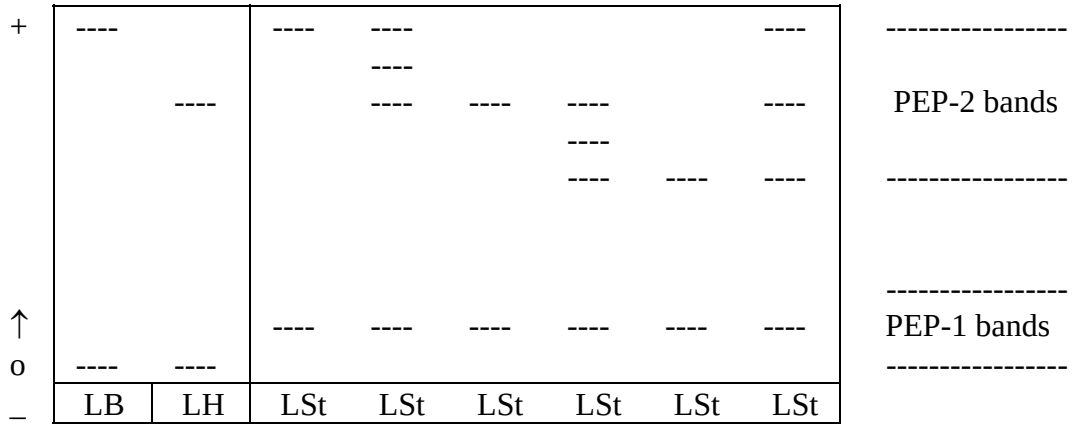
Web Fig. 5 Appendix I, Protocol A. Electrophoretic band patterns: (a) G6PDH; (b) IDH; (c) (m= migration distance of homozygotes in mm; % = percentage occurrence of genotypes in all samples together. If alleles are only found in heterozygotes, the migration distance is given I parenthesis. (str = *L. strigata;* bry = *L. bryoniae;* hui = *L. huidobrensis;* tri = *L. trifolii;* sat = *L. sativae*). Figure reproduced by kind permission of the Plant Protection Service of The Netherlands.



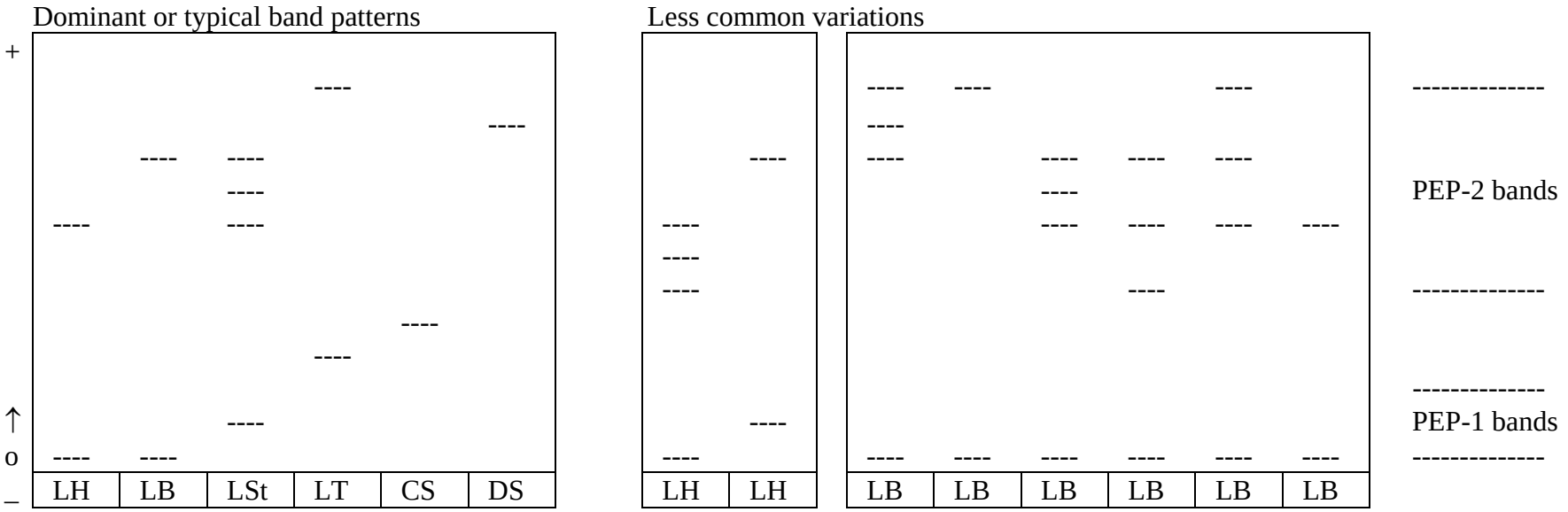
Web Fig. 6 Appendix I, Protocol B. G6PDH band patterns. LH = *Liriomyza huidobrensis*; LB = *L. bryoniae*; LSt = *L. strigata*; LT = *L. trifolii*; CS = *Chromatomyia syngenesiae*; DS = *Dacnusa sibirica*.



