

PROTOCOL FOR THE DIAGNOSIS OF QUARANTINE ORGANISMS

Liriomyza spp. (*L. bryoniae*, *L. huidobrensis*, *L. sativae*, *L. trifolii*)

IDENTITY

Name: *Liriomyza bryoniae* (Kaltenbach, 1858)
Synonyms: *Agromyza bryoniae* Kaltenbach, 1858; *Liriomyza solani* Hering, 1927; *Liriomyza citrulli* Rohdendorf, 1950
Common names: tomato leaf miner (English), Tomatenminierfliege (German)
Taxonomic position: Insecta: Diptera: Agromyzidae
EU Annex designation: I/A2

Name: *Liriomyza huidobrensis* (Blanchard, 1926)
Synonyms: *Agromyza huidobrensis* Blanchard, 1926; *Liriomyza cucumifoliae* Blanchard, 1938; *Liriomyza langei* Frick, 1951; *Liriomyza dianthi* Frick, 1958
Common names: South American leaf miner (English), pea leaf miner (USA)
Taxonomic position: Insecta: Diptera: Agromyzidae
Quarantine status: EPPO A2 list: No. 152
EU Annex designation: I/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
Common names: vegetable leaf miner (English)
Taxonomic position: Insecta: Diptera: Agromyzidae
Quarantine status: EPPO A1 list: No. 152
EU Annex designation: I/A1

Name: *Liriomyza trifolii* (Burgess, 1880)
Synonyms: *Liriomyza alliovora* Frick, 1955
Common names: American serpentine leaf miner (English), Mineuse du gerbera (French), Floridaminierfliege (German)
Taxonomic position: Insecta: Diptera: Agromyzidae
Quarantine status: EPPO A2 list: No. 131
EU Annex designation: I/A2

DIAGNOSTIC PROTOCOLS FOR ORGANISMS HARMFUL TO PLANTS

1.2: *Liriomyza* spp. (*L. bryoniae*, *L. huidobrensis*, *L. sativae*, *L. trifolii*)

Introduction

There are 376 species currently recognized in the genus *Liriomyza* (David Henshaw, personal communication, 2000), with 136 of these species found naturally in Europe (Seymour, 1994). The adult flies of all these species look very similar. They are all small, being between 1 and 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 the variation of these characters often overlap, limiting their diagnostic value.

Four species, *L. bryoniae* (Kaltenbach, 1858), *L. huidobrensis* (Blanchard, 1926), *L. sativae* (Blanchard, 1938) and *L. trifolii* (Burgess, 1880) are listed in the European Community Plant Health Directive (2000/29/EC). *L. bryoniae* is indigenous to Europe, the other three are all species that originated in the New World. All are polyphagous pests of ornamental and vegetable crops.

To identify these species the diagnostician must not only be able to distinguish between these four species but also able to distinguish any one of these species from the background fauna of indigenous *Liriomyza* species (mostly non-pest) against which it is found. This composition of this background fauna will change in different geographic locations throughout Europe and no one morphological dichotomous key has been produced that will separate each of these 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 (Figure 1), or adult flies (Figure 2). 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.

It has recently been proposed that *Liriomyza huidobrensis* is in fact a complex of two cryptic species. This follows a study of specific sequences in the mitochondrial and nuclear genomes of the leaf miner (Scheffer, 2000; Scheffer & Lewis, 2001). The name *Liriomyza langei* has been applied to North American populations, and the name *Liriomyza huidobrensis* applied to Central and South American populations. All invasive populations investigated by these authors were found to belong to *Liriomyza huidobrensis* as so defined. *Liriomyza langei* and *L. huidobrensis* cannot be separated morphologically, but a PCR-RFLP protocol for separating them has been published (Scheffer *et al.*, 2001). However, it should be noted that this protocol can only distinguish between these two taxa. *Liriomyza bryoniae* would produce a false 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 protocol, the name *Liriomyza huidobrensis* will continue to be applied to all the specimens originating from the trans-American populations that cannot currently be separated by morphological means.

Recognition of family and genus

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

Family: Agromyzidae

Agromyzids are small flies whose larvae are leaf miners, stem borers or gall-makers.

Formal description (of the adult)

The following combination of characters, 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.

(see Figure 3. for location of these characters on an adult fly)

Practical diagnosis (based on the larval stages)

In practice, agromyzids are recognisable because the larvae of all the species in the family feed in the living tissue of plants, with approximately three-quarters of all species being leaf miners. However, note that 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 sclerotised mouthparts, the mandibles with its longitudinal axis at oblique or right angles to the rest of the cephalopharyngeal skeleton and usually bearing two or more pairs of equally sized teeth, directed anteriorly, the ventral cornua (the posteriorly directed “arms”) commonly shorter than the dorsal ones. For a summary of information on the morphology and biology of the immature stages of agromyzids, with a large bibliography and illustrations of the cephalopharyngeal skeleton and posterior spiracles for a number of species, see Ferrar, 1987.

Genus: *Liriomyza*

Formal description (of the adult)

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

Practical diagnosis

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

Natural species groups

The *Liriomyza* species with which we are concerned here separate into two distinct natural species groups. The member species of each group share a similar structure to the male genitalia, indicating their common ancestry. In addition their larvae have a similar appearance with respect to both colour and the structure of the posterior spiracles. However, those external characters of the adult flies useful for identification (e.g., see 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

Damage symptom recognition

Feeding punctures and leaf mines are usually the first and most obvious sign of the presence of *Liriomyza*. 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 must be treated with caution.

Feeding punctures

Feeding punctures of *Liriomyza* species 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 discolouration 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 (see also Figures 9-10) of these species are:

1. a tightly coiled, almost blotch-like mine *L. trifolii*
2. a more loosely, irregular serpentine mine *L. bryoniae* and *L. sativae*
3. 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*
4. 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 semi-circular 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 (see also Figure 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 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 will reach 3.0 mm when full-grown. The larvae are typical of agromyzids in their gross form (see section on Agromyzidae, practical diagnosis above and Figure

12a). Pupae are oval, about 2.0 mm in length, very slightly flattened ventrally, with projecting anterior and posterior spiracles. The larvae and pupae of each species look similar to those of other species in their natural group. In practice, the different natural groups can be distinguished from each other morphologically but not the species within these groups. Species determination requires electrophoretic analysis (see Appendix A).

Group 1 (L. bryoniae, L. huidobrensis, L. strigata):

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 (figure 12b). It can be difficult to make out the number of pores, which according to Spencer, 1973, are:

<i>L. bryoniae</i>	7-12 pores
<i>L. huidobrensis</i>	about 6-9 pores
<i>L. strigata</i>	10-12 pores

Puparia are variable in colouration, 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. The form of the larval spiracles is retained in the puparium although the pores are less clearly discernible.

Group 2 (L. sativae, L. trifolii):

Larvae are translucent when newly-emerged, yellow-orange after that. Each posterior spiracle is tricorn shaped with three pores, each on a distinct projection, the outer two elongate (Figure 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 tabulated for the five species discussed in this protocol 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. Spencer produced many of the key works describing agromyzids worldwide and these are listed at the end of this protocol in the additional bibliography section.

Identification based on distiphallus structure

The distiphallus is the terminal part of the aedeagus (the intromittent organ, part of the male genitalia) (Figure 14a,d; 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 (Seymour & Collins, *in prep.*); that is 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 *L. bryoniae*, *L. huidobrensis* and *L. strigata* there are two distal bulbs side by side (Figure 14b), while in *L. sativae* and *L. trifolii* there is only one distal bulb with a medial constriction dividing distinct lower and upper sections (Figure 14c).

Separation of the five species using the distiphallus is described in Appendix B. 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.

Recording an identification

Any identification of an insect is always an exercise in probability statistics, and the opinion of the identifier will reflect his or her expertise or experience. Individuals within a species can vary from the holotype, and from one another. For any given character, the range of variation in different species may overlap wholly or in part so that for at least some individuals that character is of no value in distinguishing between those species. It is therefore important that the identifier retain specimens so that the evidence can be re-examined at a later date (a permanent record of the electrophoretic result should similarly be kept, either the plate itself or a photograph). The report on the identification should also note the number of specimens examined, the origin of the insects, the host and also include a brief justification of the level to which the identification has been taken (genus, species, etc.).

