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 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.

Subjects Agricultural Science, Entomology, Molecular Biology, Taxonomy, Zoology

Keywords *Liriomyza*, Morphological characteristics, Abdominal tergites, Multiplex PCR, Species identification, *COI*

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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*; *Spencer, 1990*; *Reitz et al., 1999*). Both larvae and

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adults cause serious damage to crops (*Musgrave, Poe & Bennett, 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, Wang & Wen, 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, Trumble & White, 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 −20 °C. After dissecting and photographing the samples, the remaining tissues were stored in 100% ethanol for DNA extraction and molecular analysis.

Table 1 Information of the primers designed 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	GAATAAATCCKGCTATAATTGCAAATAC	58 (50.9)	–	–

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 CuA_1 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^{TM} Multisource Genomic DNA Kit (Axygen, USA). A partial sequence of the mitochondrial cytochrome oxidase I (*COI*) gene was amplified with common primers F, 5′-CAACATTTATTTTGATTTTTTGG-3′ and R, 5′- TCCAATGCACTAATCTGCCATATTA-3′ (*Simon et al., 1994*; *Yang, Cao & Du, 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 ddH_2O. 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

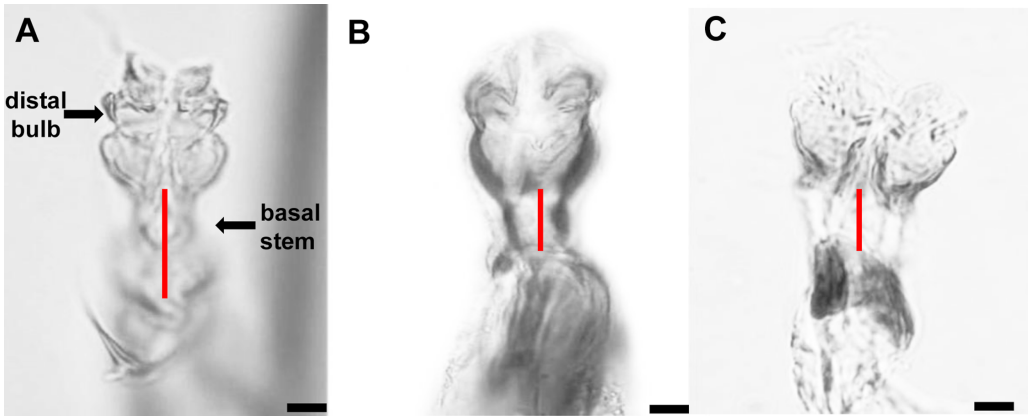

Figure 1 Photo plates of the phalluses of three *Liriomyza* species, lateral view. (A) *L. trifolii*; (B) *L. sativae*; C, *L. huidobrensis*. Arrows indicate the distiphallus. Scale bar = 0.01 mm.
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a slight constriction between upper and lower halves 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–2C). 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; Figs. 2D–2F). For *L. huidobrensis*, 100% (6/6) exhibited black inner and outer vertical setae (Table 2; Figs. 2G–2I). 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 CuA_1 divided by the penultimate section ('a' and 'b', see Figs. 3A–3C). In this study, 'a' was 2.70 $\pm$ 0.31 times the length of 'b' in *L. trifolii*, and 'a' was 2.72 $\pm$ 0.37 times the length of 'b' in *L. sativae*. For *L. huidobrensis*, 'a' was 2.20 $\pm$ 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 (Figs. 5A–5C). In *L. sativae* and *L.*

Table 2 The data of 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

Notes.
Y, yellow; B, black; U, unclear.