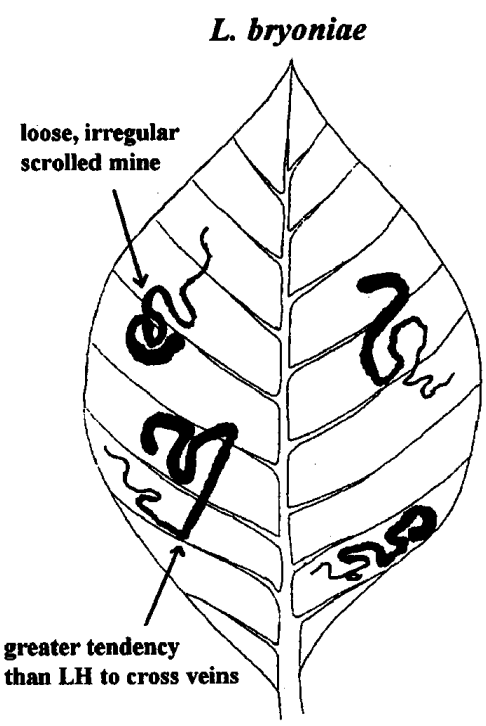
Web Fig. 7 Appendix I, Protocol B. PEP phenotypic variation for *L. strigata*. LH = *Liriomyza huidobrensis*; LB = *L. bryoniae*; LSt = *Liriomyza strigata*. nb: 1st instar larvae may not produce PEP-2 bands.



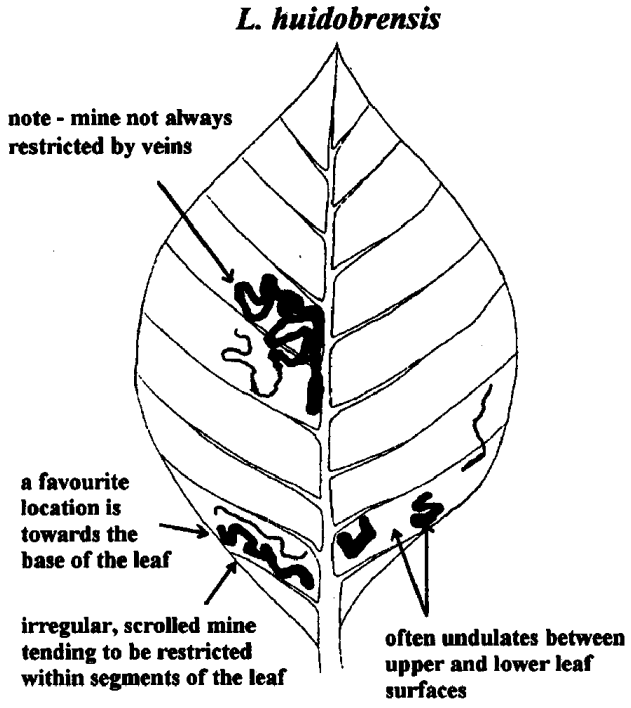
Web Fig. 8 Appendix I, Protocol B. PEP phenotypic variation for *Liriomyza huidobrensis* and *L bryoniae*. LH = *L. huidobrensis*; LB = *L. bryoniae*; LSt = *L. strigata*; LT = *L. trifolii*; CS = *Chromatomyia syngenesiae*: DS = *Dacnusa sibirica*. nb: 1st instar larvae may not produce PEP-2 bands.



