Revalidation of morphological characteristics and multiplex PCR for the identification of three congener invasive *Liriomyza* species (Diptera: Agromyzidae) in China

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Due to varietal differences, diminutive size, and similar morphological characters, it is difficult to classify and identify *Liriomyza* spp., a genus comprised of economically-important, highly-polyphagous insect pests. In this study, we reconfirmed the morphological characteristics of three closely-related invasive leafminers, *L. trifolii, L. sativae,* and *L. huidobrensis*. Morphological results showed that characteristics imparted by the male genitalia were the most reliable morphological features for identification. The colors exhibited by vertical setae were variable among species, and the ratio of the length of the ultimate section of vein CuA_1 divided by penultimate section also varied within species. Although the patterns of abdominal tergites were diverse among *Liriomyza* spp.,

L. trifolii exhibited a unique pattern with a yellow patch at the 5th black visible tergite; this pattern can be profiled as a prominent characteristic for morphological identification. In order to identify the three *Liriomyza* spp. quickly and accurately, we developed an improved molecular identification method using multiplex PCR based on the gene encoding mitochondrial cytochrome oxidase I (*COI*); this method enabled direct identification based on the size of amplified products. The results of this study provide a valuable reference for the identification of *Liriomyza* spp., which will ultimately improve our ability to control individual species.

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ABSTRACT

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21 to classify and identify *Liriomyza* spp., a genus comprised of economically-important, highly-

22 polyphagous insect pests. In this study, we reconfirmed the morphological characteristics of

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36

37 **Keywords:** *Liriomyza*, morphological characteristics, abdominal tergites, multiplex PCR, species

38 identification, *COI*

39

INTRODUCTION

Leafminer flies (Diptera: Agromyzidae), especially *Liriomyza trifolii*, *L. sativae* and *L. huidobrensis*, are invasive insect pests in many countries. They are polyphagous, economically-significant pests that cause severe damage to many ornamental and vegetable crops worldwide (Spencer, 1973, 1990; Reitz et al., 1999). Both larvae and adults cause serious damage to crops (Musgrave et al., 1975; Minkenberg & Van Lenteren, 1986). The damage caused by larval feeding on leaves can reduce photosynthetic capacity, and leaf mining activity can cause premature leaf drop resulting in reduced yields (Johnson et al., 1983; Chandler & Gilstrap, 1987). Moreover, indirect damage occurs when adults pierce leaves for feeding and oviposition, thus increasing plant susceptibility to disease (Zitter & Tsai, 1977; Motteoni & Broadbent, 1988). The rapid life cycle and high growth rate of *Liriomyza* spp. can lead to serious crop losses. Accurate identification of *Liriomyza* is important for implementing effective control strategies, because insecticide resistance and tolerance to environmental stress varies among species (Chang et al., 2017; Gao et al., 2017).

Closely-related *Liriomyza* spp. are similar in morphology at the adult stage (Oudman et al., 1995; Lei et al., 1996; Chen, 1999; Scheffer et al., 2001), and adult males can only be identified with certainty according to genitalia, which is both time-consuming and difficult. Identification at the early developmental stages of *Liriomyza* infestation is necessary for effective control; however, the absence of morphological characters makes identification difficult and larvae cannot be collected directly due to their mining behavior (Oudman et al., 1995; Chiu et al., 2000; Morgan et al., 2000; Scheffer et al., 2001).

Since morphological identification of female adults, larvae and pupae of *Liriomyza* species is

61   complex and difficult, molecular methods of identification are required. Immature developmental

62   stages are the most common forms intercepted at ports of entry, therefore, it is important to identify

63   these interceptions accurately and rapidly. With the development of mitochondrial and other

64   molecular markers (Carapelli et al., 2018; Chen et al., 2019), several molecular methods have been

65   developed to identify *Liriomyza* species (Menken & Ulenberg, 1983; Zehnder et al., 1983;

66   Oudman et al., 1995; Chiu et al., 2000; Morgan et al., 2000). Multiplex PCR is a cost-effective,

67   rapid, accurate method where identification can be determined by PCR product size with species-

68   specific primers (Nakamura et al., 2013).

69   In this study, we re-verified morphological characteristics of three leafminers, *L. trifolii*, *L.*

70   *sativae* and *L. huidobrensis*. A new morphological characteristic for detection of *L. trifolii* was

71   investigated, and an improved molecular method for identification was developed based on

72   multiplex PCR. This study provides approaches that can be deployed for identification of

73   *Liriomyza* species, which will ultimately help future control efforts.

74

75   **MATERIALS AND METHODS**

76   **Insects**

77   The three species of *Liriomyza* spp. were collected from areas where leafminers occur in China.

78   In this study, 263 individuals of three species were selected for further data analysis (Table S1).

79   These were collected at the larval stage, tagged with relevant information and transported to the

80   laboratory for pupation and emergence as adults. After preliminary morphological identification,

81   adults were labeled, immersed in 70% ethanol and stored at -20°C. After dissecting and

82   photographing the samples, the remaining tissues were stored in 100% ethanol for DNA

83   extraction and molecular analysis.

84

85   **Morphological identification**

86   Samples were examined with a stereomicroscope (Zeiss Stemi 2000c) and photographed with a

87   wide depth of field (Zeiss Smartzoom 5). Male genitalia and wings were dissected, and slides

88   were prepared and photographed with the Axio imager A2 (Zeiss, Germany).

