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DNA-based identifications reveal multiple introductions of the vegetable leafminer *Liriomyza sativae* (Diptera: Agromyzidae) into the Torres Strait Islands and Papua New Guinea

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Abstract

Leafmining flies (Diptera: Agromyzidae) can be serious economic pests of horticultural crops. Some genera such as *Liriomyza* are particularly problematic with numerous species, some of which are highly polyphagous (wide host range), which can only be confidently identified morphologically from adult males. In our study, DNA barcoding was employed to establish new locality records of the vegetable leafminer fly, *Liriomyza sativae*, from the islands of Torres Strait (Queensland, Australia) and the central highlands of Papua New Guinea (PNG). These records represent significant range extensions of this highly invasive plant pest. Specimens of immature leafminers (from leaf mines) were collected over a 5-year period during routine plant health surveys in ethanol or on FTA® filter paper cards, both methods proved effective at preserving and transporting insect DNA under tropical conditions, with FTA cards possessing some additional logistical benefits. Specimens were identified through sequencing two sections of the cytochrome oxidase I gene and the utility of each was assessed for the identification of species and intra-specific genetic lineages. Our study indicates that multiple haplotypes of *L. sativae* occur in PNG, while a different haplotype is present in the Torres Strait, with genetic regionalization between these areas apart from a single possible instance – one haplotype 'S.7' appears to be common between these two regions – interestingly this has also been the most common haplotype detected in previous studies of invasive *L. sativae* populations. The DNA barcoding methods employed here not only identified multiple introductions of *L. sativae*, but also appear generally applicable to the identification of other agromyzid leafminers (Phytomyzinae and Agromyzinae) and should decrease the likelihood of potentially co-amplifying internal hymenopteran parasitoids. Currently, *L. sativae* is still not recorded from the Australian mainland; however, further sampling of leafminer flies from Northern Australia and surrounding areas is required, as surveillance for possible *Liriomyza* incursions, as well as to characterize endemic species with which *Liriomyza* species might be confused.

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Introduction

Agromyzidae contains over 2500 species of small, morphologically similar flies whose larvae feed internally on plants, often as leaf or stem miners (Spencer, 1990; Winkler *et al.*, 2009). Nearly all species are very host-specific but a few highly polyphagous species have become important pests of agriculture and horticulture in many parts of the world. The key agromyzid pest species that pose a significant quarantine threat to agriculture and horticulture are: *Liriomyza bryoniae* (Kaltenbach), *Liriomyza cicerina* (Rondani), *Liriomyza huidobrensis* (Blanchard), *Liriomyza sativae* Blanchard, *Liriomyza strigata* (Meigen), *Liriomyza trifolii* (Burgess), *Chromatomyia horticola* (Goureau), *Phytomyza syngenesiae* Hardy and *Amauromyza maculosa* (Malloch) (Spencer, 1973, 1990; Nakamura *et al.*, 2013). Typically, these polyphagous leafminers are considered to spread via the movement of infested plants, often on ornamentals such as chrysanthemum (Spencer, 1989). While adult leafminers are free flying, immature life stages often occur within plant leaves, as eggs just below the surface, and as larvae and pupae within leaf mines or in adjacent soil (Spencer, 1973). Fully-formed mines should be readily visible to quarantine officials, however signs of early infestations are not always obvious and can be easily overlooked (Spencer, 1989).

Liriomyza sativae

Only five of the more than 300 species of the genus *Liriomyza* are considered to be truly polyphagous (Spencer, 1973). Of these one of the most serious pests of vegetable and flower crops is *L. sativae* (CABI, 2013), which can cause agricultural crop losses of up to 80% (Spencer, 1973). *Liriomyza sativae* is highly polyphagous, having been recorded from at least nine plant families, although it appears to favour Cucurbitaceae, Fabaceae and Solanaceae (Spencer, 1973, 1990). Originally *L. sativae* was restricted to the Americas (Spencer, 1973; Scheffer & Lewis, 2005), but it is now much more widespread, having been introduced to (CABI, 2013): Europe; Africa; Middle east; Asia (China, India, Indonesia, Japan, Malaysia, Sri Lanka, Thailand, Vietnam); Oceania (American Samoa, Cook Islands, Federated States of Micronesia, French Polynesia, Guam, New Caledonia, Northern Mariana Islands, Samoa, Vanuatu); but to date it has not been recorded from Papua New Guinea (PNG) or from the Australian mainland (CABI, 2013). However, *L. sativae* has previously been detected from a single island in the Torres Strait – the body of water that separates Australia from PNG (discussed in greater detail below) – from a single plant that was destroyed (IPPC, 2008; CABI, 2013).

Australia's proximity to countries with different plant health statuses represents a tangible biosecurity risk to Australia's primary industries (Maynard *et al.*, 2004; Anderson *et al.*, 2010; Anderson & Congdon, 2013). Recognition of this risk in Northern Australia led to the establishment of the Northern Australia Quarantine Strategy (NAQS). NAQS carries out plant health surveillance on Australia's northern coastline, islands of the Torres Strait, and countries to the near north, monitoring insect pests, plant diseases and weeds that might move onto the Australian mainland. On a routine NAQS plant health survey in 2008, carried out in collaboration with Papua New Guinea's National Agriculture Quarantine Inspection Authority (NAQIA), adult flies suspected to be a species of *Liriomyza* were collected from a heavily mined tomato plant on Warraber Island in the Torres Strait (IPPC, 2008). Initial identification, using external morphological characters, suggested the fly to be *L. sativae* which was later confirmed by dissection of male genitalia (examined by M. Malipatil).