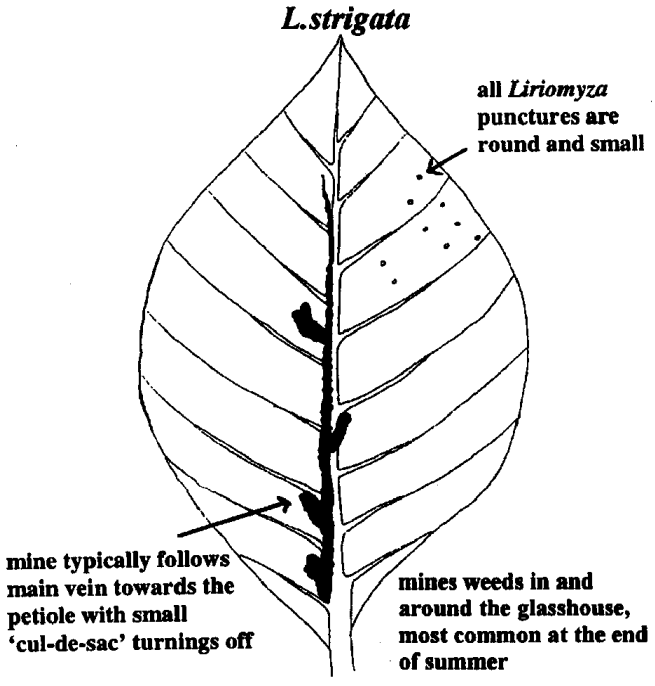
Web Fig. 9 Typical characteristics of mines from Group 1 species: (a) *L. bryoniae;* (b) *L. huidobrensis;* (c) *L. strigata.*



(a)

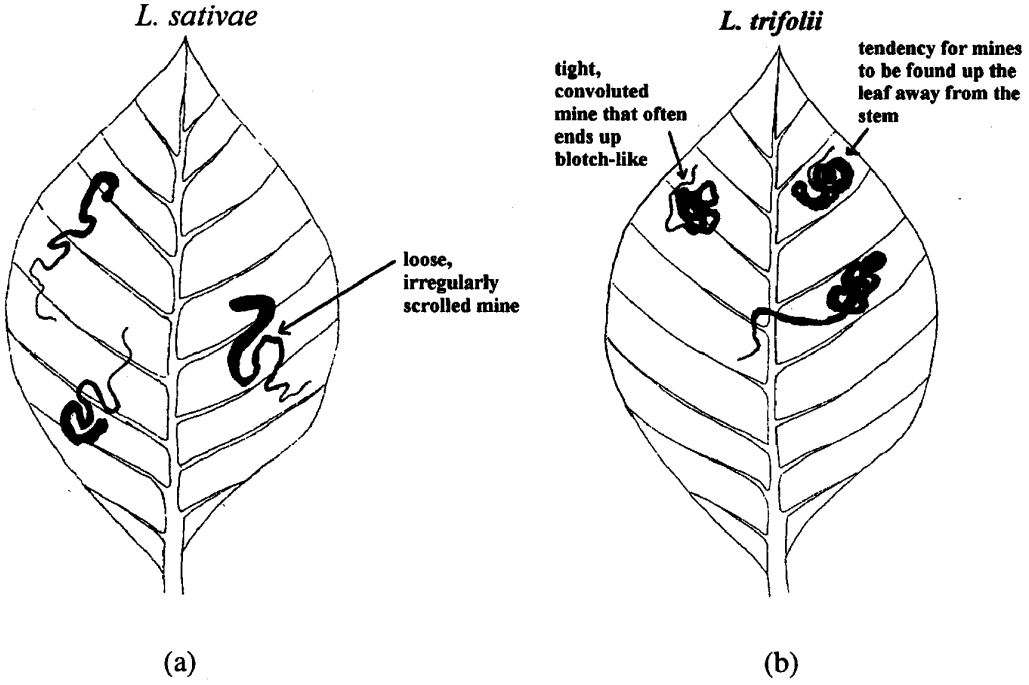


(b)

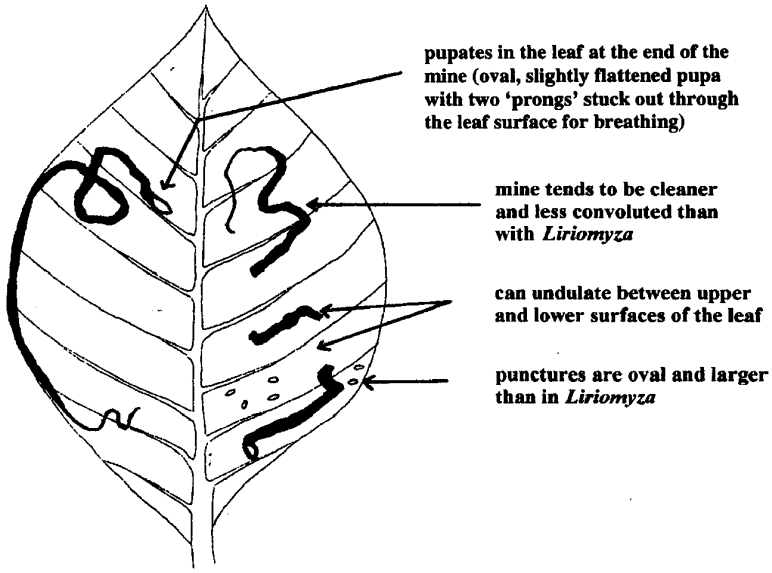


(c)

