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 CuA1 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.
Revalidation of morphological characteristics and multiplex PCR for the identification of three congener invasive *Liriomyza* species (Diptera: *Agromyzidae*) in China

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ABSTRACT

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 CuA₁ 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.

Keywords: *Liriomyza*, morphological characteristics, abdominal tergites, multiplex PCR, species identification, *COI*
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
complex and difficult, molecular methods of identification are required. Immature developmental stages are the most common forms intercepted at ports of entry, therefore, it is important to identify these interceptions accurately and rapidly. With the development of mitochondrial and other molecular markers (Carapelli et al., 2018; Chen et al., 2019), several molecular methods have been developed to identify *Liriomyza* species (Menken & Ulenberg, 1983; Zehnder et al., 1983; Oudman et al., 1995; Chiu et al., 2000; Morgan et al., 2000). Multiplex PCR is a cost-effective, rapid, accurate method where identification can be determined by PCR product size with species-specific primers (Nakamura et al., 2013).

In this study, we re-verified morphological characteristics of three leafminers, *L. trifolii*, *L. sativae* and *L. huidobrensis*. A new morphological characteristic for detection of *L. trifolii* was investigated, and an improved molecular method for identification was developed based on multiplex PCR. This study provides approaches that can be deployed for identification of *Liriomyza* species, which will ultimately help future control efforts.

**MATERIALS AND METHODS**

**Insects**

The three species of *Liriomyza* spp. were collected from areas where leafminers occur in China. In this study, 263 individuals of three species were selected for further data analysis (Table S1). These were collected at the larval stage, tagged with relevant information and transported to the laboratory for pupation and emergence as adults. After preliminary morphological identification, adults were labeled, immersed in 70% ethanol and stored at -20ºC. After disecting and
photographing the samples, the remaining tissues were stored in 100% ethanol for DNA extraction and molecular analysis.

**Morphological identification**

Samples were examined with a stereomicroscope (Zeiss Stemi 2000c) and photographed with a wide depth of field (Zeiss Smartzoom 5). Male genitalia and wings were dissected, and slides were prepared and photographed with the Axio imager A2 (Zeiss, Germany).

Differences in the ratios of ultimate section lengths of vein CuA₁ among different *Liriomyza* species were determined by one-way analysis of variance (ANOVA), followed by Tukey’s multiple comparisons. All statistical analyses were performed using SPSS v. 16.0 (SPSS, Chicago, IL, USA), and statistical significance was determined when $P < 0.05$.

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

Genomic DNA of *Liriomyza* species was extracted using the AxyPrep™ Multisource Genomic DNA Kit (Axygen, USA). A partial sequence of the mitochondrial cytochrome oxidase I (*COI*) gene was amplified with common primers F, 5’-CAACATTTATTTGATTTTTG-3′ and R, 5’-TCCAATGCACTAATCTGCCATATTA-3’ (Simon et al., 1994; Yang et al., 2010) using protocols described by Chen et al. (2019), to molecular cross-checking and verification all of *Liriomyza* species in this study using sequencing, accession number can be found in Table S1. For multiplex PCR, full-length *COI* genes of three *Liriomyza* species were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/) and aligned using Clustal X. To develop a rapid
identification method, three species-specific primers and a common reverse primer were mixed to amplify DNA from different *Liriomyza* species. The PCR conditions were as follows: 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 min; followed by extension at 72 °C for 10 min. PCR was conducted in a 25 μL reaction volume containing 2 μL (100 ng) of DNA template, 1 μL (10 μM) of each primer, 12.5 μL of 2× Taq Master mix (Vazyme Biotech Co., Ltd) and 6.5 μL ddH₂O. PCR products were separated in 1.0% agarose gels, and primers that amplified only one specific band for each species are shown in Table 1.

**RESULTS**

**Morphological identification**

The distiphallus, which is part of the male genitalia, is a very small, fragile structure enclosed by membranes located at the terminus of the aedeagus. For *L. trifolii*, the morphological characteristics of the distiphallus include one distal bulb with marked constriction between lower and upper halves in dorsoventral view; the bulb is lightly sclerotized with a long basal stem (Fig. 1A). For *L. sativae*, the distiphallus is characterized by one distal bulb with a slight constriction between upper and lower halves in the dorsoventral view; the bulb is more intensely sclerotized with a shorter basal stem (Fig. 1B). For *L. huidobrensis*, the distiphallus contains two distal bulbs; these meet at rims that extend in an anteroventral orientation (Fig. 1C).

With respect to vertical setae, *L. trifolii* exhibits inner and outer vertical setae on a yellow background; whereas vertical setae are present on a black background for *L. huidobrensis*. In *L.
sativae, outer and inner vertical setae are presented on black and yellow backgrounds,
respectively (Spencer, 1973). In this study, only 86.1% (192/223) of L. trifolii had yellow inner
and outer vertical setae; 9.9% (22/223) had yellow inner vertical setae and undetermined color
for outer setae, and 4.0% (9/223) had yellow inner and black outer vertical setae (Table 2; Figs.
2A-C). For L. sativae, 17.6% (6/34) had black inner and outer vertical setae, 58.8% (20/34) had
yellow inner and black outer vertical setae, and 23.5% (8/34) had outer black setae with an
undetermined color for inner vertical setae (Table 2; Fig. 2D-F). For L. huidobrensis, 100% (6/6)
exhibited black inner and outer vertical setae (Table 2; Fig. 2G-I). These results show that
characteristics of vertical setae are not reliable for identifying Liriomyza species.