Acknowledgement

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.

References

- Collins D. W. (1996) The separation of *Liriomyza huidobrensis* (Diptera: Agromyzidae) from related indigenous and non-indigenous species encountered in the United Kingdom using cellulose acetate electrophoresis. *Annals of Applied Biology* 128: 387-398.
- Ferrar P. A. (1987) A guide to the breeding habits and immature stages of Diptera Cyclorrhapha. Part 1: text, 478pp. Part 2: figures, 479-907. *Entomograph* Vol. 8.
- Hebert P. D. N. & Beaton M. J. (1989) Methodologies for allozyme analysis using cellulose acetate electrophoresis. Helena Laboratories, Beaumont, Texas. 32 pp.
- Hennig W. (1958) Die Familien der Diptera Schizophora und ihre phylogenetischen Verwandtschaftsbeziehungen. *Beiträge zur Entomologie* 8: 505-688.
- McAlpine J. F., Peterson B. V., Shewell G. E., Teskey H. J., Vockeroth J. R. & Wood D. M. (1981) Manual of Nearctic Diptera. Volume 1. Research Branch Agriculture Canada, Monograph No. 27. 1-674.
- Menken S. B. J. & Ulenberg S. A. (1983) Diagnosis of the agromyzids *Liriomyza bryoniae* and *L. trifolii* by means of starch gel electrophoresis. *Entomologica experimentalis et Applicata* 34: 205-208.
- Menken S. B. J. & Ulenberg S. A. (1986) Allozymatic diagnosis of four economically important *Liriomyza* species (Diptera, Agromyzidae). *Annals of Applied Biology* 109: 41-47.
- Oudman L. (1992) Identification of economically important *Liriomyza* species (Diptera: Agromyzidae) and their parasitoids using enzyme electrophoresis. *Proceedings of the section Experimental and Applied Entomology of the Netherlands Entomological Society* 3: 135-139.
- Oudman L., Aukema B., Menken S. B. J. & Ulenberg S. A. (1995) A procedure for identification of polyphagous *Liriomyza* species using enzyme electrophoresis. *Bulletin OEPP/EPPO Bulletin* 25: 349-355.
- Scheffer S. J. (2000) Molecular evidence of cryptic species within the *Liriomyza huidobrensis*

- (Diptera: Agromyzidae). *Journal of Economic Entomology* 93 (4): 1146-1151.
- Scheffer S. J. & Lewis M. L. (2001) Two nuclear genes confirm mitochondrial evidence of cryptic species within *Liriomyza huidobrensis* (Diptera: Agromyzidae). *Annals of the Entomological Society of America* 94 (5): 648-653.
- Scheffer S. J., Wijesekara A., Visser D. & Hallett R. H. (2001) Polymerase chain reaction-restriction length polymorphism method to distinguish *Liriomyza huidobrensis* from *L. langei* (Diptera: Agromyzidae) applied to three recent leafminer invasions. *Journal of Economic Entomology* 94 (5): 1177-1182.
- Seymour P. R. (1994) Taxonomy and morphological identification. In EU Contract No. 90/399005 Final Report - Evaluation and development of rapid detection and identification procedures for *Liriomyza* species: taxonomic differentiation of polyphagous *Liriomyza* species of economic importance (Aukema B., Oudman L., Menken S. B. J., Ulenberg S. A., Seymour P. R. and Martinez M.).
- Seymour P. R. & Collins D.W. (in prep.) Identification of five economically important *Liriomyza* species (Diptera: Agromyzidae) based on the structure of the distiphallus.
- Spencer K. A. (1972) Diptera: Agromyzidae. Handbooks for the Identification of British Insects 10 (5). Royal Entomological Society of London. 136 pp.
- Spencer K. A., (1973) Agromyzidae (Diptera) of economic importance. *Series Entomologica Vol. 9*. The Hague: Dr W Junk BV. 418 pp.
- Spencer K. A., (1976) The Agromyzidae (Diptera) of Fennoscandia and Denmark. *Fauna Entomologica Scandinavica* 5, Part 1: 1-304; Part 2: 305-606.
- Spencer K. A. (1987) Agromyzidae. In (McAlpine J. F., ed.) Manual of Nearctic Diptera. Volume 2. Research Branch Agriculture Canada, Monograph No. 28. 675-1332.
- Spencer K. A. (1990) Host specialization in the world Agromyzidae (Diptera). *Series Entomologica Vol. 45*. Kluwer Academic Publishers, Dordrecht. 444 pp.

Additional bibliography

Afrotropical

- Spencer K. A. (1959) A synopsis of the Ethiopian Agromyzidae (Diptera). *Trans. R. ent. Soc. Lond.* 111: 237-329.
- Spencer K. A. (1985) East African Agromyzidae (Diptera): further descriptions, revisionary notes and new records. *Journal of Natural History* 19: 969-1027.

Australian

- Spencer K. A. (1977) A revision of the Australian Agromyzidae (Diptera). *The Western Australian Museum Perth, Special Publication* 8: 1-255.

Nearctic

- Spencer K. A. (1969) The Agromyzidae of Canada and Alaska. *Mem. ent. Soc. Can.* 64: 1-311.
- Spencer K. A. & Stegmaier C. E. (1973) Agromyzidae of Florida with a supplement on species from the Caribbean. *Arthropods of Florida and neighbouring land areas* 7: 1-204. Florida Department of Agriculture, Gainesville.
- Spencer K. A. & Steyskal G. C. (1986) Manual of the Agromyzidae (Diptera) of the United States. United States Department of Agriculture Handbook No. 638. 478 pp.

Neotropical

- Spencer K. A. (1963) A synopsis of the Neotropical Agromyzidae (Diptera). *Trans. R. ent. Soc. Lond.* 115: 291-389.
- (see also Spencer & Stegmaier, 1973, above).

Oriental

- Singh S. & Ipe I. M. (1973) The Agromyzidae from India. *Agra, St. John's College (Memoirs of the School of Entomology, No. 1)*
- Spencer K. A. (1961) A synopsis of the Oriental Agromyzidae (Diptera). *Trans. R. ent. Soc. Lond.* 113: 55-100.

Palaearctic

- Hendel F. (1931-36) Agromyzidae. *Fliegen palaearkt. Reg.* 6 (59): 1-570.

(see also Spencer, 1972, 1976, above).