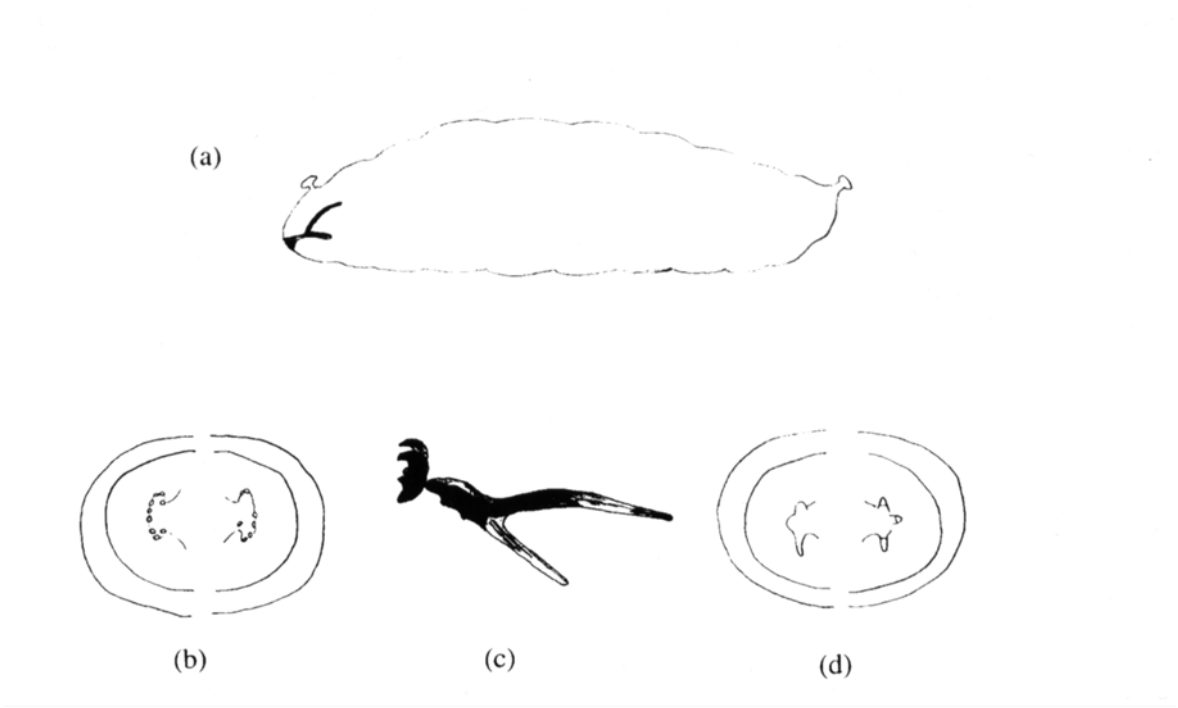
Web Fig. 10 Typical characteristics of mines from Group 2 species: (a) *L. sativae;* (b) *L. trifolii.*



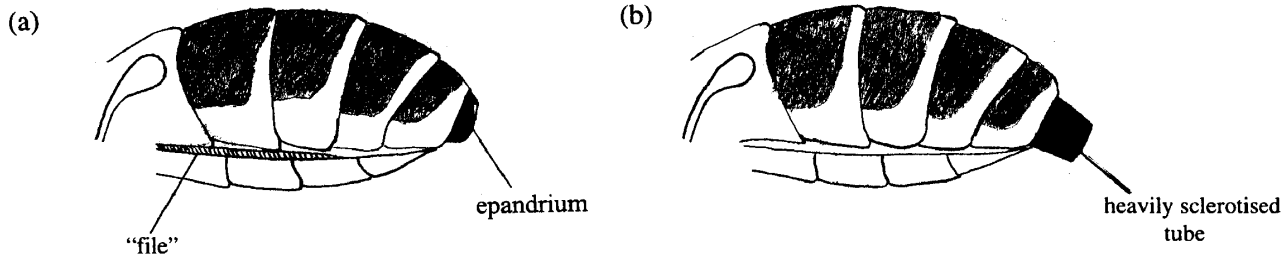
Web Fig. 11 Typical characteristics of mines of *Chromatomyia syngenesiae* (nb: the mines and punctures of *C. horticola* can appear intermediate between those of *C. syngenesiae* and *L. huidobrensis.*