Morphological identification

Generally, identification of leafminer species by morphological examination is problematic as only adult male specimens can be reliably identified, and there are no adequate diagnostic keys for the identification of eggs, larvae or pupae (Malipatil & Ridland, 2008). The primary method of identification from adult morphological characters is examination of the male genitalia; indeed, species confirmation cannot be achieved without the examination of adult males (e.g. Spencer, 1973; Lambkin *et al.*, 2008). To achieve morphological identification these characters must be examined using a high-power microscope, i.e. ×100 magnification (Malipatil & Ridland, 2008). Morphological identification of species is further complicated by the presence of cryptic species within many of the important polyphagous *Liriomyza* species (e.g. Scheffer, 2000; Takano *et al.*, 2008). Differentiation of some of these species requires the use of molecular techniques (e.g. Scheffer *et al.*, 2001, 2006).

Molecular identification

Due to the difficulties involved in morphological identification of leafminer specimens from most life stages, allozyme and restriction fragment length polymorphism (RFLP) tests have been used for identification of some *Liriomyza* species (summarized in Nakamura *et al.*, 2013). In Australia, two molecular tests, have been previously adopted (summarized in Semeraro & Malipatil, 2007) to potentially identify three exotic (to Australia) *Liriomyza* species – *L. huidobrensis*, *L. sativae* and *L. trifolii* – that have recently spread throughout Southeast Asia (these invasions are summarized in Andersen *et al.*, 2008). These tests both apply PCR–RFLP methods to distinguish these species from each other and from a limited number of Australian endemic or currently established *Liriomyza* species. The first test (Bjorksten & Hoffmann, 2005) was developed to examine the mitochondrial cytochrome oxidase I (COI) gene region (fig. 1) to distinguish between eight *Liriomyza* species. Notably, Bjorksten & Hoffmann (2005) also tested parasitoids to ensure these would not interfere with the leafminer PCR–RFLP test. Kox *et al.* (2005) developed an RFLP

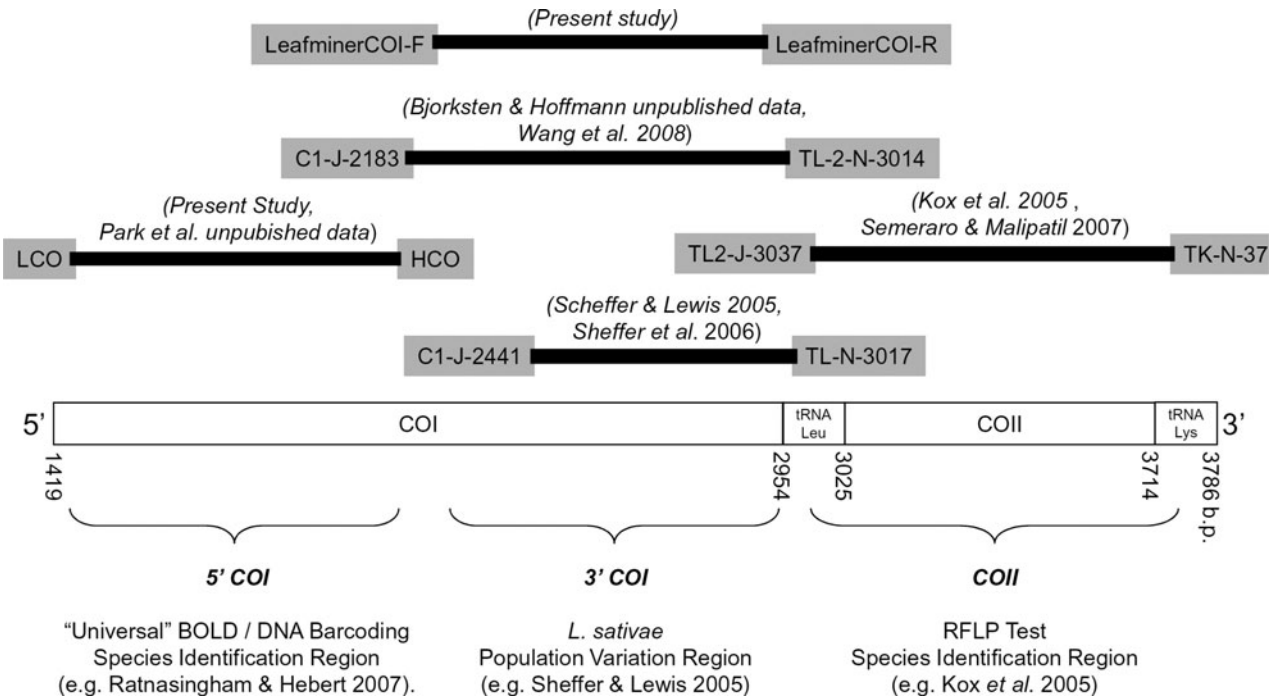

Fig. 1. Regions of COI and COII mtDNA genes employed in previous RFLP, DNA barcoding and population genetic studies (black bars). Gene boundary numbering follows the complete mtDNA genome sequence of *L. sativae* (GenBank NC_015926, Yang *et al.*, 2011); PCR primers employed in each study are indicated at the ends of each amplified region (grey boxes).