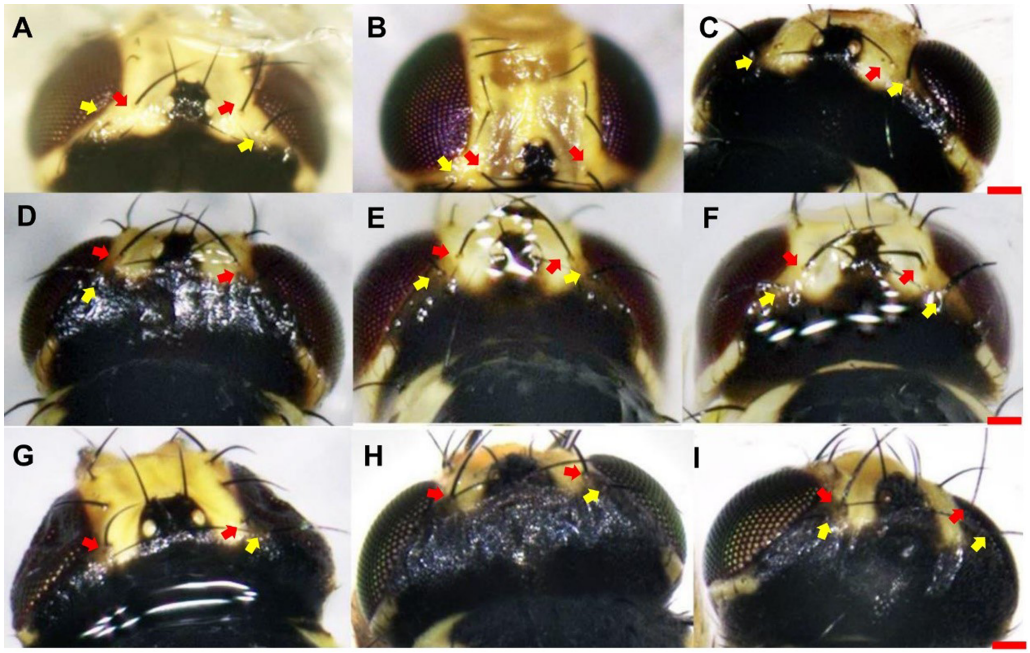

Figure 2 **The color characteristic of outer and inner vertical setae position in three *Liriomyza* species.** (A–C) *L. trifolii*; (D–F), *L. sativae*; G-I, *L. huidobrensis*. Scale bar=0.1 mm. The yellow arrow indicated the position of outer vertical setae and the red arrow indicated the position of inner vertical setae.
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huidobrensis, only the second visible tergite is divided by a yellow medial furrow and no yellow patch is evident on the 5th tergite (Figs. 5D–5I).