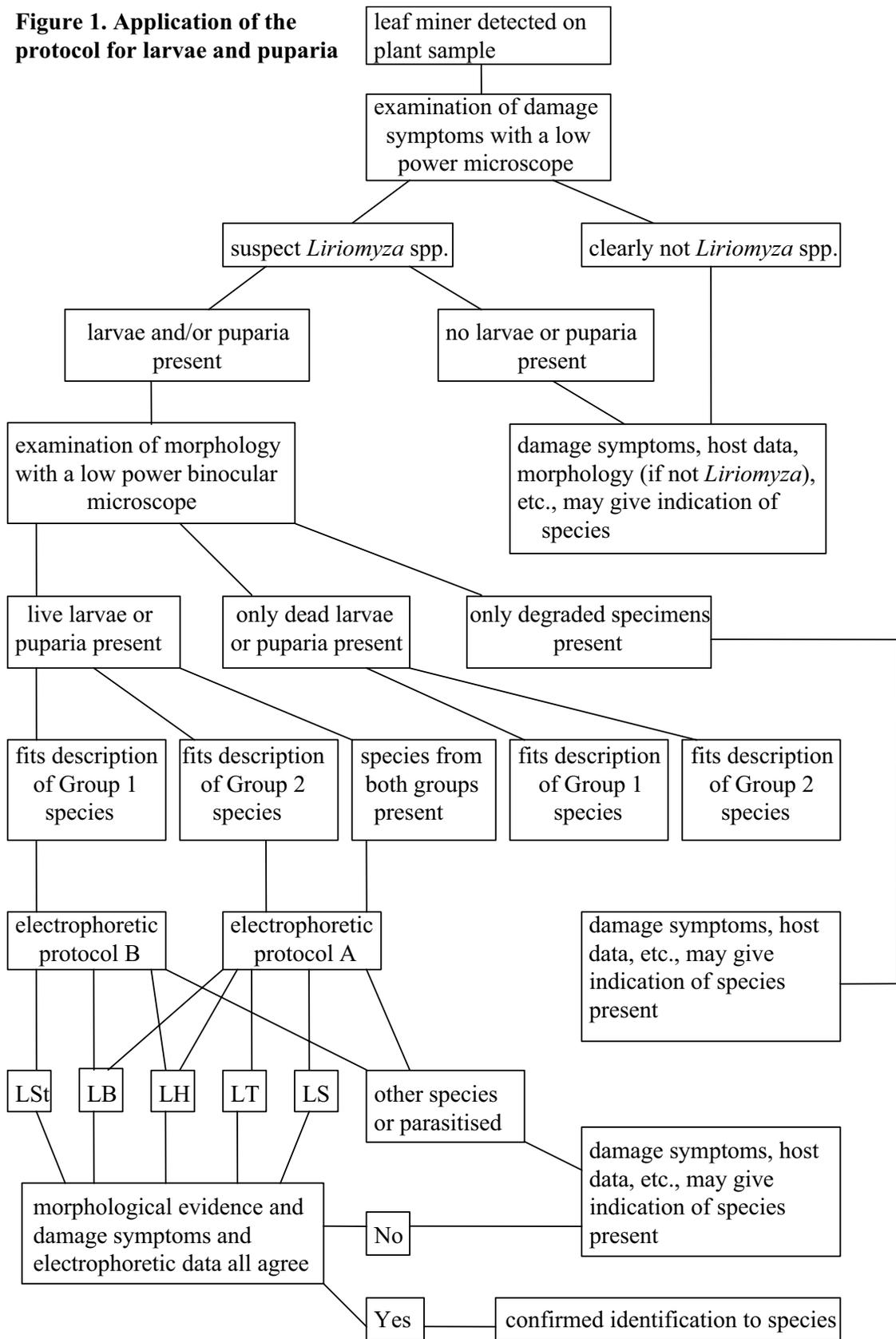
Polynesian

Spencer K. A. (1963) Diptera Agromyzidae. *Insects Micronesia* 15: 135-162.

Protocol drafted by Dominique W. Collins,
Central Science Laboratory, Sand Hutton, York YO41 1LZ, UK.

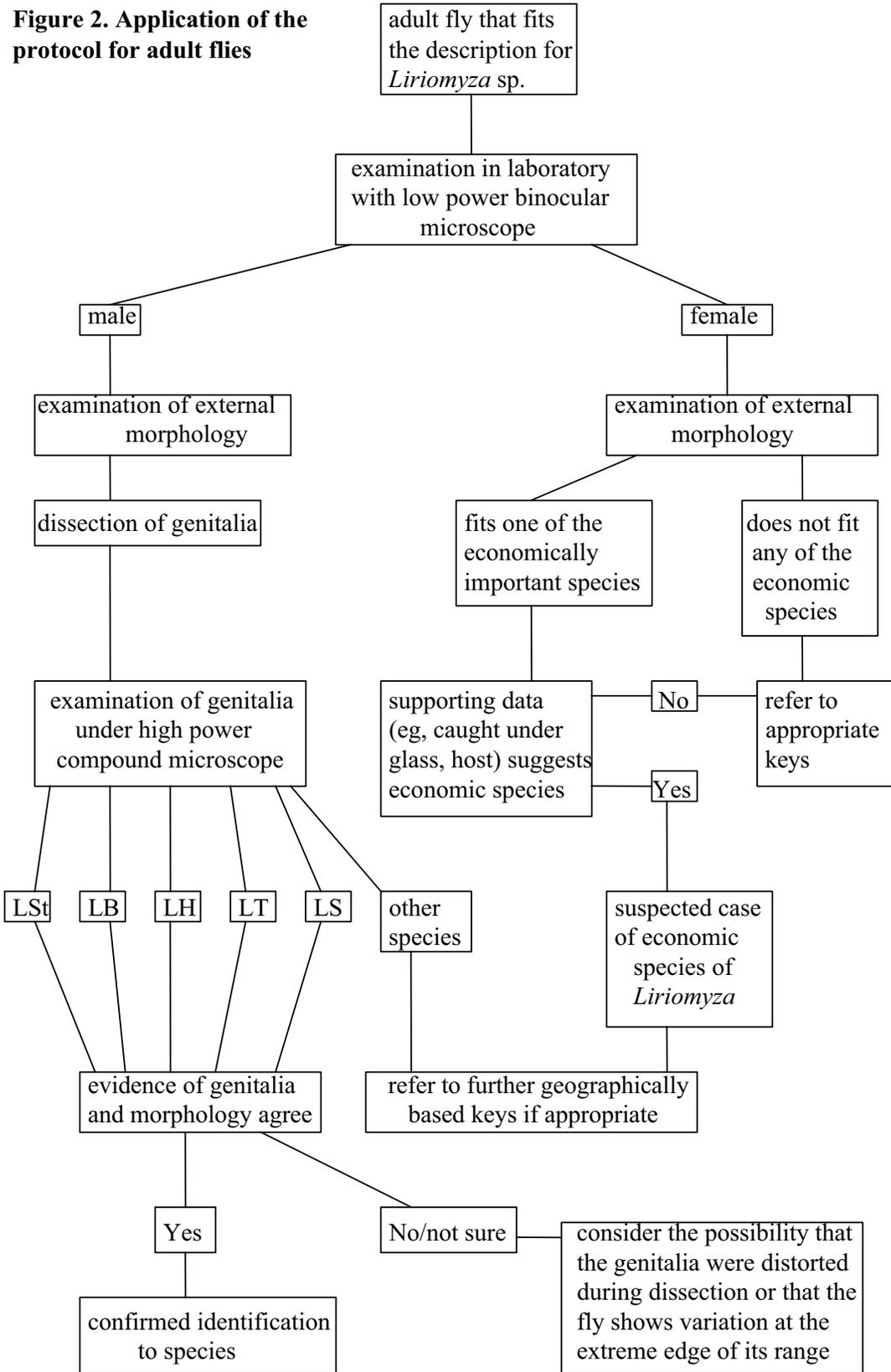
e-mail: dom.collins@csl.gov.uk

Figure 1. Application of the protocol for larvae and puparia



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

Figure 2. Application of the protocol for adult flies



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

Table 1. Morphological characters, adult.

	<i>Male distiphallus</i>	<i>vertical setae</i> (see Figure 3)	<i>Anepisternum</i> (see Figure 3)	<i>Vein Cu 1A</i> (see Figure 4)
<i>L. bryoniae</i>	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
<i>L. huidobrensis</i>	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
<i>L. sativae</i>	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
<i>L. strigata</i>	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
<i>L. trifolii</i>	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

Table 1 (cont.). Morphological characters, adult

	<i>Third antennal segment</i>	<i>Frons & orbits</i>	<i>Femur</i>	<i>Mesonotum</i>	<i>wing length</i>
<i>L. bryoniae</i>	Small, yellow	Frons bright yellow, orbits slightly paler	Bright yellow with some brownish striations	Black, largely shining but with distinct matt undertone	1.75-2.1mm
<i>L. huidobrensis</i>	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	1.7-2.25mm
<i>L. sativae</i>	Small, yellow	Frons and orbits bright yellow	Bright yellow	Black, shining	1.3-1.7mm
<i>L. strigata</i>	Small, yellow	Frons and orbits yellow	Yellow with some brownish striations	Black, shining but slightly matt	1.8-2.1mm
<i>L. trifolii</i>	Small, yellow	Frons and orbits yellow	Yellow, occasional slight brownish striations	Matt black with grey undertone	1.3-1.7mm

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

Appendix A

Electrophoretic identification of larvae and puparia to species

A diagrammatic representation of the successive steps undertaken in this procedure is presented as Figure 1.

The use of allozyme electrophoresis to identify the immature stages of selected *Liriomyza* species 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.

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.

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.