test for the mitochondrial cytochrome oxidase II (COII) gene region (fig. 1) to distinguished eight *Liriomyza* species of economic concern; an additional exotic pest species *L. cicerina* (chickpea leafminer) and endemic *Liriomyza chenopodii* (beet leafminer) were added to this test by Semeraro & Malipatil (2007). Additional alternative molecular identification PCR-based protocols employing species-specific primers (for the COI gene) have also been developed for a limited number of *Liriomyza* species (Miura *et al.*, 2004; Nakamura *et al.*, 2013).

DNA barcoding (DNA sequence species identification) is potentially a much more informative method of molecular identification, as it directly characterizes a greater number of variable sites than the other molecular methods outlined above (e.g. Armstrong & Ball, 2005), and can utilize the large number of reference specimens that are now present on DNA sequence databases (e.g. Blacket *et al.*, 2012). In common with other invasive insect species (Boykin *et al.*, 2012) a variety of DNA regions have previously been utilized for DNA sequence identification of leafminers. The 3′ region of the mitochondrial COI gene (fig. 1) has been used both to identify agromyzid species in a DNA barcoding approach (Scheffer *et al.*, 2006) and assess genetic variation within *L. sativae* populations (Scheffer & Lewis, 2005; Scheffer *et al.*, 2006; Wang *et al.*, 2008); while the 5′ end of the mitochondrial COI (fig. 1), generally considered the 'Universal' DNA sequence identification region employed in DNA barcoding (Ratnasingham & Hebert, 2007), is beginning to be employed for leafminer identification (Bhuiya *et al.*, 2011; Maharjan *et al.*, 2014), with a large number of species (>300) now with reference DNA barcodes (BOLD, accessed March 2015).

All molecular identification tests outlined above employ PCR amplification of mitochondrial DNA (fig. 1) and rely on adequately preserved samples. Mitochondrial DNA is relatively robust to degradation and can often be obtained from old preserved (pinned) reference specimens (e.g. Strutzenberger *et al.*, 2012). However, preservation of field collected soft bodied invertebrate samples (i.e. larvae/pupae) can prove difficult, relying on freezing or storing in ethanol which can often cause logistical issues (Moreau *et al.*, 2013), collecting in tropical environments adds even more challenges. Filter paper cards (FTA® cards) are an alternative that have been successfully employed in collecting invertebrate DNA (e.g. Régnier *et al.*, 2011) that is robust to storage conditions; however, FTA cards have not yet been widely utilized for field collection of larval insects.

Current study – objectives

The objectives of the present study were: (1) To confirm the initial morphological species identification of *L. sativae* from the Torres Strait, through DNA barcoding, and provide new locality/host records for additional specimens collected from recent NAQS surveys in the Torres Strait and PNG. (2) To demonstrate the effectiveness of preserving and transporting DNA from field collected insects on FTA cards sampled under tropical conditions. (3) To assess genetic variation present in any new *L. sativae* incursions detected compared with previously sampled populations throughout the world. (4) To compare the sequence similarity of a pair of novel agromyzid-specific DNA barcoding primers, and other previously employed universal primers, against agromyzid and hymenopteran parasitoid DNA sequences and (5) To test the DNA barcoding methods employed here on some other agromyzid species to assess their general applicability.

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Materials and methods

Specimens examined

Initially, several adult male specimens were aspirated from the foliage of an infested tomato plant in August 2008 from Warraber Island in the Torres Strait and identified morphologically as *L. sativae* (determined by M. Malipatil), through dissecting and examining the male terminalia (table 1). Further NAQS surveys conducted over 5 years from eight islands across the Torres Strait as well as from the highlands of PNG (>1500 m elevation) resulted in additional *Liriomyza* (larvae and pupae, table 1), which could not be identified to species morphologically. Immature specimens collected from the field were preserved (table 1) in ethanol or squashed onto Whatman FTA® (FTA cards) and identified from DNA sequences (i.e. DNA barcoding), as outlined below. Duplicate adult and immature specimens have been lodged in insect reference collections (table 1). The DNA barcoding identification methods employed here (see below) were also tested on a number of other agromyzid species (table 2).

DNA extraction

DNA was extracted from immature agromyzid specimens using a commercially available kit (DNeasy® Blood and Tissue Kit; Qiagen) following the manufacturers protocol. FTA card extractions included some extra steps modified from a Qiagen protocol: 'Isolation of Total DNA from FTA and Guthrie Card' (Qiagen, 2010). Briefly, a 2 mm^2 paper square containing part of the specimen was removed from the FTA card using a single-use sterile disposable scalpel blade; care was taken to try to not remove the entire sample, to retain some for potential future extractions/PCR assays. The excised FTA card sample was incubated in ATL buffer (280 µl) and Proteinase K (20 µl) at 56°C for 1 h (vortexing every 15 min), then buffer AL (300 µl) was added and samples were incubated for 10 min at 70°C (vortexing every 3 min). Finally, ethanol (150 µl) was added and the sample was processed in a QIAGEN column according to the manufacturers DNeasy protocol, including a final 'double' DNA elution step, resulting in a final volume of 200 µl of DNA in AE buffer.