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

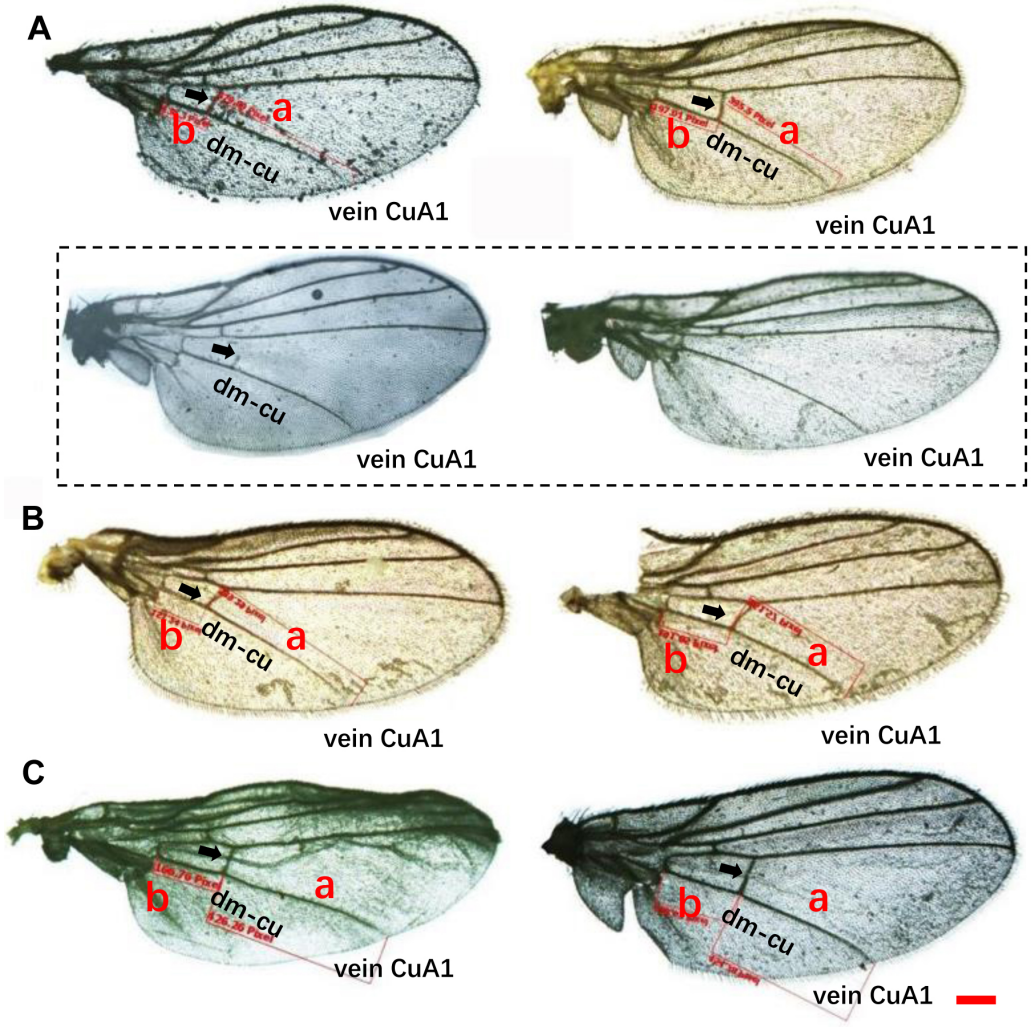

Figure 3 Comparison of the wing patterns of three *Liriomyza* species. The length of ultimate section of vein CuA_1 divided by penultimate section (a and b sections). (A), *L. trifolii*; (B), *L. sativae*; (C), *L. huidobrensis*. Scale bar=0.1 mm. Dot box represents abnormal wing pattern in *L. trifolii*.
Full-size DOI: 10.7717/peerj.10138/fig-3

one reverse primer, 1181 R, that was common to all three *Liriomyza* species. The position of forward primers was selected to produce < 1,000 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

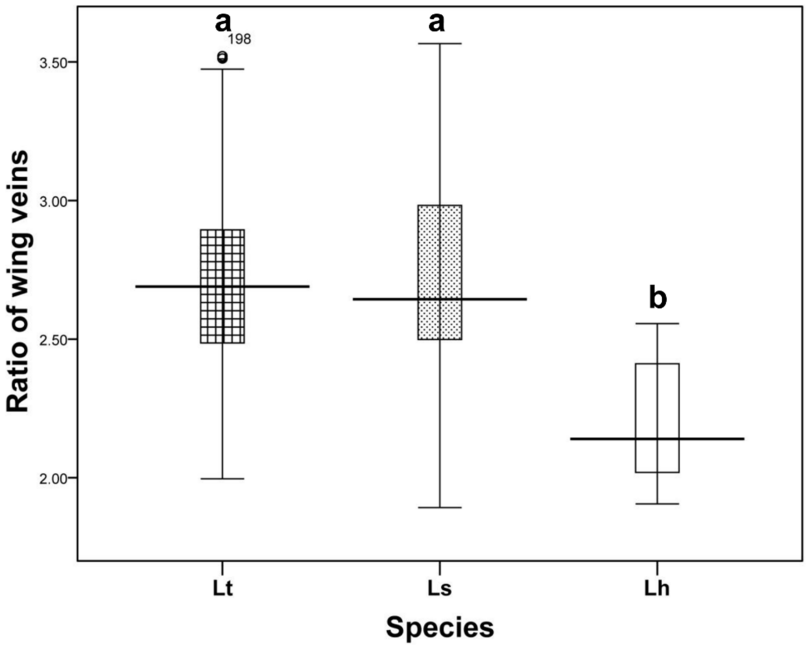

Figure 4 The ratio of the length of ultimate section of vein CuA1 divided by penultimate section. Differences in the ratio length of ultimate section of vein CuA1 among different *Liriomyza* species were determined by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison ($P < 0.05$). The data in the figure is the average ± standard deviation.

Full-size DOI: 10.7717/peerj.10138/fig-4

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, 1996*; *Shiao, 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.