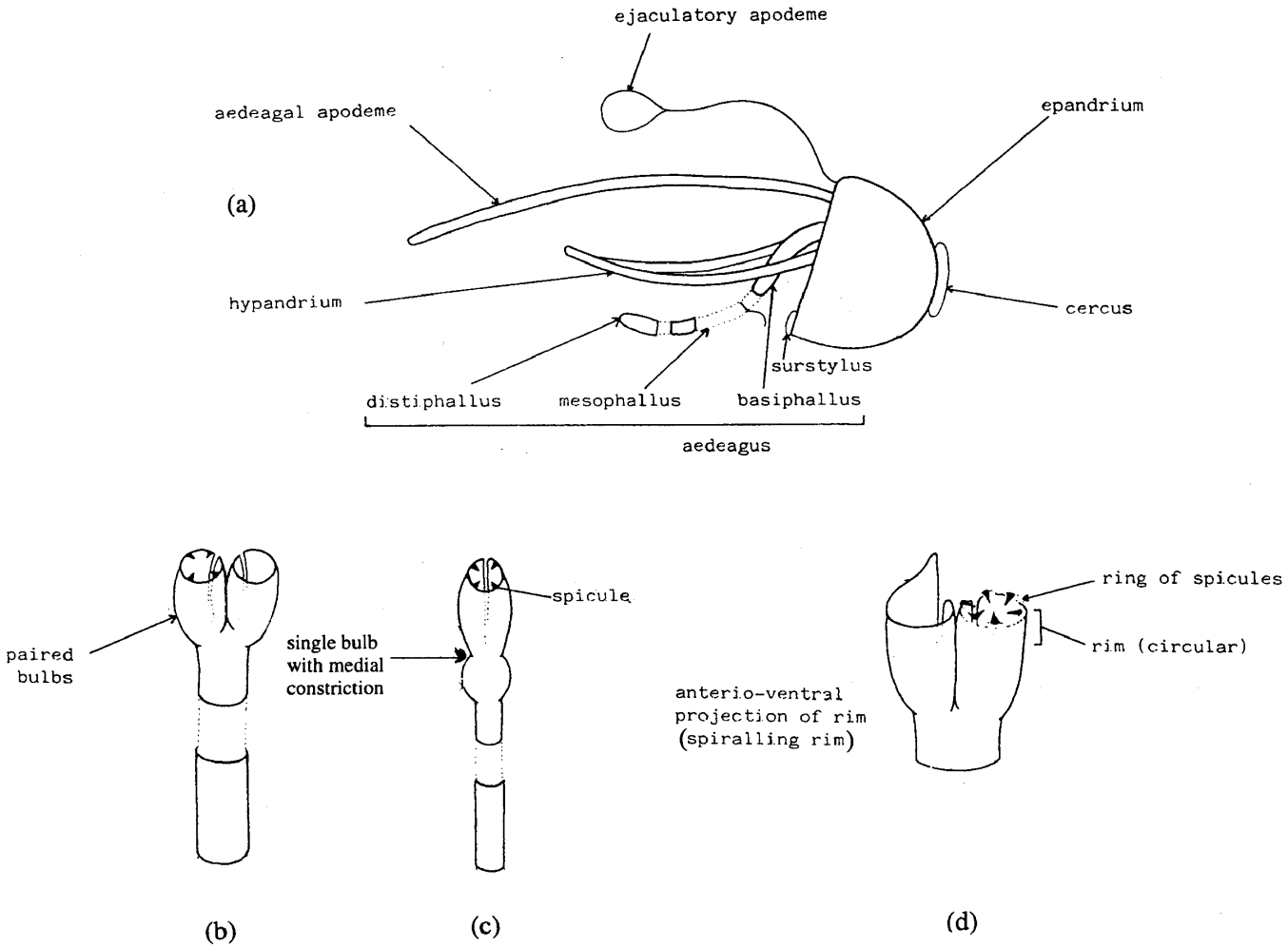
Web Fig. 12 *Liriomyza* immature stages: (a) larva, gross form; (b) posterior spiracles. Group 1 (larvae – left; pupa – right); (c) cephalopharyngeal skeleton; (d) posterior spiracles, Group 2 (larva – left; pupa – right)