PCR amplification and sequencing

Two sections of the COI gene were examined in this study (fig. 1): (1) The 'Universal' DNA barcoding region – the 5′ region of COI – amplified using standard Folmer *et al.* (1994) primers, LCO/HCO (size 703 b.p.); (2) A non-overlapping second region – the 3′ region of COI – which has previously been examined in a large number of *L. sativae* (Scheffer & Lewis, 2005; Scheffer *et al.*, 2006) was amplified using novel primers that almost fully overlap with the previously published sequence data (size 738 b.p.). The new primers LeafminerCOI-F 5′-CATTATTAGYCAHGAATCWGG-3′ and LeafminerCOI-R 5′-TCTGCDGGAGGNGTATTTTG-3′, were designed using Primer 3 (Rozen & Skaletsky, 2000) to match agromyzid sequences present on GenBank (including representatives of *Chromatomyia*, *Ophiomyia*, *Liriomyza*, *Napomyza*, *Phytomyza:* from Scheffer *et al.*, 2007; Winkler *et al.*, 2009; and *Liriomyza:* from Scheffer *et al.*, 2006; Wang *et al.*, 2008, 2011).

The new agromyzid-specific primers used in the present study for the amplifications of the 3′ region of COI (fig. 1) have an advantage over universal PCR primers, such as LCO/HCO (see Supplementary table 1), in being less likely to co-amplify parasitoid DNA that may be present within the agromyzid pupae/larvae and might be co-amplified during PCR (e.g. Nakamura *et al.*, 2013), potentially interfering with a DNA-barcoding specimen identification approach. Previous studies of leafminer parasitoids in Australia (Bjorksten *et al.*, 2005; Lambkin *et al.*, 2008) and Southeast Asia (Rauf *et al.*, 2000; Prijono *et al.*, 2004; Fisher *et al.*, 2005; Tran, 2009) have shown that the most common hymenopteran parasitoids belong to Chalcidoidea, Cynipoidea and Ichneumonoidea, with Eulophidae (Chalcidoidea) wasps being particularly abundant. The sequences of four pairs of primers for amplifying sections of COI in leafminers (fig. 1) were compared with previously obtained leafminer and hymenopteran parasitoid DNA sequences (from Chalcidoidea and Ichneumonoidea, available on GenBank) to assess primer and DNA sequence similarity (Supplementary table 1). However, these primers were not trialled experimentally on parasitoid DNA samples in the present study.

All PCR amplifications in the present study used 5 µl of template DNA in 25 µl PCR reactions (including: 1 × BSA, 1 × NEB ThermoPol Reaction Buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of each primer & 1 unit of NEB Taq DNA Polymerase) performed in an Eppendorf thermocycler (epgradientS). PCR profiles for both primer pairs included an initial denaturing step of 94°C for 2 min, followed by 40 cycles of 30 s steps at 94, 50 and 72°C, with a final extension step of 72°C for 2 min. These primers and PCR conditions were employed to successfully amplify and sequence the two sections of COI (5′ & 3′) in a range of agromyzids, including both Phytomyzinae and Agromyzinae (table 2). DNA sequencing was conducted commercially on an ABI sequencer through Micromon (Monash University) and Macrogen (Korea).

Sequence comparison and phylogenetic analysis

DNA barcoding species identification of agromyzid species (identifying them from their best matches on public databases) was conducted using both the 5′ and 3′ regions of COI (fig. 1). Sequences from the 5′ region of COI (i.e. LCO/HCO) were compared with 'All Barcode Records on Bold', i.e. sequences >500 bp in length on the BOLD database (Ratnasingham & Hebert, 2007); while sequences from the 3′ region of COI (i.e. LeafminerCOI-F/R) were compared with previously published leafminer sequences on the NCBI GenBank database through Blastn Searches. All of the DNA sequences from the present study ($n = 84$) have been submitted to GenBank (tables 1 and 2).

Comparisons of DNA sequences and phylogenetic analyses were performed in MEGA 5.0 (Tamura *et al.*, 2011). Genetic variation within *L. sativae* populations in the Torres Strait ($n = 8$) and PNG ($n = 3$) was assessed through generating a neighbour-joining tree (Supplementary fig. 1) that included previously published sequences (from the 3′ region of COI, which currently has the largest number of reference sequences available) from '*L. sativae*-W' group (i.e. the group of *L. sativae* that has expanded its range beyond the Americas) specimens; from Scheffer & Lewis (2005) $n = 82$, Scheffer *et al.* (2006) $n = 107$, Wang *et al.* (2008) $n = 6$, Yang *et al.* (2011) $n = 1$. Sequences were compared using pairwise comparisons to account for sequence length differences between datasets (which varied from approximately 500 to 700 bp). The frequency and geographic distribution of haplotypes detected in the Torres Strait and PNG in the present study were compared with

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Table 1. *Liriomyza sativae* specimens from the Torres Strait and Papua New Guinea examined through DNA barcoding.