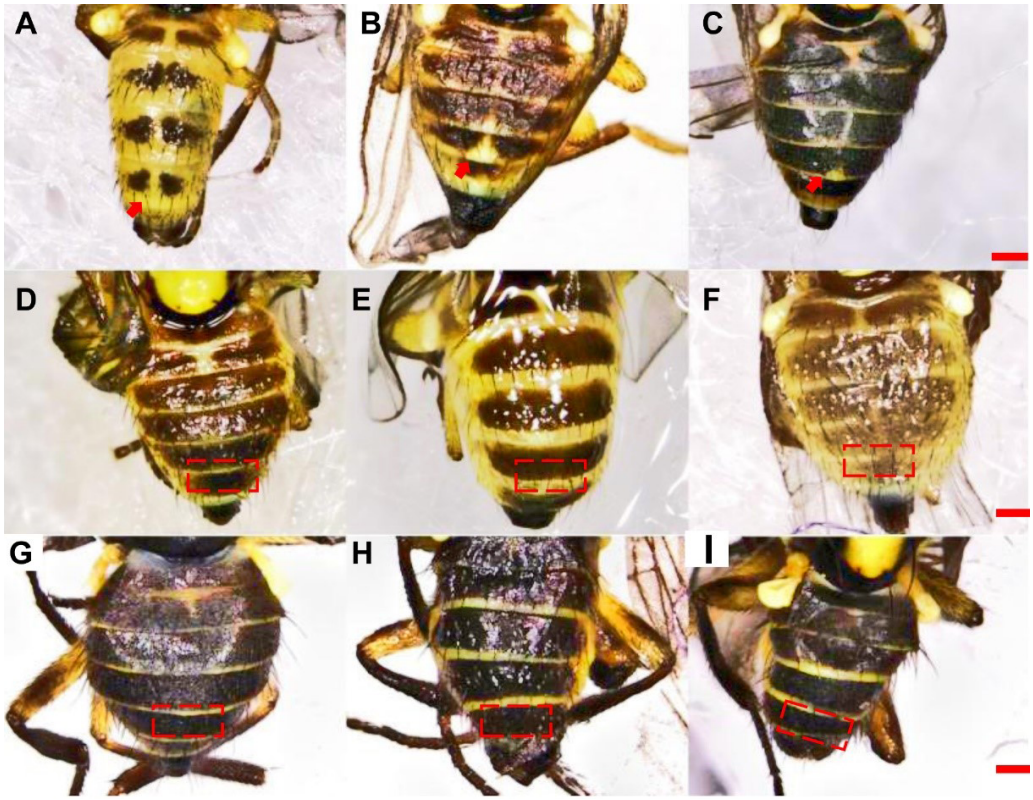

Figure 5 **Diagrams of abdominal color patterns of three *Liriomyza* species.** (A–C) *L. trifolii*; (D–F), *L. sativae*; (G–I), *L. huidobrensis*. Scale bar = 0.1 mm.

Full-size DOI: 10.7717/peerj.10138/fig-5

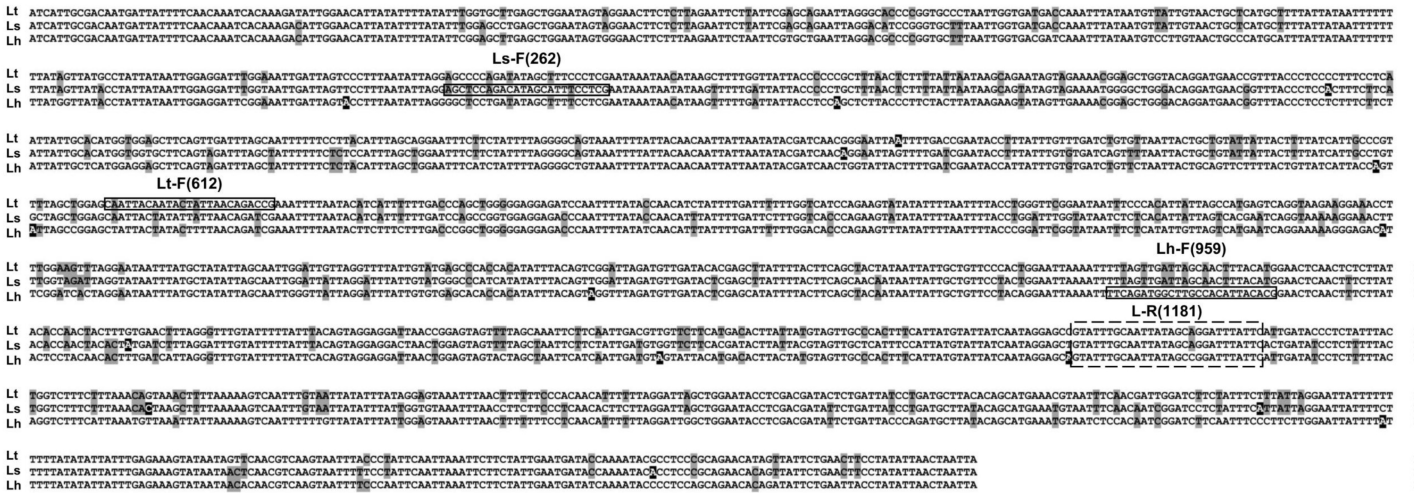

Figure 6 **Alignment of *COI* sequences. Boxs indicate primer positions used in this paper.** Base substitutions are indicated by the shadow. Lt, *L. trifolii*; Ls, *L. sativae*; Lh, *L. huidobrensis*.