Wing pattern ratios were calculated as the length of the ultimate section of vein CuA₁
divided by the penultimate section (‘a’ and ‘b’, see Fig. 3A-C). In this study, ‘a’ was 2.70 ± 0.31
times the length of ‘b’ in L. trifolii, and ‘a’ was 2.72 ± 0.37 times the length of ‘b’ in L. sativae.
For L. huidobrensis, ‘a’ was 2.20 ± 0.24 times the length of ‘b’ ($F_{2,237} = 7.345$, $P < 0.05$) (Fig. 4).
Although the ratio of L. huidobrensis was significantly different from the other two species ($P <
0.05$), there was no significant difference between L. trifolii and L. sativae ($P = 0.907$). Many L.
trifolii individuals exhibited truncated or missing dm-cu cross veins. Furthermore, we noted
inconsistency between left and right forewing patterns within individual samples (Fig. 3A, with
dashed lines).

In L. trifolii, the 2nd-5th visible tergites were generally divided by a yellow medial furrow in
male adults; furthermore, there was a yellow patch at the 5th black visible tergite that can
distinguish L. trifolii from other Liriomyza species (Figure 5A-C). In L. sativae and L.
huidobrensis, only the second visible tergite is divided by a yellow medial furrow and no yellow patch is evident on the 5th tergite (Fig. 5D-I).

**Molecular detection of Liriomyza spp.**

Candidate primers for species-specific detection of Liriomyza were based on the alignment of 262 (L. sativae), 612 (L. trifolii), and 959 (L. huidobrensis) COI sequences. We designed one reverse primer, 1181 R, that was common to all three Liriomyza species. The position of forward primers was selected to produce <1000 bp amplicons when paired with the reverse primer with at least 300 bp nucleotides between species. In addition, sites were selected where the number of differential nucleotides was >2 bp to increase the specificity of the primers (Fig. 6).

The three Liriomyza species could be differentiated by specific PCR products in 1.0% agarose gels, and the resulting PCR products were 569, 919, and 222 bp for L. trifolii, L. sativae and L. huidobrensis, respectively (Fig. 7A). The validity of multiplex PCR for identification was further confirmed by using the system with different developmental stages; the approach worked equally well for larvae, pupae and adults of the three Liriomyza species (Fig. 7B). Populations from different geographical regions were also obtained to evaluate the reliability of species-specific primers. The results obtained by multiplex PCR (Fig. S1) and subsequent sequence analysis of COI (Fig. S2) showed that geography did not impact the reliability of primers.

**DISCUSSION**

The morphological characteristics used for Liriomyza identification have primarily followed...
Spencer's (1973) criteria. However, variability in life stages, emergence times and sample preservation result in large differences in body color and markings, which can make current morphological criteria unreliable for identification (Spencer, 1973; Kang et al., 1996; Shao, 2004).

Currently, the identification of *Liriomyza* spp. based on morphology is restricted to male adults because there are no reliable features for species-level identification of female adults or immature developmental stages (EPPO, 2005). The identification of adults requires the examination of the male adult genitalia. In general, the distiphallus provides reliable detection of the three *Liriomyza* species and has considerable diagnostic value (Spencer, 1973; Shiao, 2004). However, differences in distiphalluses between species are subtle and dissection is difficult for nonprofessionals. Consequently, features of distiphallic structure should be cross-checked with other external morphological characteristics to ensure that identification is valid.

According to Spencer (1973), coloration of the vertical setae is an important external feature that can distinguish *L. trifolii* and *L. sativae* without dissection; however, this feature is unstable and lacks clear interspecific boundaries. Results of the current study show that reliance on coloration of vertical setae can result in misidentification of *L. trifolii* and *L. sativae*; thus, this feature should only be used as a supplement for identification. The ratio of the length of the ultimate section of vein CuA₁ is unreliable since most ratio values overlapped among *Liriomyza* species. In this study, we also evaluated the patterns of abdominal tergites and discovered that the yellow patch at the 5th black visible tergite of *L. trifolii* is a new, reliable morphological characteristic for identification. Similar findings were reported for abdominal color patterns for
six *Liriomyza* species (Shiao, 2004).