Species	Specimen collected	Host	Collector	Date	Specimen[1] preservation	*n*	MtDNA[2] haplotype	GenBank accession
Liriomyza sativae	Torres Strait, Warraber (Sue) Island	Tomato	A. Rice, D. Tenakanai, B. Waterhouse	Aug 2008	Ethanol[3]	2	S.27	KR476579
L. sativae	Torres Strait, Masig (Yorke) Island	Pumpkin & Scarlet Wisteria	L. Halling	7 Jun 2010	Ethanol	2	S.27	KR476579
L. sativae	Torres Strait, Mabuiag Island	Siratro	L. Halling	2 Jun 2010	Ethanol	1	S.27	KR476579
L. sativae	Torres Strait, Erub (Darnley) Island	Tomato	A.D. Rice & S. Cowan	Mar 2011	Ethanol[3]	1	S.7	KR476573
L. sativae	Papua New Guinea, Goroka	Tomato	A.D. Rice & S. McKenna	24 Jun 2011	FTA card[3]	4	S.7	KR476573
L. sativae	Papua New Guinea, Hati (near Korn)	Tomato	A.D. Rice	24 Jun 2011	FTA card	4	S.7	KR476573
L. sativae	Papua New Guinea, Minj Village (Hagen)	Tomato	A.D. Rice	24 Jun 2011	FTA card	4	S.7 & S.28	KR476573 KR476580
L. sativae	Torres Strait, Poruma (Coconut) Island	Tomato	A.D. Rice	17 Aug 2011	Ethanol	2	S.27	KR476579
L. sativae	Torres Strait, Poruma (Coconut) Island	Yellow Bells	A.D. Rice	31 Jul 2012	FTA card	1	S.27	KR476579
L. sativae	Torres Strait, Warraber (Sue) Island	Siratro	A.D. Rice	31 Jul 2012	FTA card	5	S.27	KR476579
L. sativae	Torres Strait, Iama (Yam) Island	Castor Oil Plant	A.D. Rice	23 Mar 2012	FTA card	2	S.27	KR476579
L. sativae	Torres Strait, Ngurapai (Horn) Island	Siratro	A.D. Rice	26 May 2012	FTA card	4	S.27	KR476579
L. sativae	Torres Strait, Waiben (Thursday) Island	Curcurbitae	S. Cowan	16 Jun 2013	FTA card	1	S.27	KR476579
L. sativae	Torres Strait, Waiben (Thursday) Island	Tomato	S. Cowan	17 Jun 2013	FTA card	1	S.27	KR476579

[1]Immature specimens (larvae/pupae) preserved in ethanol or FTA cards.
[2]Haplotype names follow Scheffer & Lewis (2005) for the 3′ region of COI.
[3]Duplicate associated specimens: adults pinned and larvae/pupae in 70% ethanol in NAQS & VAIC (Victorian Agricultural Invertebrate Collection, DEDJTR Victoria) insect reference collections.

Table 2. DNA barcoding identification of agromyzid leafminers.

Species[1]	Subfamily	Specimen code	Specimen collected	Host	Collector	Date	Specimen preservation	BOLD[2] identification	GenBank[3] identification	GenBank acccession
Liriomyza chenopodii	Phytomyzinae	Lcp50	Australia, Vic., Knoxfield	Chickweed	P. Ridland	6 Sep 2005	Ethanol	<90% Phytomyzinae	<89% Phytomyzinae	KR476569
Liriomyza brassicae	Phytomyzinae	Lb137	Australia, Vic., Knoxfield	Laboratory colony	M. Robinson	24 May 2004	Ethanol	100% *L. brassicae*	99% *L. brassicae*	KR476570
Liriomyza cicerina	Phytomyzinae	Lc118	Syria, Aleppo, Te-Hadya	Chickpea	A. Joubi	16 June 2005	Ethanol	<91% Agromyzidae	<89% Phytomyzinae	KR476571
Liriomyza huidobrensis	Phytomyzinae	Lh101	Indonesia, Sulawesi, Malino	Chinese Cabbage	La Daha	19 May 2003	Ethanol	>99% *L. huidobrensis*	99% *L. huidobrensis*	KR476572
Liriomyza sativae	Phytomyzinae	VAITC2841	Papua New Guinea, Goroka	Tomato	A. Rice & S. McKenna	24 Jun 2011	FTA card	>99% *L. sativae*	100% L sativae	KR476573
Liriomyza trifolii	Phytomyzinae	Lt112	USA, Southern California	Laboratory colony	J. Trumble	Jul 2005	Ethanol	100% *L. trifolii*	99% *L. trifolii*	KR476574
Phytomyza syngenesiae	Phytomyzinae	VAITC2844	Australia, Vic., Ringwood East	Milkweed	M. Blacket	18 Nov 2010	FTA card	>99% *P. syngenesiae*	99% *P. syngenesiae*	KR476575
P. syngenesiae	Phytomyzinae	VAITC2845	Australia, Vic., Doncaster	Milkweed	M. Malipatil	21 Nov 2010	FTA card	>99% *P. syngenesiae*	99% *P. syngenesiae*	KR476576
Agromyzidae undet.	Agromyzinae?	VAITC3596	Australia, Qld., Weipa	Oval-leafed Alysicarpus	A. Postle	16 Apr 2012	FTA card	>93% Agromyzinae	90% Agromyzidae	KR476577
Agromyzidae undet.	Agromyzinae?	VAITC3705	Australia, Qld., Cairns	Capsicum	M. Berridge	20 Aug 2012	FTA card	>98% Agromyzinae	91% Agromyzinae	KR476578