Full-size DOI: 10.7717/peerj.10138/fig-6

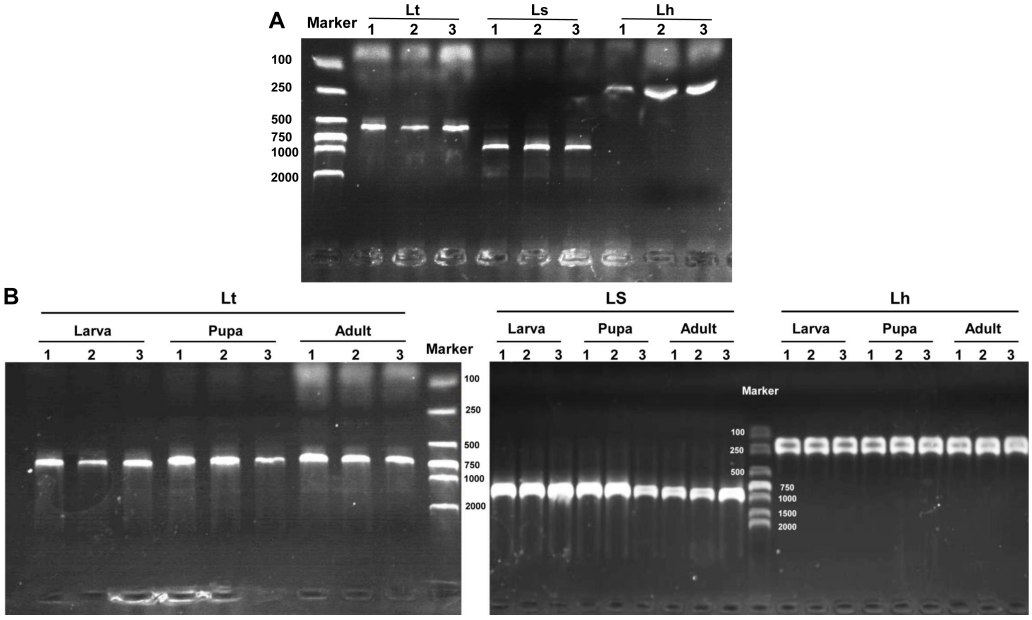

Figure 7 Agarose gel electrophoresis image of multiplex PCR products. (A) DNA from different *Liriomyza* adults. (B) DNA from different developmental stages of *L. trifolii*. Each experiment has three biological repeats. Lt, *L. trifolii*; Ls, *L. sativae*; Lh, *L. huidobrensis*.

Full-size DOI: 10.7717/peerj.10138/fig-7

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 CuA_1 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, Lewis & Ravindra, 2006*; *Blacket et al., 2015*; *Sooda et al., 2017*); furthermore, multiplex PCR assays are more sensitive than enzyme electrophoresis

methods (*Zehnder, Trumble & White, 1983*; *Menken & Ulenberg, 1983*; *Minkenberg & Van Lenteren, 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 CuA_1 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.

ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

Jing-Yun Chen, Si-Zhu Zheng, Yuan Gao, Yun-Fang Chen, and Yan-Feng Deng are employed by Suzhou Customs.

Author Contributions

- Ya-Wen Chang and Jing-Yun Chen conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Si-Zhu Zheng, Yuan Gao, Yunfang Chen and Yanfeng Deng analyzed the data, prepared figures and/or tables, and approved the final draft.

- Yu-Zhou Du conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.

DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:

The COI sequences amplified by pecies-specific primer designed in this study are available at GenBank: MT919718 (*L. trifolii*), MT919719 (*L. sativae*) and MT919720 (*L. huidobrensis*).

Data used for molecular cross-checking and verification all of Liriomyza species in this study were MT932588–MT932810 (*L. trifolii*), MT926413–MT926446 (*L. sativae*), and MT926447–MT926452 (*L. huidobrensis*).

Data Availability

The following information was supplied regarding data availability:

The raw measurements are available in the Supplemental Files.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.10138#supplemental-information.