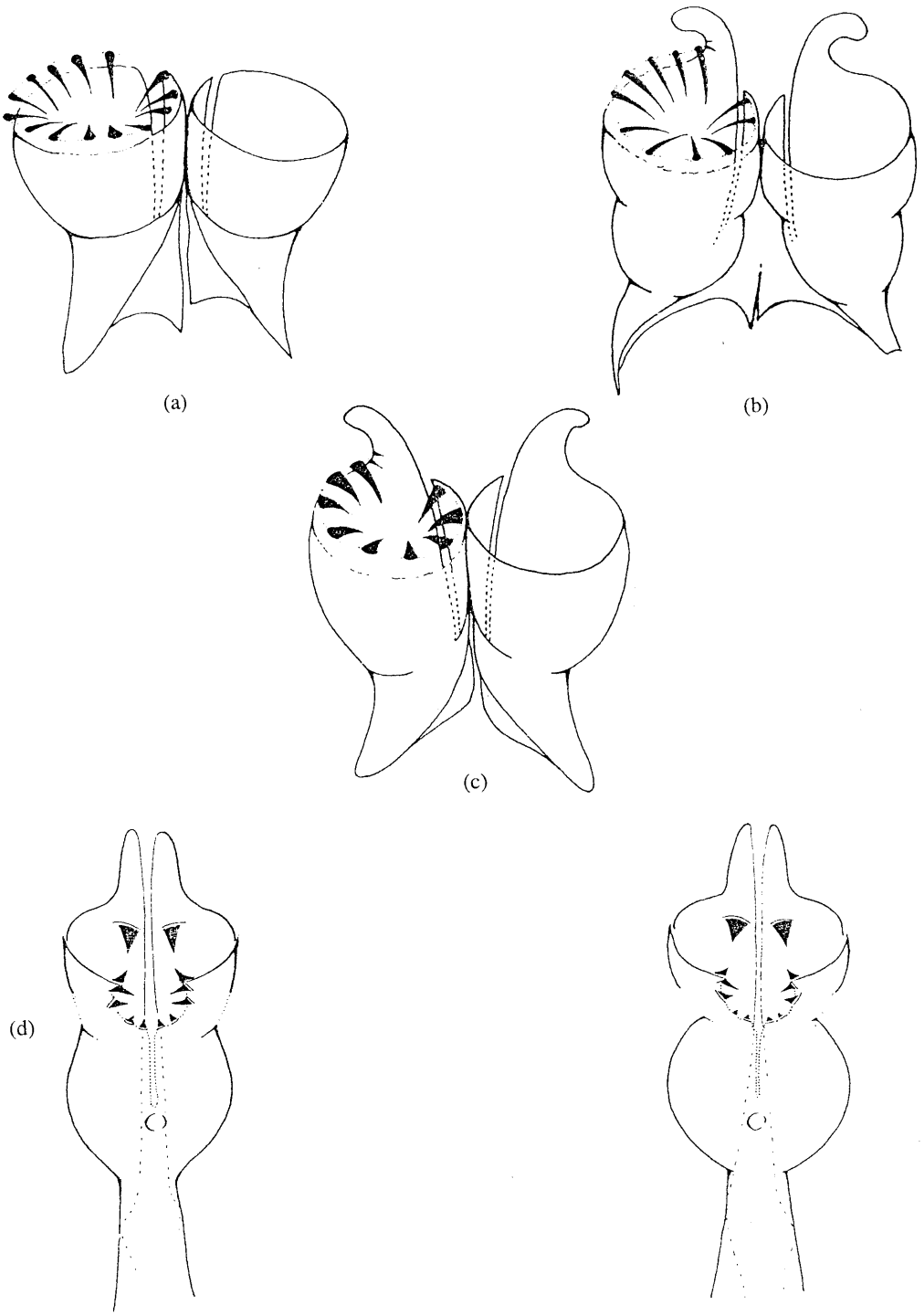
Web Fig. 13 *Liriomyza abdomen: (a) male; (b) female*



Web Fig. 14 Generalized diagrams of the male genitalia of *Liriomyza:* (a) genital complex; (b) distiphallus, Group 1; (c) distiphallus, Group 2; (d) distiphallic parts