[1]Morphological species identification.
[2]Best match (% similarity indicated) for 5′ section of COI amplified using PCR primers LCO/HCO (approx. 660 bp).
[3]Best match (% similarity indicated) for 3′ section of COI amplified using PCR primers Leafminer F/R (approx. 700 bp).

Table 3. *Liriomyza sativae* haplotypes detected from the Torres Strait and Papua New Guinea compared with previously reported matching *L. sativae* haplotypes.

	Scheffer & Lewis (2005)	Scheffer *et al.* (2006)	Wang *et al.* (2008)	Torres Strait & PNG (Present Study)
Haplotype S.7 (AY697777)	North America, Middle East, Asia (23)*	Southeast Asia (52)*	Asia (8)*	PNG (11), Torres Strait (1)
Haplotype S.27 (AY697761)	Southeast Asia (1)	– (0)	– (0)	Torres Strait (21)*
Haplotype S.28 (AY697760)	Southeast Asia (3)	Southeast Asia (23)	– (0)	PNG (1)

Haplotype names follow Scheffer & Lewis (2005) (GenBank number in brackets), collection locations are indicated (haplotype frequency in brackets), '–' indicates that haplotype was not detected in that study, an asterisk indicates that haplotype was the most common of all the *L. sativae* haplotypes collected in that study.

previously published examinations of genetic variation in *L. sativae* populations worldwide (table 3).

Results

DNA preservation

Preservation of DNA from immature leafminer samples collected from the field on FTA cards (Objective 2) proved highly effective in the present study, with successful amplification and DNA sequencing of all samples tested (tables 1 and 2). This included samples (e.g. *P. syngenesiae*, table 2) that were preserved on FTA cards stored at room temperature for up to 2 years, prior to DNA extraction. Field samples stored in ethanol appeared equally effective in preserving DNA, but created additional logistical issues during collection, requiring additional conditions for safe transport of ethanol in the field and postage of samples. Dry pinned adult material was not extensively tested here, however a DNA extraction from a leg of a single adult female, from Erub Island Torres Strait (collected at the same time as the larva, table 1), was trialled without success.

Molecular identification of L. sativae*/genetic variation within populations*

Sequencing two sections of COI (5′ and 3′ regions) in individuals of *L. sativae* collected from PNG and the Torres Strait allowed the larval/pupal field samples to be accurately identified to species (Objective 1). Haplotypes detected matched (100%) with three COI *L. sativae* haplotypes previously detected (fig. 2, tables 1 and 3). Two of the haplotypes 'S.7' and 'S.28' (haplotype names follow Scheffer & Lewis, 2005) were found in PNG (fig. 2), whereas the third haplotype, 'S.27', occurred solely in the Torres Strait (fig. 2), accounting for >95% of the Torres Strait samples (a single Torres Strait sample, from Erub Island, was found to possess haplotype S.7). A comparison with previous results (Objective 3) shows that Haplotype S.7 has been found from multiple locations in North America, Asia and the Middle East, where it was the most common haplotype detected (Scheffer & Lewis, 2005; Scheffer *et al.*, 2006; Wang *et al.*, 2008); Haplotype S.28 has previously only been detected from Southeast Asia (Scheffer & Lewis, 2005; Scheffer *et al.*, 2006); while Haplotype S.27 has previously been detected only once before, from Southeast Asia (Scheffer & Lewis, 2005).

DNA barcoding of leafminers

DNA barcoding species identification of a number of other agromyzid leafminers was conducted using both the 5′ and 3′ regions of COI (table 2). Each of these regions successfully amplified in all of the leafminer species tested here (Objective 5). Generally, both regions of COI appear similar in utility for DNA sequence species identification (table 2), with identifications using BOLD for the 5′ region of COI providing closer DNA matches in some cases (e.g. Agromyzinae, table 2). Interestingly, there are currently a large number of recently deposited DNA sequences on BOLD for the 'Universal' 5′ region of COI (that are not yet publically available) from various agromyzid species (many appear to be awaiting formal taxonomic identification) some of which are similar to the currently undetermined Agromyzinae species included in the present study (table 2).

A comparison of universal primers and the new leafminer primers (Objective 4) used in the present study (fig. 1, Supplementary table 1), against some previously sequenced dipteran leafminer and hymenopteran parasitoid DNA sequences for which the COI-tRNAleu region is available on GenBank (Supplementary table 1) indicates that commonly employed universal primers have >80% sequence match and high 3′ primer sequence similarity (the latter being especially important for primer specificity, see Qu *et al.*, 2012) to both leafminer and parasitoid DNA, while the new leafminer primers (fig. 1) are highly specific (>95% match/high 3′ similarity) for dipteran leafminers (both Agromyzidae and Drosophilidae), with low similarity to hymenopteran parasitoid COI sequences (<70% match/generally low 3′ sequence similarity). A comparison of Eulophidae COI DNA sequences (data from Burks *et al.*, 2011), for a much shorter section of COI available for many of the parasitoid genera previously detected in *Liriomyza* leafminers (Rauf *et al.*, 2000; Bjorksten *et al.*, 2005; Lambkin *et al.*, 2008; Tran, 2009) (data not shown), show the same general patterns of primer/DNA similarity as indicated in Supplementary table 1.