REFERENCES

Blacket MJ, Rice AD, Semeraro L, Malipatil MB. 2015. DNA-based identifications reveal multiple introductions of the vegetable leafminer *Liriomyza sativae* (Diptera, Agromyzidae) into the Torres Strait Islands and Papua New Guinea. *Bulletin Entomology Research* **105**:533–544 DOI 10.1017/S0007485315000383.

Carapelli A, Soltani A, Leo C, Vitale M, Amri M, Mediouni-BenJemâa J. 2018. Cryptic diversity hidden within the leafminer genus *Liriomyza* (Diptera: Agromyzidae). *Genes* **9**:Article 554 DOI 10.3390/genes9110554.

Chandler LD, Gilstrap FE. 1987. Seasonal fluctuation and age structure of *Liriomyza trifolii* (Diptera, Agromyzidae) larval populations on bell peppers. *Journal of Economic Entomology* **80**:102–106 DOI 10.1093/jee/80.1.102.

Chang YW, Chen JY, Lu MX, Gao Y, Tian ZH, Gong WR, Dong CS, Du YZ. 2017. Cloning and expression of genes encoding heat shock proteins in *Liriomyza trifolii* and comparison with two congener leafminer species. *PLOS ONE* **12**:e0181355 DOI 10.1371/journal.pone.0181355.

Chen JY, Chang YW, Tang XT, Zheng SZ, Du YZ. 2019. Population genetics of *Liriomyza trifolii* (Diptera: Agromyzidae) and comparison with four *Liriomyza* species in China based on *COI*, *EF-1a* and microsatellites loci. *Scientific Reports* **9**:17856 DOI 10.1038/s41598-019-53886-9.

Chen NZ. 1999. Identification of *Liriomyza sativae* and other important *Liriomyza*. *Entomology Knowledge* **36**:222–226.

Chiu YC, Wu WJ, Shiao SF, Shih CJ. 2000. The application of RAPD-PCR to develop a rapid diagnostic technique for identification of 6 species of *Liriomyza*. *Chinese Journal of Entomology* **20**:293–309.

EPPO. 2005. *Liriomyza* spp. *EPPO Bulletin* **35**:335–344 DOI 10.1111/j.1365-2338.2005.00827.x.

Gao YL, Reitz SR, Xing ZL, Ferguson S, Lei ZR. 2017. A decade of leafminer invasion in China, lessons learned. *Pest Management Science* **73**:1775–1779 DOI 10.1002/ps.4591.

Guan W, Wang Z, Cai X, Wang Y, Chen D. 2006. Molecular identification of *Liriomyza trifolii* and *Liriomyza sativae*. *Chinese Journal of Entomology* **43**:558–561.

Johnson MW, Welter SC, Toscano NC, Tingi P, Trumble JT. 1983. Reduction of tomato leaflet photosynthesis rates by mining activity of *Liriomyza sativae* (Diptera, Agromyzidae). *Journal of Economic Entomology* **76**:1061–1063 DOI 10.1093/jee/76.5.1061.

Kang L. 1996. *Ecology and sustainable control of serpentine leafminers*. Beijing: Science Press.

Kox LFF, Van-den Beld HE, Lindhout BI, De Goffau LJW. 2005. Identification of economically important *Liriomyza* species by PCR-RFLP analysis. *EPPO Bulletin* **35**:79–85 DOI 10.1111/j.1365-2338.2005.00807.x.

Lei ZR, Wang Y, Wen JZ. 1996. Identification of 11 kinds of leafminers on vegetables. *Plant Protection* **22**:40–43.

Menken SBJ, Ulenberg SA. 1983. Diagnosis of the agromyzids *Liriomyza bryoniae* and *L. trifolii* by means of starch gel electrophoresis. *Entomologia Experimentalis et Applicata* **34**:205–208 DOI 10.1111/j.1570-7458.1983.tb03320.x.

Minkenberg DPTM, Van Lenteren JC. 1986. The leafminers *Liriomyza bryoniae* and *L. trifolii* (Diptera, Agromizae), their parasites and host plants, a review. *Agricultural University Wageningen Paper* **86**:1–50.

Miura K, Tagami Y, Ohtaishi M, Iwasaki A. 2004. Application of molecular techniques to distinguish *Liriomyza trifolii* from *L. sativae* on tomato cultivation in Japan. *Journal of Economic Entomology* **97**:964–969 DOI 10.1603/0022-0493(2004)097[0964:AOMTTD]2.0.CO;2.