89   Differences in the ratios of ultimate section lengths of vein CuA_1 among different *Liriomyza*

90   species were determined by one-way analysis of variance (ANOVA), followed by Tukey's

91   multiple comparisons. All statistical analyses were performed using SPSS v. 16.0 (SPSS,

92   Chicago, IL, USA), and statistical significance was determined when $P < 0.05$.

93

94   **Molecular identification and primer selection for multiplex PCR**

95   Genomic DNA of *Liriomyza* species was extracted using the AxyPrep^{TM} Multisource Genomic

96   DNA Kit (Axygen, USA). A partial sequence of the mitochondrial cytochrome oxidase I (*COI*)

97   gene was amplified with common primers F, 5′-CAACATTTATTTTGATTTTTTGG-3′ and R,

98   5′- TCCAATGCACTAATCTGCCATATTA-3′ (Simon et al., 1994; Yang et al., 2010) using

99   protocols described by Chen et al. (2019), to molecular cross-checking and verification all of

100   *Liriomyza* species in this study using sequencing, accession number can be found in Table S1.

101   For multiplex PCR, full-length *COI* genes of three *Liriomyza* species were downloaded

102   from NCBI (https://www.ncbi.nlm.nih.gov/) and aligned using Clustal X. To develop a rapid

103   identification method, three species-specific primers and a common reverse primer were mixed

104   to amplify DNA from different *Liriomyza* species. The PCR conditions were as follows:

105   denaturation at 94 °C for 3 min; 35 cycles at 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1

106   min; followed by extension at 72 °C for 10 min. PCR was conducted in a 25 μL reaction volume

107   containing 2 μL (100 ng) of DNA template, 1 μL (10 μM) of each primer, 12.5 μL of 2× Taq

108   Master mix (Vazyme Biotech Co., Ltd) and 6.5 μL ddH$_2$O. PCR products were separated in

109   1.0% agarose gels, and primers that amplified only one specific band for each species are shown

110   in Table 1.

111

112   **RESULTS**

113   **Morphological identification**

114   The distiphallus, which is part of the male genitalia, is a very small, fragile structure enclosed by

115   membranes located at the terminus of the aedeagus. For *L. trifolii*, the morphological

116   characteristics of the distiphallus include one distal bulb with marked constriction between lower

117   and upper halves in dorsoventral view; the bulb is lightly sclerotized with a long basal stem (Fig.

118   1A). For *L. sativae*, the distiphallus is characterized by one distal bulb with a slight constriction

119   between upper and lower halves in the dorsoventral view; the bulb is more intensely sclerotized

120   with a shorter basal stem (Fig. 1B). For *L. huidobrensis*, the distiphallus contains two distal

121   bulbs; these meet at rims that extend in an anteroventral orientation (Fig. 1C).

122   With respect to vertical setae, *L. trifolii* exhibits inner and outer vertical setae on a yellow

123   background; whereas vertical setae are present on a black background for *L. huidobrensis*. In *L.*

124   *sativae,* outer and inner vertical setae are presented on black and yellow backgrounds,

125   respectively (Spencer, 1973). In this study, only 86.1% (192/223) of *L. trifolii* had yellow inner

126   and outer vertical setae; 9.9% (22/223) had yellow inner vertical setae and undetermined color

127   for outer setae, and 4.0% (9/223) had yellow inner and black outer vertical setae (Table 2; Figs.

128   2A-C). For *L. sativae*, 17.6% (6/34) had black inner and outer vertical setae, 58.8% (20/34) had

129   yellow inner and black outer vertical setae, and 23.5% (8/34) had outer black setae with an

130   undetermined color for inner vertical setae (Table 2; Fig. 2D-F). For *L. huidobrensis*, 100% (6/6)

131   exhibited black inner and outer vertical setae (Table 2; Fig. 2G-I). These results show that

132   characteristics of vertical setae are not reliable for identifying *Liriomyza* species.

133   Wing pattern ratios were calculated as the length of the ultimate section of vein CuA_1

134   divided by the penultimate section ('a' and 'b', see Fig. 3A-C). In this study, 'a' was 2.70 ± 0.31

135   times the length of 'b' in *L. trifolii*, and 'a' was 2.72 ± 0.37 times the length of 'b' in *L. sativae*.

136   For *L. huidobrensis*, 'a' was 2.20 ± 0.24 times the length of 'b' ($F_{2,237} = 7.345$, $P < 0.05$) (Fig. 4).

137   Although the ratio of *L. huidobrensis* was significantly different from the other two species ($P <$

138   0.05), there was no significant difference between *L. trifolii* and *L. sativae* ($P = 0.907$). Many *L.*

139   *trifolii* individuals exhibited truncated or missing dm-cu cross veins. Furthermore, we noted

140   inconsistency between left and right forewing patterns within individual samples (Fig. 3A, with

141   dashed lines).

142   In *L. trifolii*, the 2nd-5th visible tergites were generally divided by a yellow medial furrow in

143   male adults; furthermore, there was a yellow patch at the 5th black visible tergite that can

144   distinguish *L. trifolii* from other *Liriomyza* species (Figure 5A-C). In *L. sativae* and *L.*

145   *huidobrensis*, only the second visible tergite is divided by a yellow medial furrow and no yellow

146   patch is evident on the 5th tergite (Fig. 5D-I).