Discussion

Liriomyza sativae *populations in the Torres Strait and PNG*

Previous invasive introductions of *Liriomyza* species into new areas vary genetically, from diverse (Philippines – Scheffer *et al.*, 2006) to very limited (China – He *et al.*, 2002). Prior studies on introduced *L. sativae* populations have detected a great deal of genetic variation with a large number of haplotypes found worldwide (Scheffer & Lewis, 2005; Scheffer *et al.*, 2006; Wang *et al.*, 2008); however, the present study detected only three haplotypes, and these appear to be generally geographically localized (fig. 2, table 3). It seems that the two geographic areas surveyed here for *L. sativae* – PNG highlands and the Torres Strait islands – generally do

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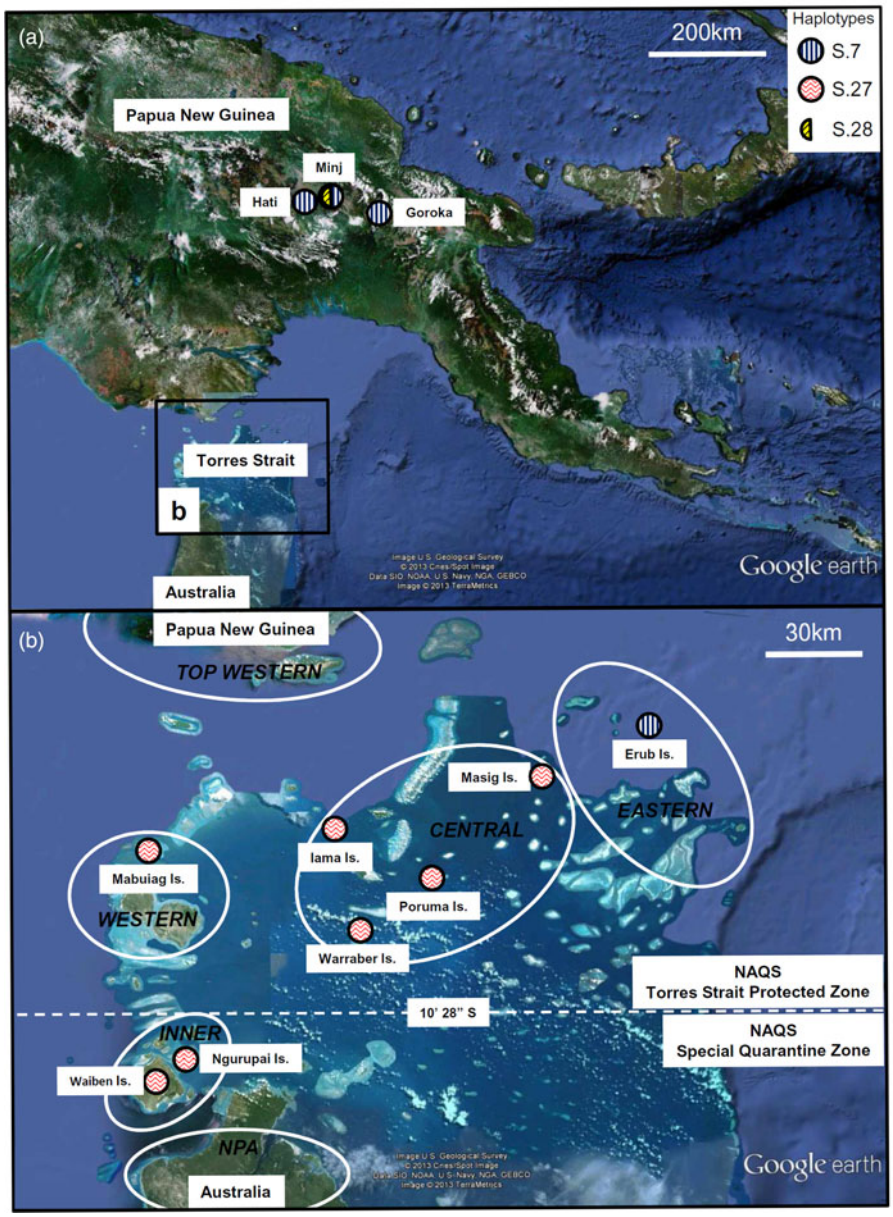

Fig. 2. (a) Map of Papua New Guinea and the Torres Strait indicating geographic sampling and *L. sativae* haplotypes detected (patterned circles, named in key at top right). (b) Map of the Torres Strait enlarged, indicating geographic sampling and *L. sativae* haplotypes detected (patterned circles); traditional island community groups (indicated by ovals) follow Anderson *et al.* (2010); the boundary of NAQS quarantine control zones (see text) is also indicated (dashed line).