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, Texas). 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 minutes in 800 ml 25 mM Tris Glycine, pH 8.5 buffer solution to which NADP (70 mg l⁻¹) and MgCl₂ (70 mg l⁻¹) have been added (Table 3). Three gels are required for protocol A, two gels for protocol B.

Sample preparation

Individual larvae or puparia are homogenised 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 need to 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 minutes at 200V (1 mA per gel).

Protocol B: The gels are initially run simultaneously for 18 minutes at 200V (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 minutes, still at 200V.

Staining

Staining schedules essentially follow those outlined by Hebert & Beaton (1989). Staining solutions are prepared fresh from stock solutions whilst 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 immediately before they are to be used). Recipes for the different staining solutions are given in Table 2. 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. It takes about a minute before the mixture sets over the gel plate. 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 minutes at 37°C. The staining reaction may be brought to a halt at any time by placing the agar-overlain gel plate in a 7% (v/v) solution of acetic acid.

Protocol A: The three gels are respectively stained for glucose-6-phosphate dehydrogenase (G6PDH), isocitrate dehydrogenase (IDH) and malic enzyme (ME).

Protocol B: The first gel to be removed from the electrophoresis tank is stained for G6PDH, the second for leucine-glycine peptidase (PEP).

Table 2. Recipes for staining solutions of G6PDH, IDH, ME and PEP

Chemical (stock solution)	G6PDH	IDH	ME	PEP	
Tris-HCl, 0.1M, 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. Recipes for the gel/electrode and stain buffers.

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 buffer	1.21 g Tris 100 ml distilled water titrate to pH 8.0 with 1M HCl

Interpretation of band patterns

Interpretation of the band patterns is achieved using the biochemical keys presented below.

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 (see Figure 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).....*L. bryoniae*
or only one of the *L. bryoniae* homozygote bands present

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

Protocol B (see Figures 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.....*L. trifolii*; *L. sativae*
band located between 20 and 30 mm *C. syngenesiae*
C. horticola
PEP-1 band absent or heavy streaking associated.....indicative of parasitism
with it by *D. sibirica*

Appendix B

Identification to species using the male distiphallus

A diagrammatic representation of the successive steps undertaken in this procedure is presented as Figure 2. The male's distiphallus is a very small, fragile structure enclosed by membranes and requires careful dissection before examination under a high power microscope. It is recommended that the evidence of the distiphallic structure is correlated with the evidence of external morphology (see Table 1) in order to confirm the identification.

Determining the sex of flies

male: the lobes of the epandrium, which are dark and pubescent and not so heavily sclerotised as the female tube, curve around and down at the rear of the abdomen, from the dorsal to the ventral sides (Figure 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.

female: the abdominal segments beyond segment 6 form a black, heavily sclerotised tube which extends out beyond the 6th tergite (Figure 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

1. Using fine mounted needles, carefully separate the abdomen from the rest of the fly.
2. Briefly wet the abdomen in absolute ethanol.
3. Bring the abdomen to the boil in 10% potassium hydroxide (or 10% sodium hydroxide) and boil for 60-90 seconds.
4. Transfer the abdomen to cold glacial acetic acid and leave for 3 minutes.
5. Blot off excess glacial acetic acid and transfer the abdomen to a drop of Heinz mounting medium* on a cavity slide.
6. 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 Plate 1).
7. Using fine mounted needles, position the genital complex for lateral viewing under a compound light microscope (recommended at 400 × magnification).
8. Re-position the genital complex for ventral viewing of the distiphallus (again at 400 × magnification)

*or a similar semi-viscous mounting fluid such as Berlese solution or Hoyer's solution.

Diagnostic key using the male distiphallus

(to be used in conjunction with Figure 15 and 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 antero-ventrally); evenly sclerotized *L. bryoniae*
 With bulb rims spiralled (i.e., drawn out antero-ventrally): strongly sclerotized antero-
 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*

Figure 3. Generalised diagrams of an adult male *Liriomyza* illustrating the morphological characters mentioned in this protocol