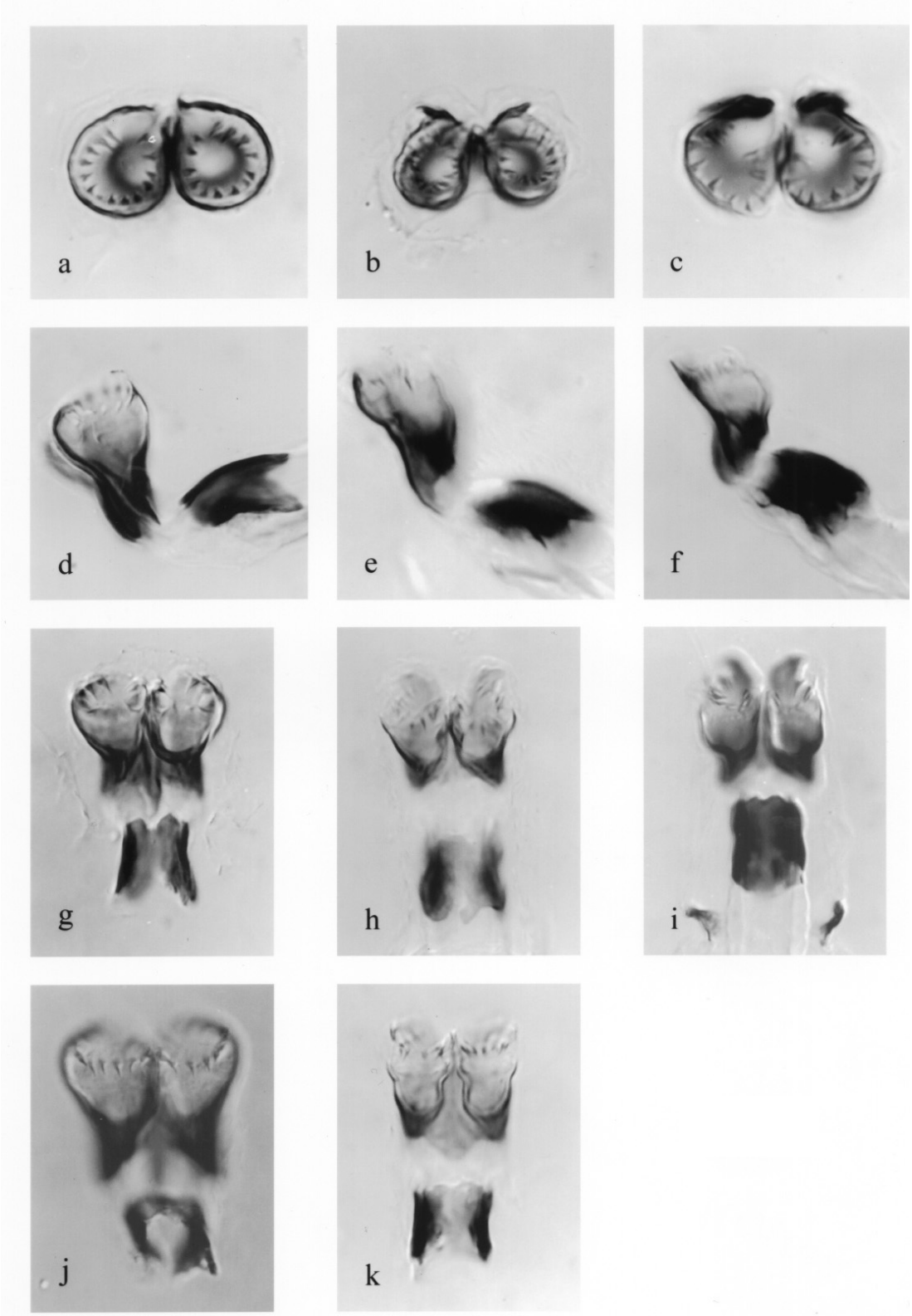


Web Fig. 15 Generalized diagrams of the distiphallus of each species, dorsal view (spicules not shown on right side, a-c); (a) *L. bryoniae;* (b) *L. huidobrensis;* (c) *L. strigata;* (d) *L. sativae;* (e) *L. trifolii*