147

148   **Molecular detection of *Liriomyza* spp.**

149   Candidate primers for species-specific detection of *Liriomyza* were based on the alignment of

150   262 (*L. sativae*), 612 (*L. trifolii*), and 959 (*L. huidobrensis*) *COI* sequences. We designed one

151   reverse primer, 1181 R, that was common to all three *Liriomyza* species. The position of forward

152   primers was selected to produce <1000 bp amplicons when paired with the reverse primer with at

153   least 300 bp nucleotides between species. In addition, sites were selected where the number of

154   differential nucleotides was >2 bp to increase the specificity of the primers (Fig. 6).

155   The three *Liriomyza* species could be differentiated by specific PCR products in 1.0%

156   agarose gels, and the resulting PCR products were 569, 919, and 222 bp for *L. trifolii*, *L. sativae*

157   and *L. huidobrensis,* respectively (Fig. 7A). The validity of multiplex PCR for identification was

158   further confirmed by using the system with different developmental stages; the approach worked

159   equally well for larvae, pupae and adults of the three *Liriomyza* species (Fig. 7B). Populations

160   from different geographical regions were also obtained to evaluate the reliability of species-

161   specific primers. The results obtained by multiplex PCR (Fig. S1) and subsequent sequence

162   analysis of *COI* (Fig. S2) showed that geography did not impact the reliability of primers.

163

164   **DISCUSSION**

165   The morphological characteristics used for *Liriomyza* identification have primarily followed

166   Spencer's (1973) criteria. However, variability in life stages, emergence times and sample

167   preservation result in large differences in body color and markings, which can make current

168   morphological criteria unreliable for identification (Spencer, 1973; Kang et al., 1996; Shao,

169   2004).

170   Currently, the identification of *Liriomyza* spp. based on morphology is restricted to male

171   adults because there are no reliable features for species-level identification of female adults or

172   immature developmental stages (EPPO, 2005). The identification of adults requires the

173   examination of the male adult genitalia. In general, the distiphallus provides reliable detection of

174   the three *Liriomyza* species and has considerable diagnostic value (Spencer, 1973; Shiao, 2004).

175   However, differences in distiphalluses between species are subtle and dissection is difficult for

176   nonprofessionals. Consequently, features of distiphallic structure should be cross-checked with

177   other external morphological characteristics to ensure that identification is valid.

178   According to Spencer (1973), coloration of the vertical setae is an important external feature

179   that can distinguish *L. trifolii* and *L. sativae* without dissection; however, this feature is unstable

180   and lacks clear interspecific boundaries. Results of the current study show that reliance on

181   coloration of vertical setae can result in misidentification of *L. trifolii* and *L. sativae*; thus, this

182   feature should only be used as a supplement for identification. The ratio of the length of the

183   ultimate section of vein CuA_1 is unreliable since most ratio values overlapped among *Liriomyza*

184   species. In this study, we also evaluated the patterns of abdominal tergites and discovered that

185   the yellow patch at the 5th black visible tergite of *L. trifolii* is a new, reliable morphological

186   characteristic for identification. Similar findings were reported for abdominal color patterns for

187   six *Liriomyza* species (Shiao, 2004).

188   Molecular methods for insect identification can be used with different developmental

189   stages, including immature stages where morphological features may be lacking. Furthermore,

190   molecular assays may facilitate identification of atypical or damaged samples. However, the

191   specificity of molecular assays may be limited because they were developed for a particular

192   purpose and evaluated against a restricted number of species (Nakamura et al., 2013). Multiplex

193   PCR assays were recently developed for identification of *Liriomyza* species (Miura et al., 2004;

194   Guan et al., 2006; Nakamura et al., 2013) and are based on amplification of a target gene region

195   using species-specific primer combinations. Multiplex PCR assays are easier and faster than

196   other molecular methods, such as RAPD-PCR, PCR-RFLP, DNA barcoding and real-time PCR

197   (Chiu et al., 2000; Morgan et al., 2000; Scheffer et al., 2001; Kox et al., 2005; Scheffer et al.,

198   2006; Blacket et al., 2015; Sooda et al., 2017); furthermore, multiplex PCR assays are more

199   sensitive than enzyme electrophoresis methods (Zehnder et al. 1983; Menken & Ulenberg, 1983,

200   1986; Oudman et al., 1995). In general, the reliability and sensitivity of multiplex PCR

201   represents a great improvement in molecular identification protocols and will enable us to

202   manage invasive pests more effectively.

203

204   **CONCLUSIONS**

205   Invasive *Liriomyza* spp. comprise a group of insect pests that cause considerable economic loss

206   and serious quarantine problems. In this study, morphological features were re-evaluated for *L.*

207   *trifolii*, *L. sativae*, and *L. huidobrensis*, and the discriminative ability of traditional

208   morphological characteristics, such as male genitalia, abdominal color patterns, length of CuA_1

209   and abdominal tergite patterns were reevaluated. Furthermore, we developed an improved

210   molecular identification method using multiplex PCR based on *COI* to identify the three

211   *Liriomyza* species quickly and accurately. This study provides valuable tools for the

212   identification of *Liriomyza* spp. using both morphological and molecular criteria.

213

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306

307

308   **FIGURE LEGENDS**

309

310   **Figure 1.** Lateral view of phalluses in three *Liriomyza* species. A, *L. trifolii*; B, *L. sativae*; C, *L. huidobrensis*.

311   Arrows indicate the distiphallus. Scale bar = 0.01 mm.

312

313   **Figure 2.** Color characteristics of outer and inner vertical setae in three *Liriomyza* species. A-C, *L. trifolii*; D-F,

314   *L. sativae*; G-I, *L. huidobrensis*. Scale bar = 0.1 mm. Yellow arrows indicate the position of outer vertical setae,

315   and red arrows indicate the position of inner vertical setae.