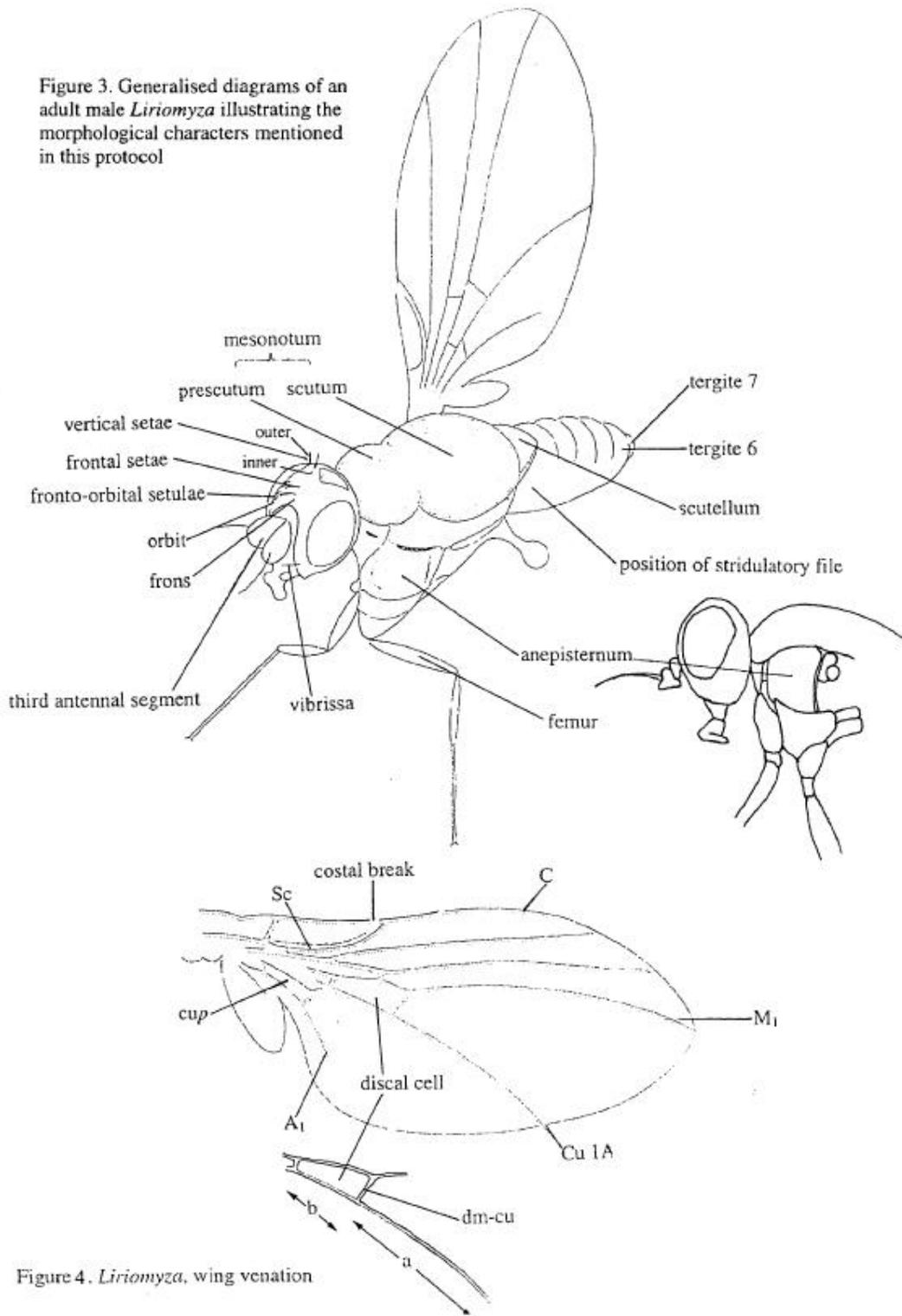


Figure 4. *Liriomyza*, wing venation

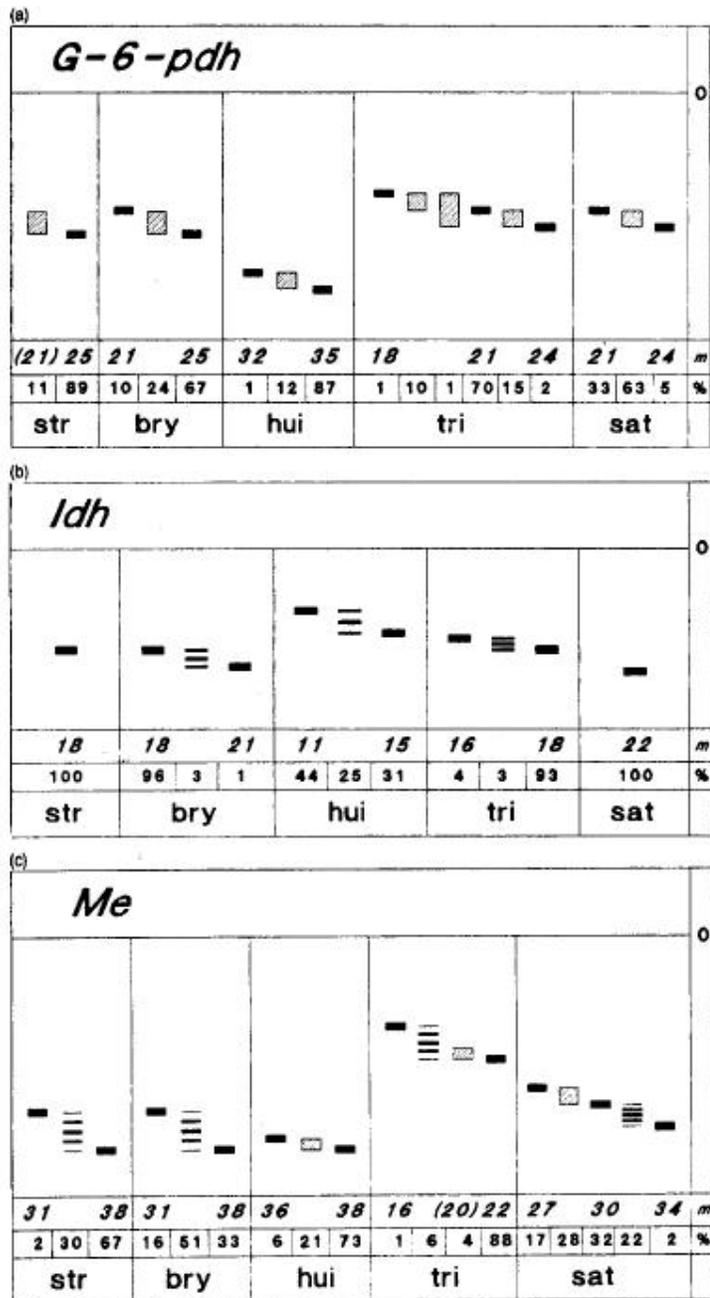


Figure 5. Protocol A.

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 in 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.

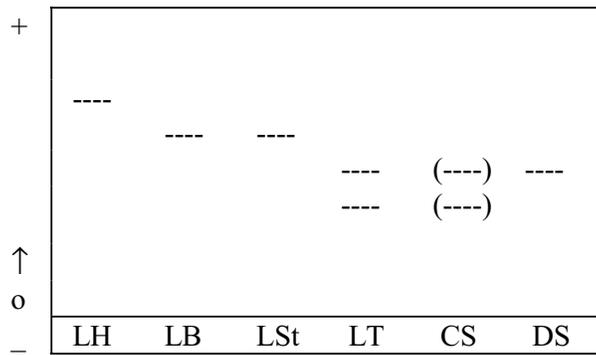


Figure 6. Protocol B.
 G6PDH band patterns. LH = *Liriomyza huidobrensis*;
 LB = *L. bryoniae*; LSt = *L. strigata*; LT = *L. trifolii*;
 CS = *Chromatomyia syngenesiae*; DS = *Dacnusa sibirica*.

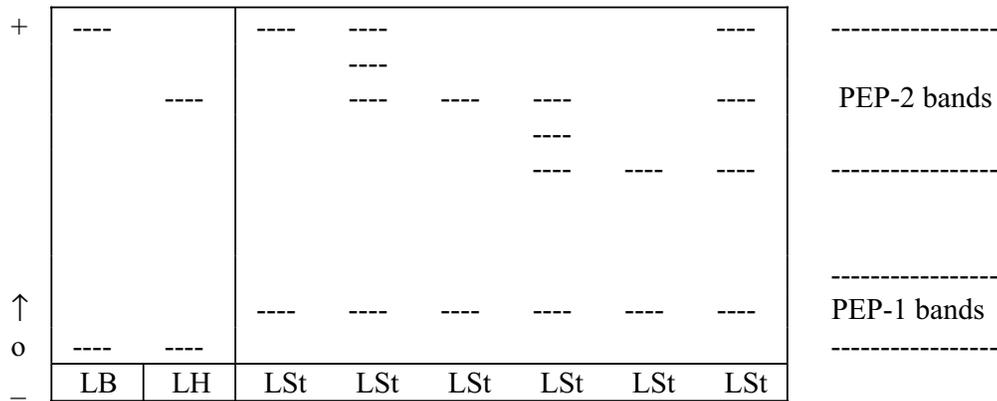


Figure 7. Protocol B.
 PEP phenotypic variation for *L. strigata*. LH = *Liriomyza huidobrensis*; LB = *L. bryoniae*;
 LSt = *Liriomyza strigata*. nb: 1st instar larvae may not produce PEP-2 bands.

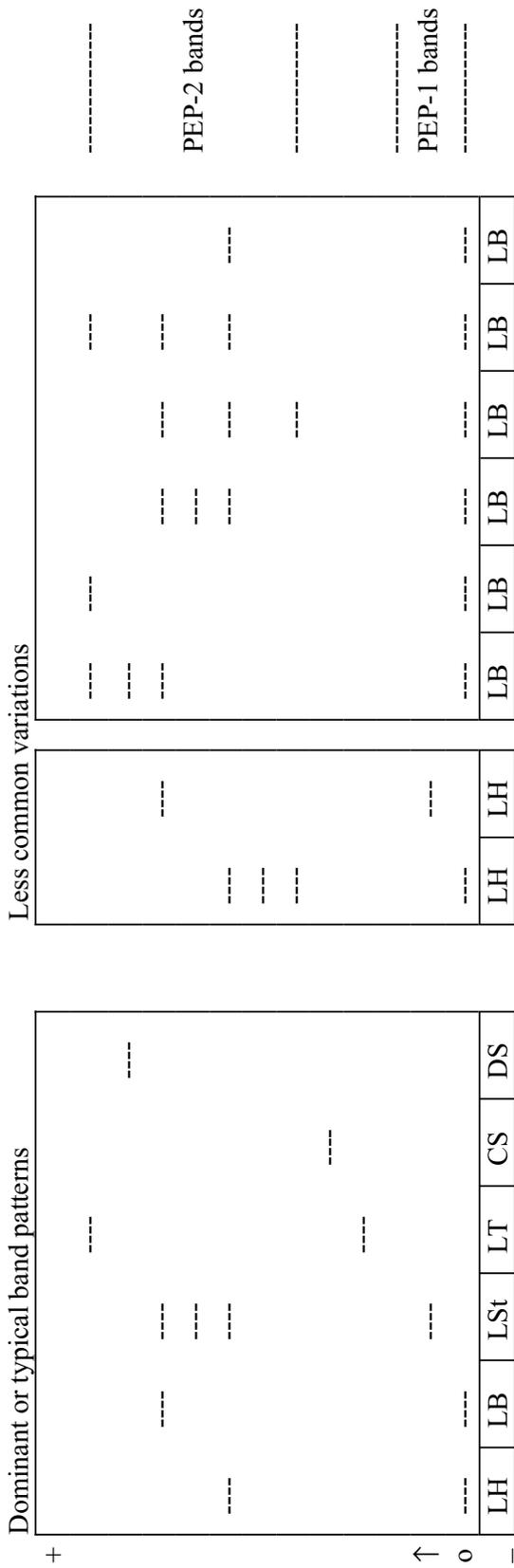


Figure 8. Protocol B. PEP phenotypic variation for *Liriomyza huidobrensis* and *L. bryoniae*. LH = *L. huidobrensis*; LB = *L. bryoniae*; LSt = *L. strigata*; LT = *L. trifolii*; CS = *Chromatomyia syngenesiae*; DS = *Dacnusa sibirica*. nb: 1st instar larvae may not produce PEP-2 bands.