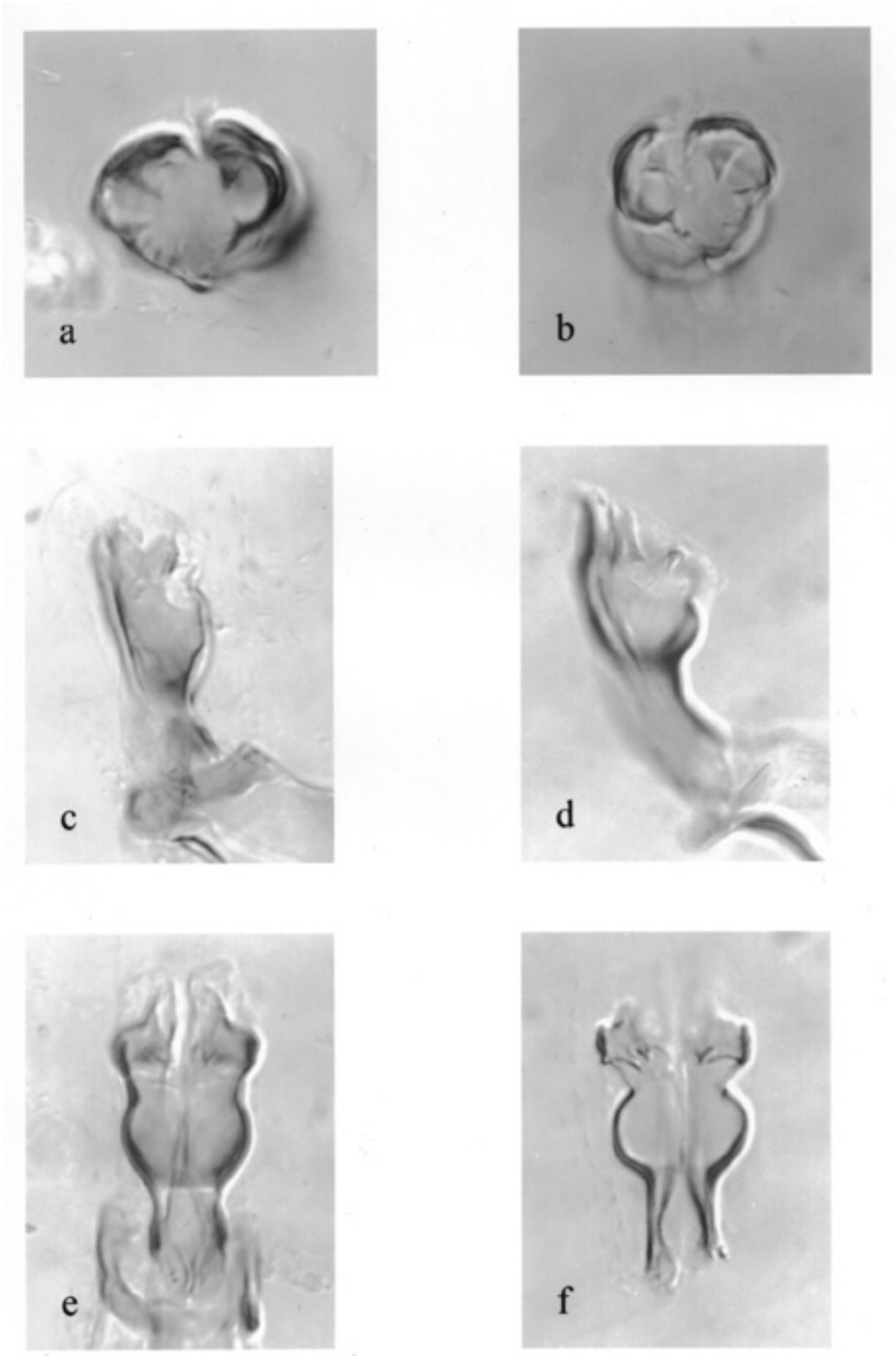
Web Fig. 16 Distiphalli at × 400 microscope magnification

(a) *L. bryoniae*, anterior
(b) *L. huidobrensis*, anterior
(c) *L. strigata*, anterior
(d) *L. bryoniae*, lateral
(e) *L. huidobrensis*, lateral
(f) *L. strigata*, lateral
(g) *L. bryoniae*, dorso-ventral
(h) *L. huidobrensis*, dorso-ventral
(i) *L. strigata*, dorso-ventral
(j) *L. bryoniae*, dorso-ventral (different plane to (g))
(k) *L. huidobrensis*, dorso-ventral (different plane to (h))



Web Fig. 17 Distiphalli at × 400 microscope magnification:

(a) *L.sativae*, anterior
(b) *L. trifolii*, anterior
(c) *L. sativae*, lateral
(d) *L. trifolii*, lateral
(e) *L. sativae*, dorso-ventral
(f) *L. trifolii*, dorso-ventral



Web Fig. 18 Genital complex *(L. huidobrensis)*, lateral