316

317   **Figure 3.** Comparison of wing pattern ratios for three *Liriomyza* species. Ratios were calculated as the length of

318   the ultimate section of vein CuA_1 divided by the penultimate section ('a' and 'b' sections). A, *L. trifolii*; B, *L.*

319   *sativae*; C, *L. huidobrensis*. Scale bar = 0.1 mm. The panel outlined with dashed lines represents an abnormal

320   wing pattern in *L. trifolii*. Black arrows indicate the position of dm-cu cross veins.

321

322   **Figure 4.** Ratios showing lengths of the ultimate section of vein CuA_1 divided by the penultimate section.

323   Differences among three *Liriomyza* species were determined by one-way analysis of variance (ANOVA),

324   followed by Tukey's multiple comparison ($P < 0.05$). The data represent mean ± SD. The interval in box plot

325   means median value. Abbreviations: Lt, *L. trifolii*; Ls, *L. sativae*, and Lh, *L. huidobrensis*.

326

327   **Figure 5.** Abdominal color patterns of three *Liriomyza* species. A-C, *L. trifolii*; D-F, *L. sativae*; G-I, *L.*

328   *huidobrensis*. Scale bar = 0.1 mm.

329

330   **Figure 6.** Alignment of *COI* sequences in *Liriomyza* spp. Sequences surrounded by rectangular boxes represent

331   primers used in this paper. Base substitutions are indicated by shading. Lt, *L. trifolii*; Ls, *L. sativae*; Lh, *L.*

332   *huidobrensis*.

333

334   **Figure 7.** Agarose gel electrophoresis of multiplex PCR products. (A) PCR products in three different

335   *Liriomyza* species. (B) PCR products from different developmental stages of the three *Liriomyza* species. Each

336   experiment contained three biological samples. Abbreviations: Lt, *L. trifolii*; Ls, *L. sativae*; Lh, *L.*

337   *huidobrensis*.

338

339   **Figure S1.** Agarose gel electrophoresis of multiplex PCR products from different geographical populations of

340   *Liriomyza*. Lt Lanes 1-10 indicate *L. trifolii* populations from: (1) Hengshui, (2) Hangzhou, (3) Dongguan, (4)

341   Zhangzhou, (5) Qionghai, (6), Nanning, (7) Changzhou, (8) Nanchang, (9), Huizhou, and (10) Huzhou. Ls lanes

342   1-2 indicate *L. sativae* populations from Shangqiu and Luoyang, respectively. Lh lanes 1-2 represent *L.*

343   *huidobrensis* populations from Kunming and the laboratory, respectively.

344

345   **Figure S2.** Alignment of COI sequences from different geographical population using species-specific primers.

346   The shaded nucleotides represent divergent sites. Nucleotides bounded by red and blue dashed rectangles

347   represent forward and reverse primers, respectively. Lt sequences represent the following *L. trifolii* populations:

348   Lt 1, Hengshui; Lt 2, Hangzhou; Lt 3, Dongguan; Lt 4, Zhangzhou; Lt 5, Qionghai; Lt 6, Nanning; Lt 7,

349   Changzhou; Lt 8, Nanchang; Lt 9, Huizhou; and Lt 10, Huzhou. Ls sequences represent *L. sativae* populations

350   from Shangqiu (Ls 1) and Luoyang (Ls 2). Lh sequences represent *L. huidobrensis* populations from Kunming

351   (Lh 1) and the laboratory (Lh 2).

352

Table 1. Information of the primers designed in this study.

Table 1. Primers used in this study.

Primer name	Nucleotide sequence (5′–3′)	Ta (Tm) ºC	Product size (bp)	GenBank number
Lt612	CAATTACAATACTATTAACAGACCG	58 (48.5)	569	MT919718
Ls262	AGCTCCAGACATAGCATTTCCTCG	58 (58.9)	919	MT919719
Lh959	TTCAGATGGCTTGCCACATTACACG	58 (59.9)	222	MT919720
LR1181	GAATAAATCCKGCTATAATTGCAAATAC	58 (50.9)	-	-

Table 2. The data of color characteristic of outer and inner vertical setae position in three *Liriomyza* species.

2 **Table 2.** Color characteristics of outer and inner vertical setae in three *Liriomyza* species

Species	Vertical setae position (Inner/ Outer)	Individual phenotypes	Vertical setae position (Inner/ Outer)	Individual phenotypes	Vertical setae position (Inner/ Outer)	Individual phenotypes
L. trifolii	Y / Y	192	B / Y	0	U / Y	0
	Y / B	9	B / B	0	U / B	0
	Y / U	22	B / U	0	U / U	0
L. sativae	Y / Y	0	B / Y	0	U / Y	0
	Y / B	20	B / B	6	U / B	8
	Y / U	0	B / U	0	U / U	0
L. huidobrensis	Y / Y	0	B / Y	0	U / Y	0
	Y / B	0	B / B	6	U / B	0
	Y / U	0	B / U	0	U / U	0

3 Abbreviations: Y, yellow; B, black; and U, unclear.

Figure 1

Photo plates of the phalluses of three *Liriomyza* species, lateral view.