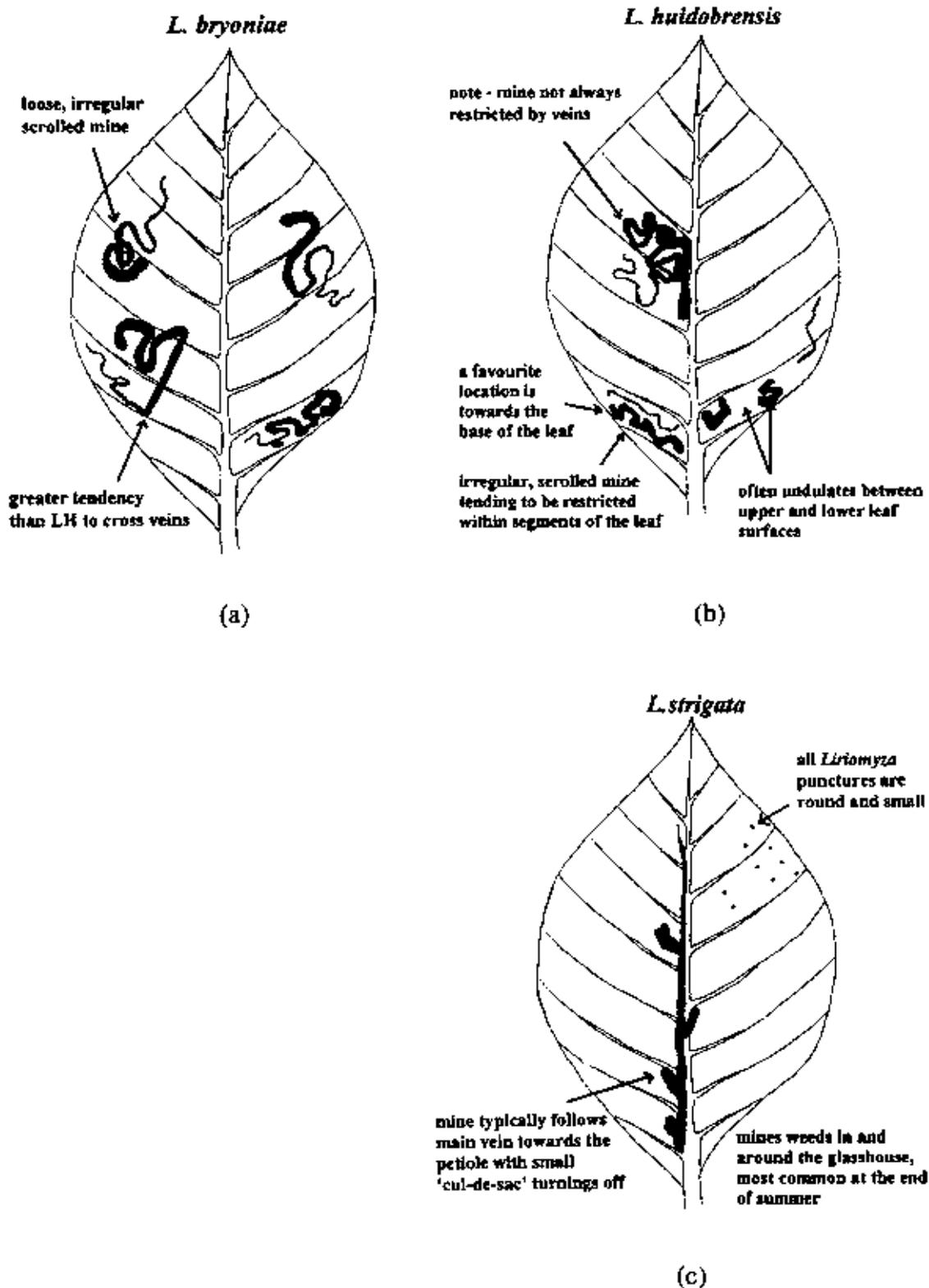


Figure 9. Typical characteristics of mines from Group 1 species: (a) *L. bryoniae*; (b) *L. huidobrensis*; (c) *L. strigata*.

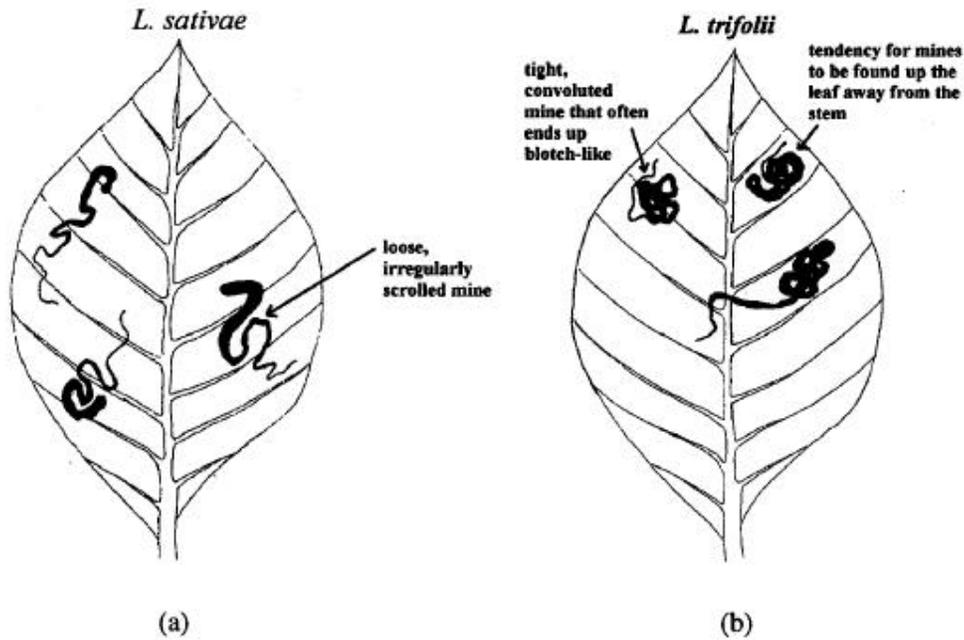


Figure 10. Typical characteristics of mines from Group 2 species: (a) *L. sativae*; (b) *L. trifolii*.

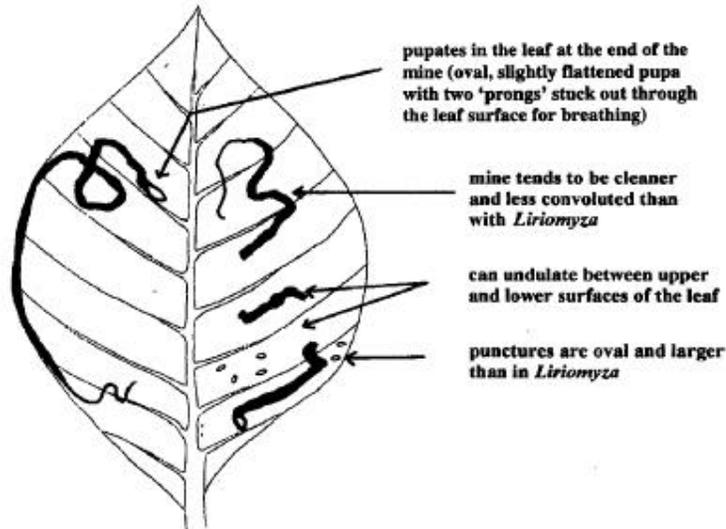


Figure 11. Typical characteristics of mines of *Chromatomyia syngenesiae* (nb: the mines and punctures of *C. horticola* can appear intermediate between those of *C. syngenesiae* and *L. huidobrensis*).