Molecular methods for insect identification can be used with different developmental stages, including immature stages where morphological features may be lacking. Furthermore, molecular assays may facilitate identification of atypical or damaged samples. However, the specificity of molecular assays may be limited because they were developed for a particular purpose and evaluated against a restricted number of species (Nakamura et al., 2013). Multiplex PCR assays were recently developed for identification of *Liriomyza* species (Miura et al., 2004; Guan et al., 2006; Nakamura et al., 2013) and are based on amplification of a target gene region using species-specific primer combinations. Multiplex PCR assays are easier and faster than other molecular methods, such as RAPD-PCR, PCR-RFLP, DNA barcoding and real-time PCR (Chiu et al., 2000; Morgan et al., 2000; Scheffer et al., 2001; Kox et al., 2005; Scheffer et al., 2006; Blacket et al., 2015; Sooda et al., 2017); furthermore, multiplex PCR assays are more sensitive than enzyme electrophoresis methods (Zehnder et al. 1983; Menken & Ulenberg, 1983, 1986; Oudman et al., 1995). In general, the reliability and sensitivity of multiplex PCR represents a great improvement in molecular identification protocols and will enable us to manage invasive pests more effectively.

**CONCLUSIONS**

Invasive *Liriomyza* spp. comprise a group of insect pests that cause considerable economic loss and serious quarantine problems. In this study, morphological features were re-evaluated for *L. trifolii*, *L. sativae*, and *L. huidobrensis*, and the discriminative ability of traditional
morphological characteristics, such as male genitalia, abdominal color patterns, length of CuA₁ and abdominal tergite patterns were reevaluated. Furthermore, we developed an improved molecular identification method using multiplex PCR based on COI to identify the three *Liriomyza* species quickly and accurately. This study provides valuable tools for the identification of *Liriomyza* spp. using both morphological and molecular criteria.
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FIGURE LEGENDS

Figure 1. Lateral view of phaluses in three Liriomyza species. A, L. trifolii; B, L. sativae; C, L. huidobrensis.
Arrows indicate the distiphallus. Scale bar = 0.01 mm.

Figure 2. Color characteristics of outer and inner vertical setae in three Liriomyza species. A-C, L. trifolii; D-F, L. sativae; G-I, L. huidobrensis. Scale bar = 0.1 mm. Yellow arrows indicate the position of outer vertical setae, and red arrows indicate the position of inner vertical setae.

Figure 3. Comparison of wing pattern ratios for three Liriomyza species. Ratios were calculated as the length of the ultimate section of vein CuA1 divided by the penultimate section (‘a’ and ‘b’ sections). A, L. trifolii; B, L. sativae; C, L. huidobrensis. Scale bar = 0.1 mm. The panel outlined with dashed lines represents an abnormal wing pattern in L. trifolii. Black arrows indicate the position of dm-cu cross veins.

Figure 4. Ratios showing lengths of the ultimate section of vein CuA1 divided by the penultimate section. Differences among three Liriomyza species were determined by one-way analysis of variance (ANOVA), followed by Tukey’s multiple comparison (P < 0.05). The data represent mean ± SD. The interval in box plot means median value. Abbreviations: Lt, L. trifolii; Ls, L. sativae, and Lh, L. huidobrensis.

Figure 5. Abdominal color patterns of three Liriomyza species. A-C, L. trifolii; D-F, L. sativae; G-I, L. huidobrensis. Scale bar = 0.1 mm.
Figure 6. Alignment of COI sequences in Liriomyza spp. Sequences surrounded by rectangular boxes represent primers used in this paper. Base substitutions are indicated by shading. Lt, L. trifolii; Ls, L. sativae; Lh, L. huidobrensis.

Figure 7. Agarose gel electrophoresis of multiplex PCR products. (A) PCR products in three different Liriomyza species. (B) PCR products from different developmental stages of the three Liriomyza species. Each experiment contained three biological samples. Abbreviations: Lt, L. trifolii; Ls, L. sativae; Lh, L. huidobrensis.

Figure S1. Agarose gel electrophoresis of multiplex PCR products from different geographical populations of Liriomyza. Lt Lanes 1-10 indicate L. trifolii populations from: (1) Hengshui, (2) Hangzhou, (3) Dongguan, (4) Zhangzhou, (5) Qionghai, (6), Nanning, (7) Changzhou, (8) Nanchang, (9), Huizhou, and (10) Huzhou. Ls lanes 1-2 indicate L. sativae populations from Shangqiu and Luoyang, respectively. Lh lanes 1-2 represent L. huidobrensis populations from Kunming and the laboratory, respectively.

Figure S2. Alignment of COI sequences from different geographical population using species-specific primers. The shaded nucleotides represent divergent sites. Nucleotides bounded by red and blue dashed rectangles represent forward and reverse primers, respectively. Lt sequences represent the following L. trifolii populations: Lt 1, Hengshui; Lt 2, Hangzhou; Lt 3, Dongguan; Lt 4, Zhangzhou; Lt 5, Qionghai; Lt 6, Nanning; Lt 7, Changzhou; Lt 8, Nanchang; Lt 9, Huizhou; and Lt 10, Huzhou. Ls sequences represent L. sativae populations
from Shangqiu (Ls 1) and Luoyang (Ls 2). Lh sequences represent *L. huidobrensis* populations from Kunming (Lh 1) and the laboratory (Lh 2).
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) °C | 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      | GAATAAATCCKGCTATAATGCAAATAC  | 58 (50.9)  | -                 | -             |
Table 2 (on next page)

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

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*. 