A, *L. trifolii*; B, *L. sativae*; C, *L. huidobrensis*. Arrows indicate the distiphallus. Scale bar=0.01 mm.
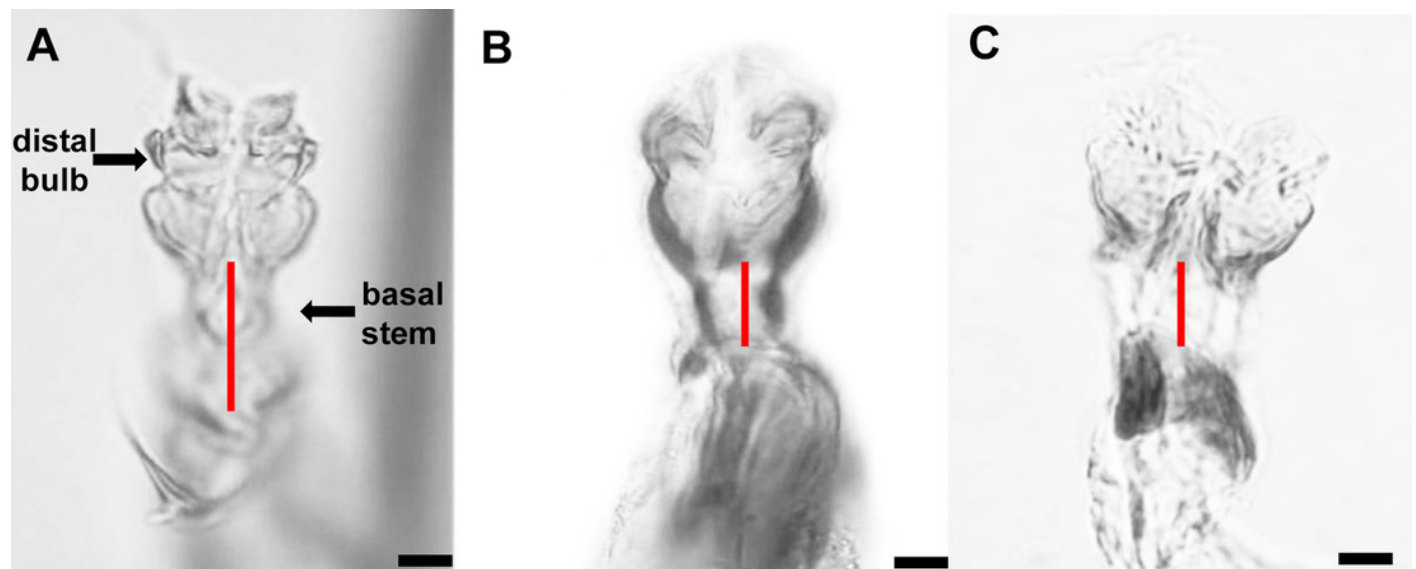
Figure 2

The color characteristic of outer and inner vertical setae position in three *Liriomyza* species.

A-C, *L. trifolii*; D-F, *L. sativae*; G-I, *L. huidobrensis*. Scale bar=0.1 mm. The yellow arrow indicated the position of outer vertical setae and the red arrow indicated the position of inner vertical setae.
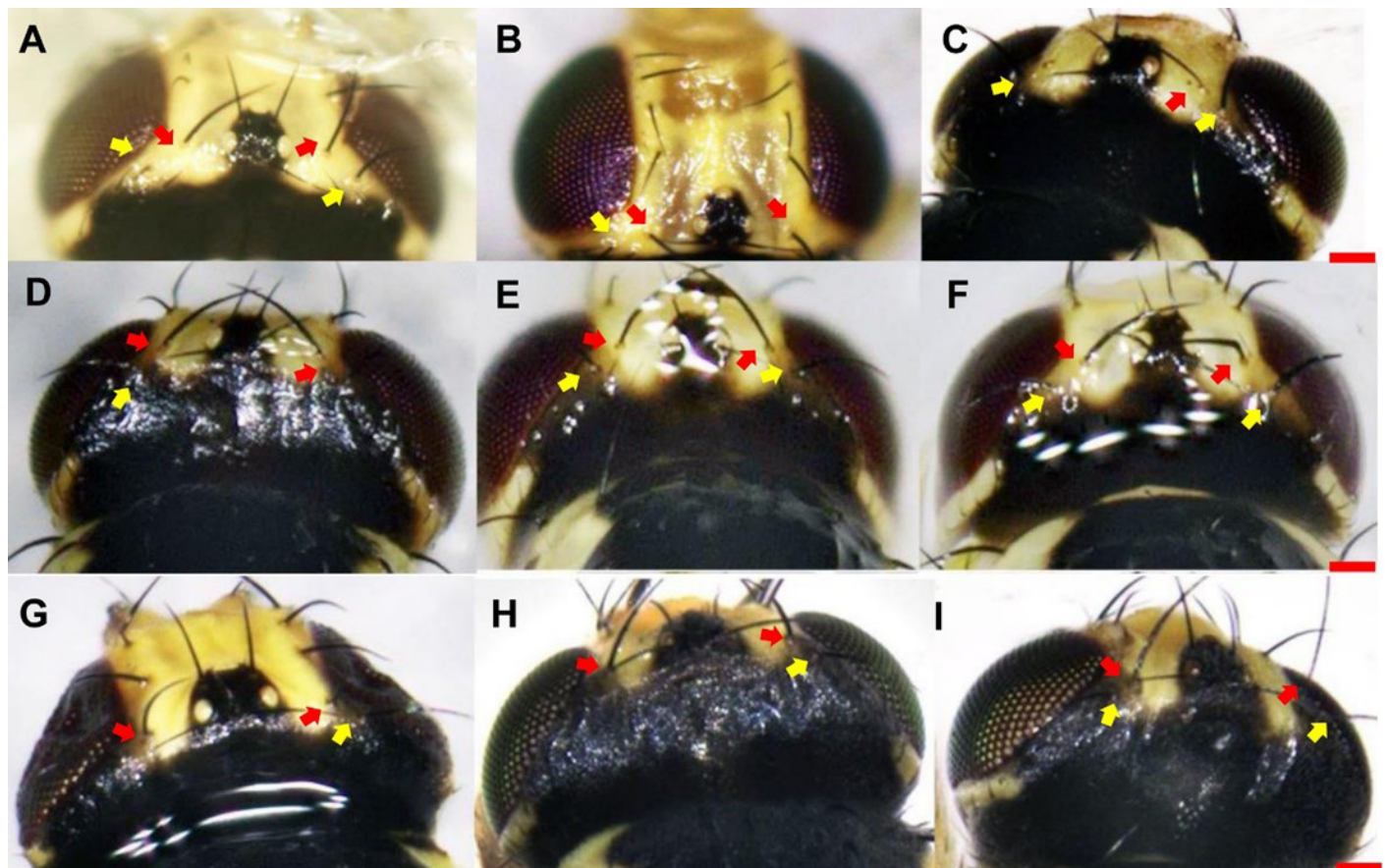
Figure 3