Morgan DJW, Reitz SR, Atkinson PW, Trumble JT. 2000. The resolution of Californian populations of *Liriomyza huidobrensis* and *Liriomyza trifolii* (Diptera, Agromyzidae) using PCR. *Heredity* **85**:53–61 DOI 10.1046/j.1365-2540.2000.00731.x.

Motteoni JA, Broadbent AB. 1988. Wounds caused by *Liriomyza trifolii* (Diptera, Agromyzidae) as sites for infection of Chrysanthemum by *Pseudomonas cichorii*. *Canadian Journal of Plant Pathology* **10**:47–52 DOI 10.1080/07060668809501763.

Musgrave CA, Poe SL, Bennett DR. 1975. Leaf miner population estimation in polycultured vegetables. *Proceedings of the Florida State Horticultural Society* **88**:156–160.

Nakamura S, Masuda T, Mochizuki A, Konishi K, Tokumaru S, Ueno K, Yamaguchi T. 2013. Primer design for identifying economically important *Liriomyza* species (Diptera, Agromyzidae) by multiplex PCR. *Molecular Ecology Resources* **13**:96–102 DOI 10.1111/1755-0998.12025.

Oudman L, Aukema B, Menken SBJ, Ulenberg SA. 1995. A procedure for identification of polyphagous *Liriomyza* species using enzyme electrophoresis. *EPPO Bulletin* **25**:349–355 DOI 10.1111/j.1365-2338.1995.tb01477.x.

Reitz SR, Kund GS, Carson WG, Phillips PA, Trumble JT. 1999. Economics of reducing insecticide use on celery through low-input pest management strategies. *Agriculture, Ecosystems and Environment* **73**:185–197 DOI 10.1016/S0167-8809(99)00016-X.

Scheffer SJ, Lewis ML, Ravindra CJ. 2006. DNA barcoding applied to invasive leafminers (Diptera, Agromyzidae) in the Philippines. *Annals of the Entomological Society of America* **99**:204–210 DOI 10.1603/0013-8746(2006)099[0204:DBATIL]2.0.CO;2.

Scheffer SJ, Wijesekara A, Visser D, Hallet RH. 2001. Polymerase chain reaction-restriction fragment-length polymorphism method to distinguish *Liriomyza huidobrensis* from *L. langei* (Diptera, Agromyzidae) applied to three recent leafminer invasions. *Journal of Economic Entomology* **94**:1177–1182 DOI 10.1603/0022-0493-94.5.1177.

Shiao SF. 2004. Morphological diagnosis of six *Liriomyza* species (Diptera, Agromyzidae) of quarantine importance in Taiwan. *Applied Entomology and Zoology* **39**:27–39 DOI 10.1303/aez.2004.27.

Simon C, Frati F, Beckenbaeh AT, Crespi B, Liu H, Flook P. 1994. Evolution, weighting and phylogeneties utility of mitoehondrial gene sequences and compilation of conserved polymerase chain reaction Primers. *Annals of the Entomological Society of America* **87**:651–701 DOI 10.1093/aesa/87.6.651.

Sooda A, Gunawardana D, Li DM, Kumarasinghe L. 2017. Multiplex real-time PCR assay for the detection of three invasive leafminer species, *Liriomyza huidobrensis*, *L. sativae* and *L. trifolii* (Diptera, Agromyzidae). *Austral Entomology* **56**:153–159 DOI 10.1111/aen.12237.

Spencer KA. 1973. *Agromyzidae (Diptera) of economic importance 9, series entomologica*. Bath: The Hague Publishers.

Spencer KA. 1990. *Host specialization in the world Agromyzidae (Diptera) 45, series entomologica*. Dordrecht: Kluwer.

Yang F, Cao JM, Du YZ. 2010. Survey and molecular identification of *Liriomyza trifolii* in Jiangsu, China. *Plant Protection* **36**:108–111.

Zehnder GW, Trumble JT, White WR. 1983. Discrimination of *Liriomyza* species (Diptera, Agromyzidae) using electrophoresis and scanning microscopy. *Proceedings of the Entomological Society of Washington* **85**:564–574.

Zitter TA, Tsai JH. 1977. Transmission of three potyviruses by the leafminer *Liriomyza sativa* (Diptera, Agromzidae). *Plant Disease Reporter* **61**:1052–1029.