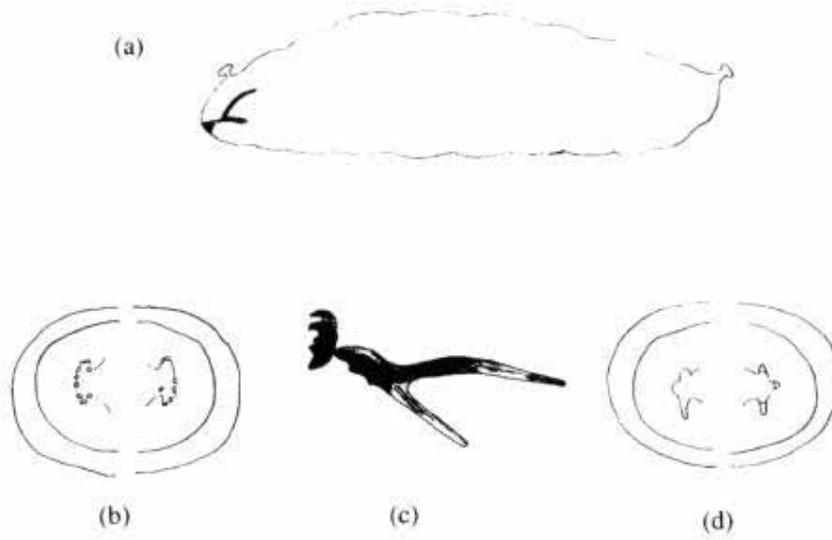


Figure 12. *Liriomyza* immature stages: (a) larva, gross form; (b) posterior spiracles, Group 1 (larva, left; pupa, right); (c) cephalopharyngeal skeleton; (d) posterior spiracles, Group 2 (larva, left; pupa, right)



Plate 1. Genital complex (*L. huidobrensis*), lateral

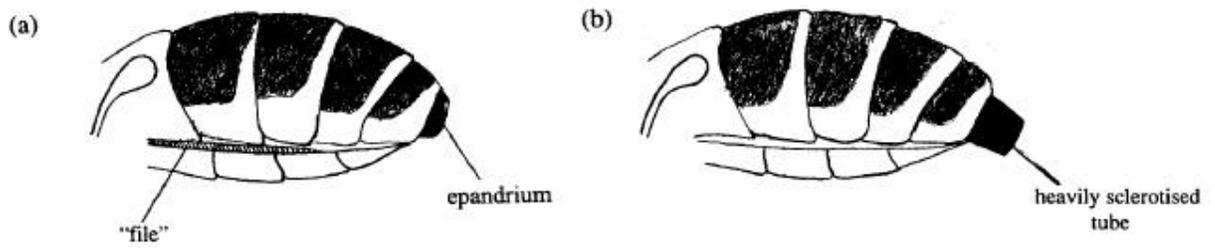


Figure 13. *Liriomyza* abdomen: (a) male; (b) female

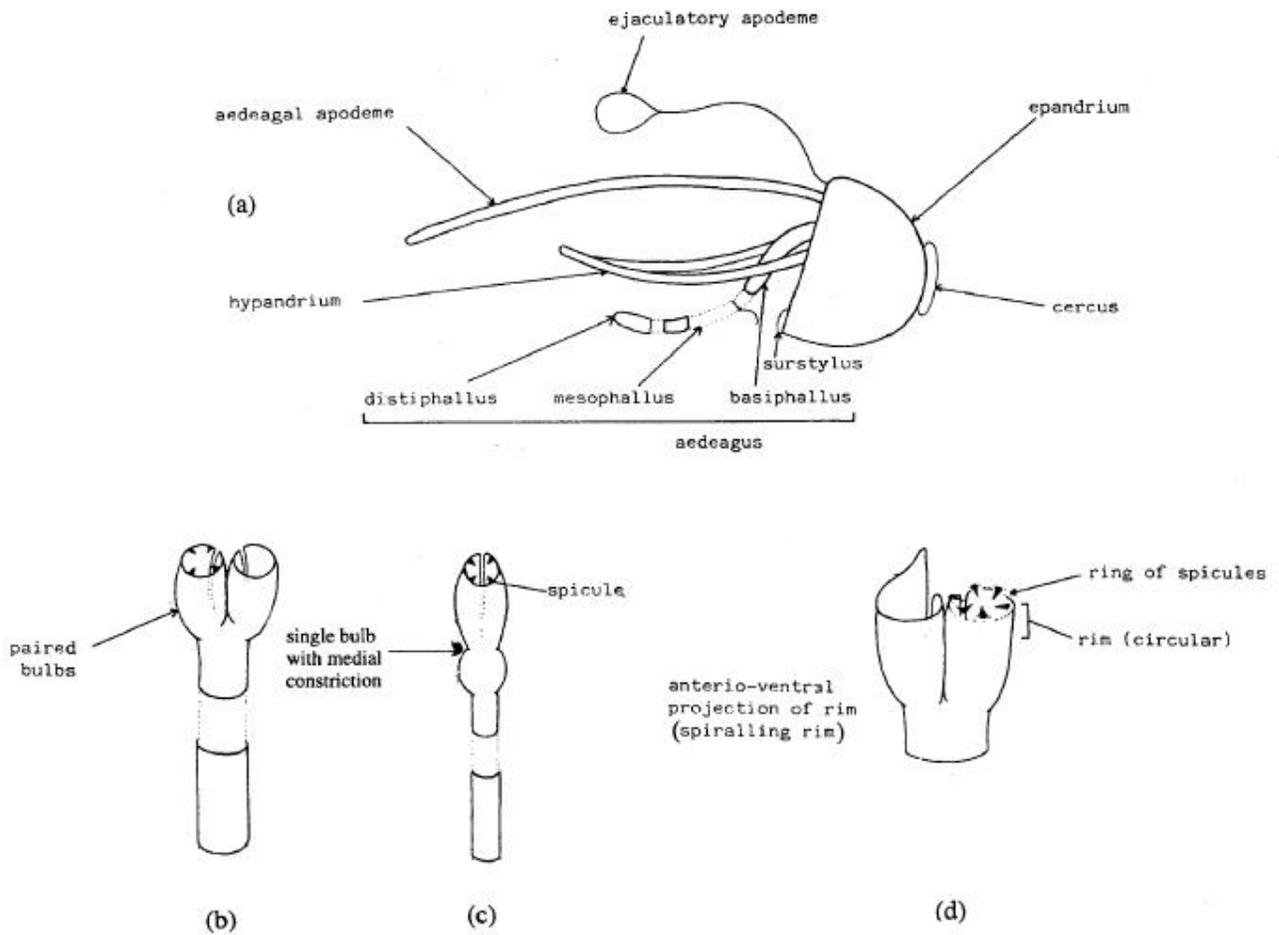


Figure 14. Generalised diagrams of the male genitalia of *Liriomyza*: (a) genital complex; (b) distiphallus, Group 1; (c) distiphallus, Group 2; (d) distiphallic parts