Comparison of the wing patterns of three *Liriomyza* species. The length of ultimate section of vein CuA_1 divided by penultimate section (a and b sections).

A, *L. trifolii*; B, *L. sativae*; C, *L. huidobrensis*. Scale bar=0.1 mm. Dot box represents abnormal wing pattern in *L. trifolii.*
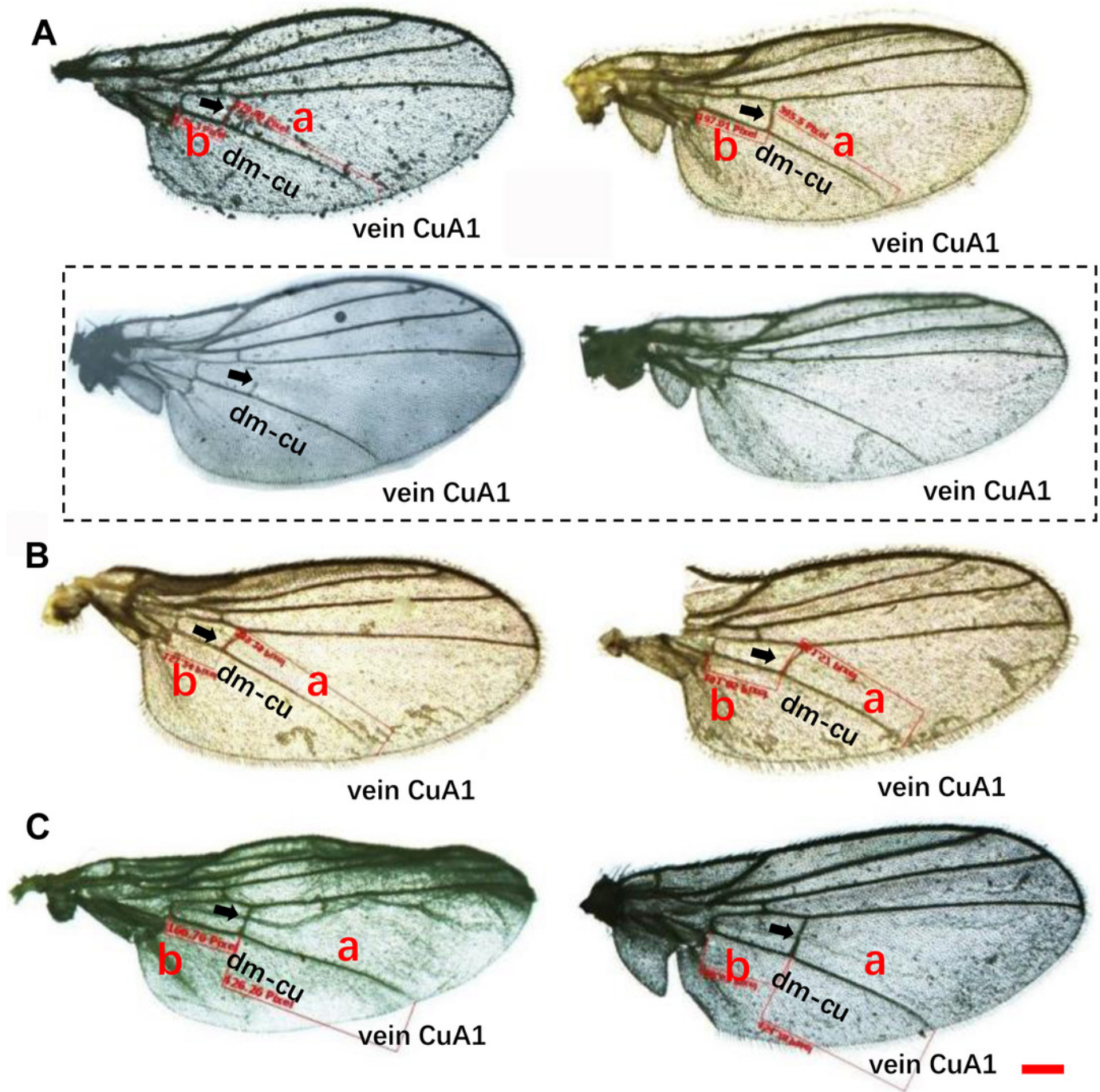
Figure 4

The ratio of the length of ultimate section of vein CuA1 divided by penultimate section.

Differences in the ratio length of ultimate section of vein CuA1 among different *Liriomyza* species were determined by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison ($P < 0.05$). The data in the figure is the average ± standard deviation.
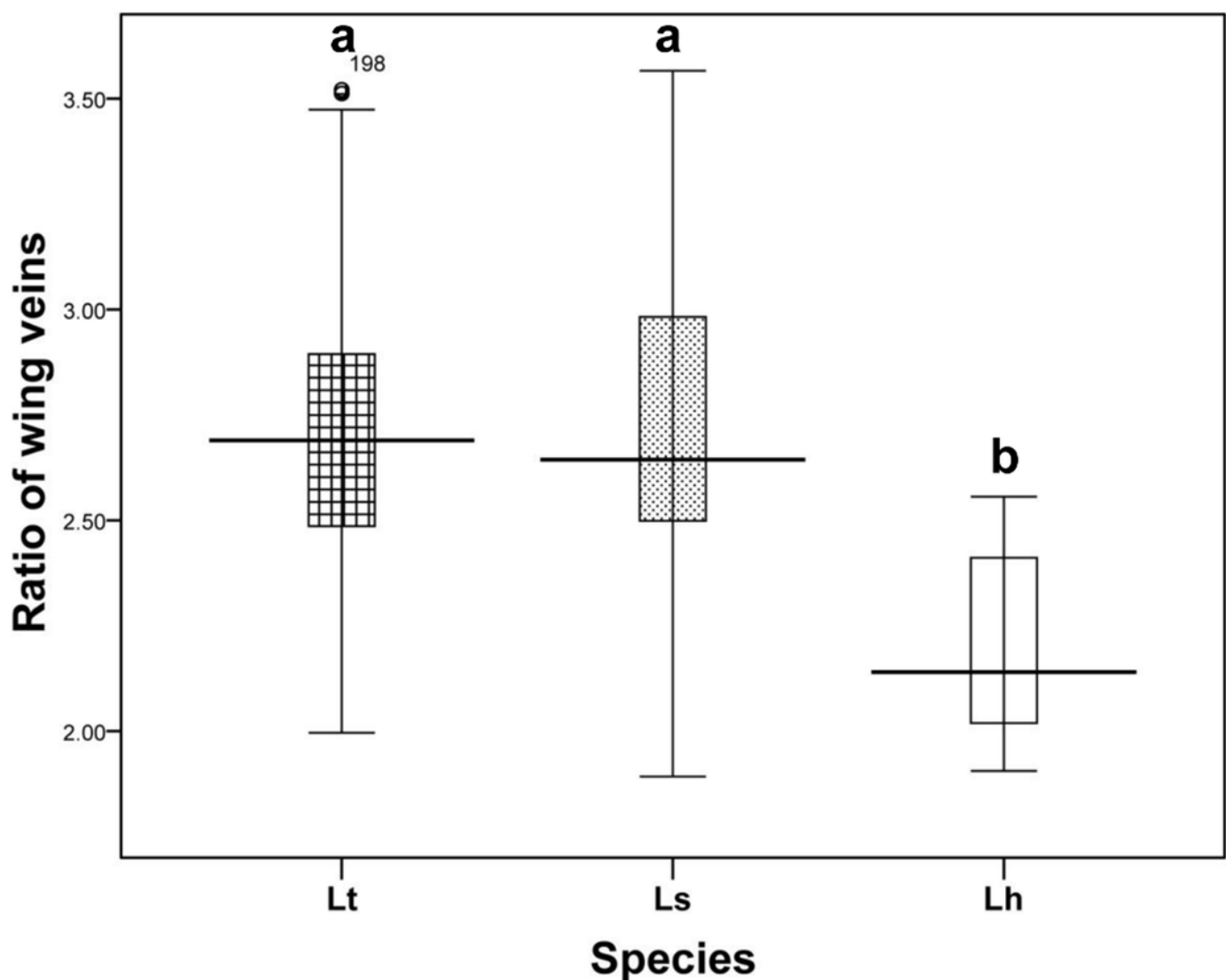
Figure 5

Diagrams of abdominal color patterns of three *Liriomyza* species.