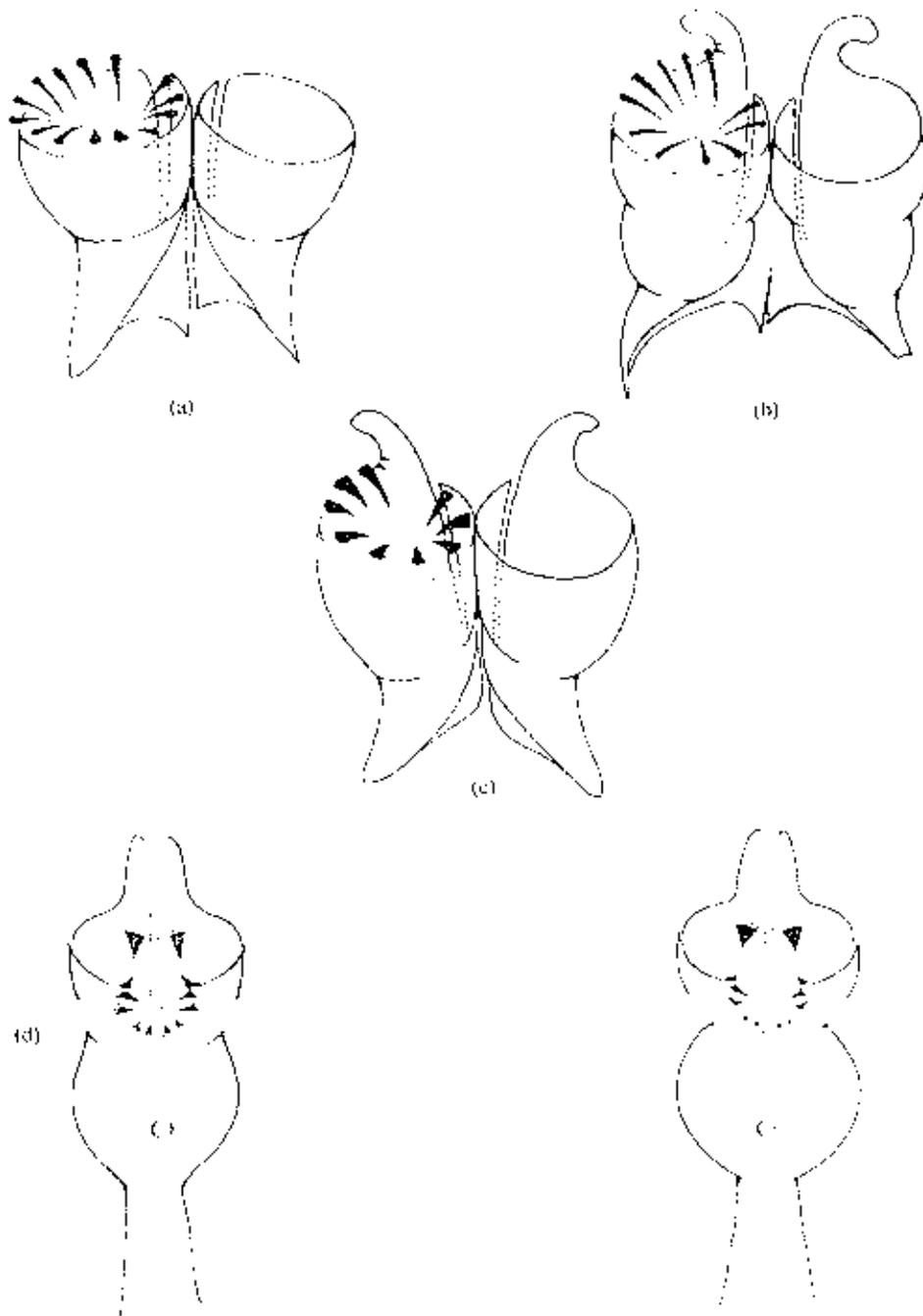


Figure 15. Generalised diagrams of the distyphallus of each species, dorsal view (sucklets not shown on right side, a-c); (a) *I. beyonae*; (b) *I. haidobrovska*, (c) *I. serigata*; (d) *I. salivae*; (e) *I. trifida*

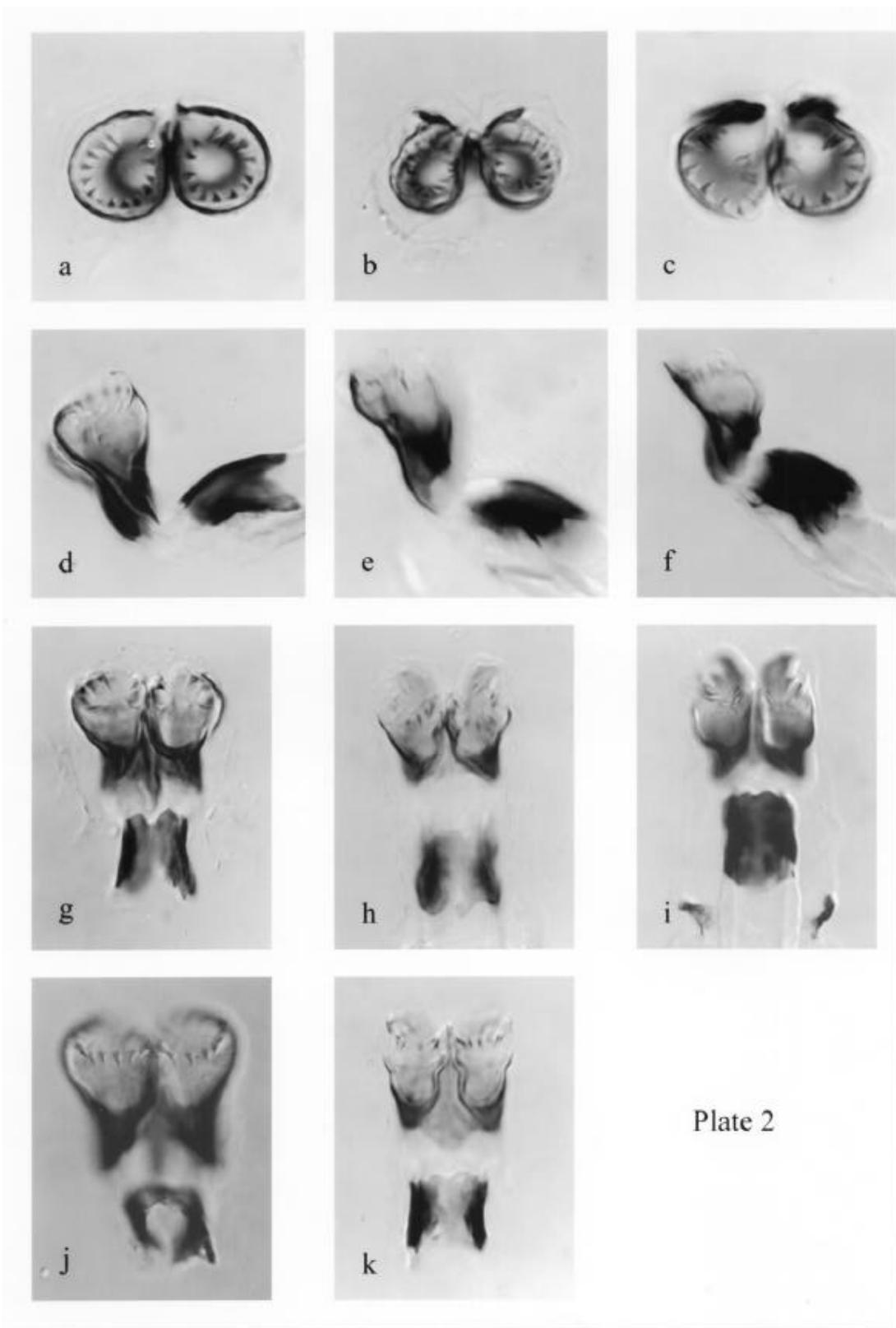


Plate 2. Distiphalli at $\times 400$ microscope magnification

- (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))

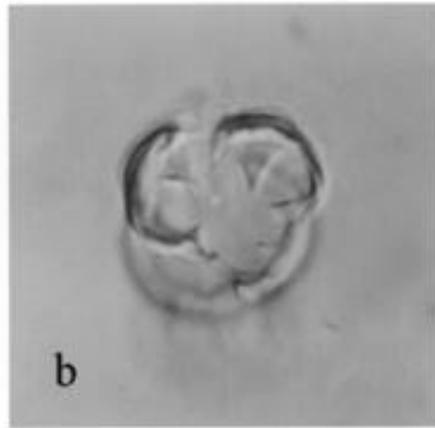
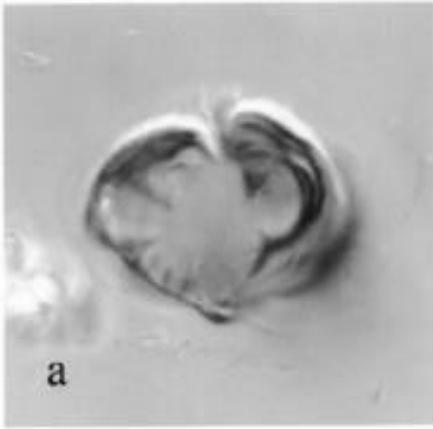


Plate 3

Plate 3. *Distiphalli* at $\times 400$ 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

END