A, *L. trifolii*; B, *L. sativae*; C, *L. huidobrensis*. Scale bar=0.1 mm.
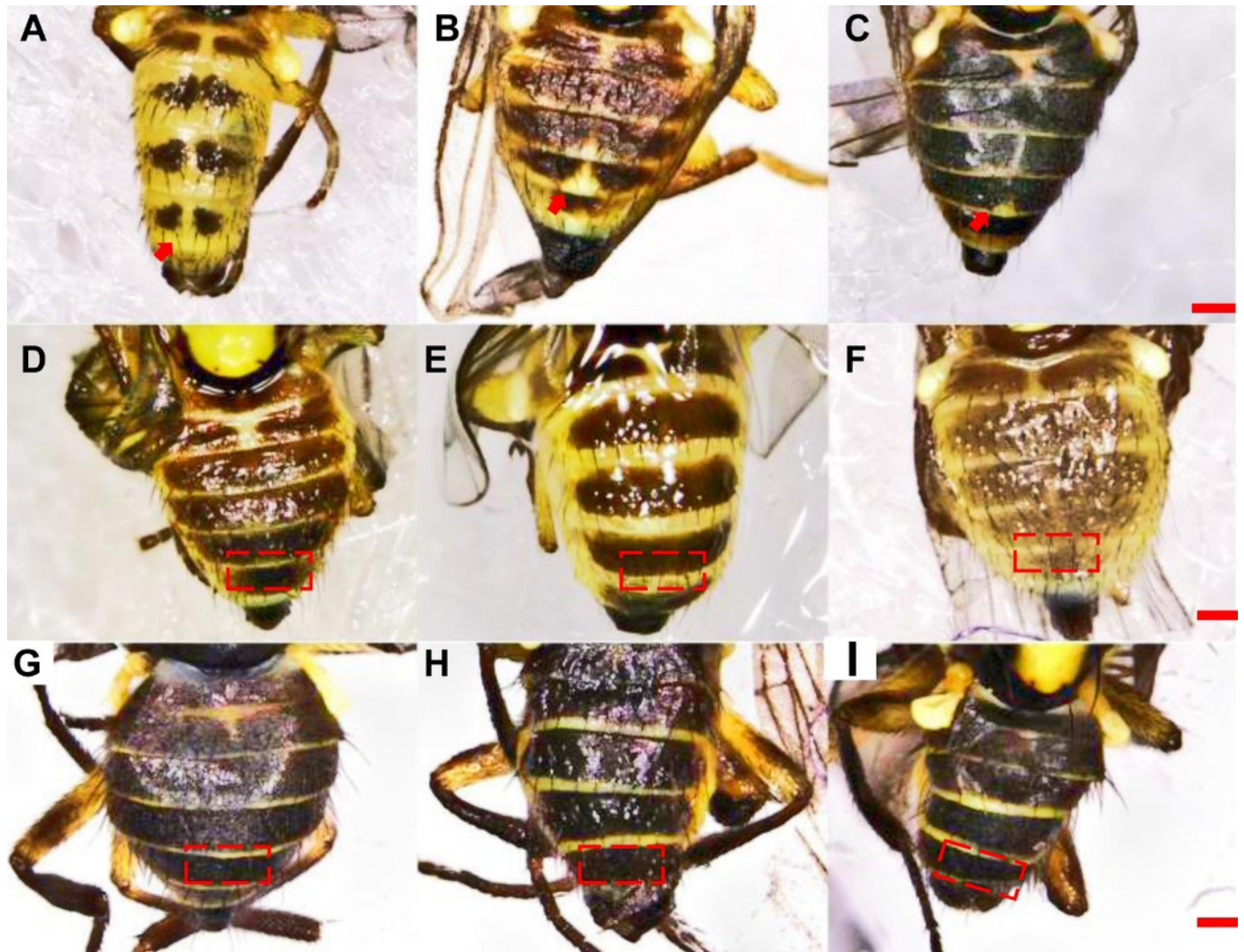
Figure 6

Alignment of *COI* sequences. Boxs indicate primer positions used in this paper.

Base substitutions are indicated by the shadow. Lt, *L. trifolii*; Ls, *L. sativae*; Lh, *L. huidobrensis*.
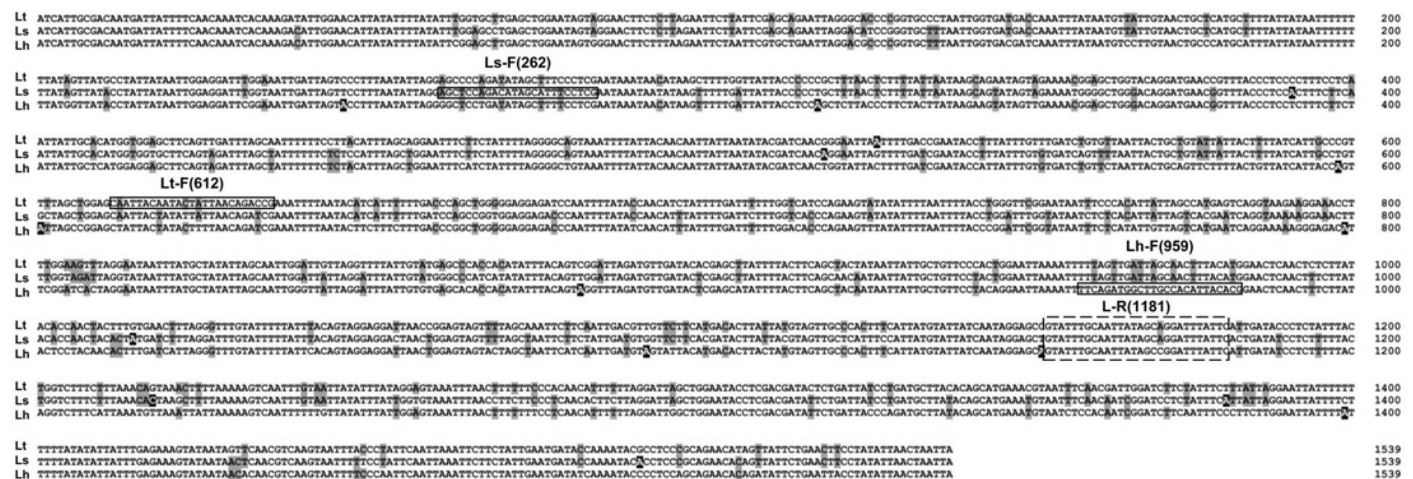
Figure 7

Agarose gel electrophoresis image of multiplex PCR products.

(A) DNA from different *Liriomyza* adults. (B) DNA from different developmental stages of *L. trifolii*. Each experiment has three biological repeats. Lt, *L. trifolii*; Ls, *L. sativae*; Lh, *L. huidobrensis*.