not share a common source, with very little overlap of haplotypes between them. Indeed, across most of the Torres Strait (sampled in the present study from multiple sites over a 5-year period) it appears the introduction of *L. sativae* could have occurred from a single source, possibly even from a single individual, which has now spread (multiple times) to eight islands in the Torres Strait (fig. 2b). However, despite equivalent survey effort (from NAQS) *L. sativae* has still not yet been detected from certain sections of the Torres Strait, such as the 'Top Western' zone which lies within 4 km of PNG. The S.27 haplotype found in Torres Strait has previously only been detected once before (Scheffer & Lewis, 2005). Interestingly, the single possible exception to the Torres Strait/PNG genetic regionalization observed in the present study (i.e. Erub Island) comes from an area separated from the rest of Torres Strait by a deep water channel from a distinct community group (i.e. 'Eastern', fig. 2b). However, the haplotype detected (S.7) has previously been found to be among the most widespread and common haplotype detected around the world (Scheffer & Lewis, 2005; Scheffer *et al.*, 2006; Wang *et al.*, 2008); see table 3. Before drawing any conclusions regarding possible source populations additional genetic screening work on geographically adjacent possible *L. sativae* sources that have not yet been examined from Southeast Asia/Oceania is required

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to determine if the haplotypes detected in the present study are regionally common.

In Southeast Asia three common polyphagous invasive vegetable leafminer species of (*L. sativae*, *L. trifolii* and *L. huidobrensis*) have recently spread (summarized in Andersen *et al.*, 2008), where they often co-occur within a single geographic region, but generally differ in their patterns of distribution (Andersen *et al.*, 2008; Tantowijoyo & Hoffmann, 2011; Xiang *et al.*, 2012). In the present study, it might have been predicted that *L. huidobrensis*, present in the highlands of Indonesia (Tantowijoyo & Hoffmann, 2010), may have been the first *Liriomyza* species detected from the highlands of PNG, however we actually found *L. sativae*, which is generally more common in lowlands (Rauf *et al.*, 2000; Tantowijoyo & Hoffmann, 2010, 2011). One explanation for this may be that unlike some insect species that are likely to disperse primarily through natural means, such as wind, within the surveyed region (e.g. Anderson *et al.*, 2010; Anderson & Congdon, 2013), introductions of *Liriomyza* species into new areas are most likely associated with human trade (Spencer, 1989; Scheffer & Lewis, 2005). Therefore the species of *Liriomyza* that invades an area appears highly dependent on the movement of host plant material between areas by humans. If more than one polyphagous *Liriomyza* species is present within a region, species distributions appear to shift due to competition mediated through insecticide resistance (Gao *et al.*, 2012) or differences in environmental tolerances (Huang & Kang, 2007; Tantowijoyo & Hoffmann, 2010; Wang *et al.*, 2014).

DNA barcoding identification

Species identification from immature specimens was possible in the present study through the collection of well-preserved DNA samples. It was not necessary to amplify the PCR target regions (fig. 1) in small sections which is often an indication of DNA degradation (e.g. Strutzenberger *et al.*, 2012). Field sampling on FTA cards proved very effective for preservation of soft bodied immature leafminer specimens in the tropics, without requiring freezing or the transport of ethanol. FTA cards have previously been commonly employed in the preservation and identification of pathogens (e.g. Becker *et al.*, 2004) and have proven useful for preserving insect DNA (e.g. Harvey, 2005; Gómez & Uribe, 2007; Karimian *et al.*, 2011), including under extreme field conditions (i.e. present study). An extra advantage of using FTA cards for DNA preservation is that the samples become partially processed, helping to reduce associated biosecurity/biohazard risks (Karimian *et al.*, 2011). The major disadvantage with using FTA cards is that specimens are morphologically destroyed. DNA barcoding should ideally obtain DNA using relatively nondestructive techniques to ensure a voucher specimen is available for future morphological examinations (Floyd *et al.*, 2010). Therefore, best practice should be to try to also preserve morphological specimens for new range records (e.g. Martin, 2004; Scheffer *et al.*, 2006), if possible, to provide reference specimens lodged in museum reference collections (as in the present study, table 1). The latter point being especially important as many agromyzid reference DNA sequences do not currently appear to be formally identified to species on public databases (e.g. see table 2).

Traditional *Liriomyza* species identification involves rearing immature specimens to adults, with only male specimens reliably identifiable morphologically (Parrella & Keil, 1984; Malipatil & Ridland, 2008); DNA barcoding provides an extremely useful alternative identification method that can be used for any lifestage. The present study provides the first DNA barcode reference specimen information for two *Liriomyza* species: *L. chenopodii* and *L. cicerina* (table 2); the DNA barcoding methods presented here appear generally applicable to identification of agromyzid leafminers (table 2), and should prove valuable in identifying leafminer species in the future. Indeed, the new primers presented here (LeafminerCOI-F/R) may even prove useful for DNA barcoding identification of other dipteran leafminers, such as *Scaptomyza* (Drosophilidae) (Supplementary table 1), with which immature agromyzid leafminers could potentially be confused, and should also help avoid potential co-amplification of parasitoid DNA (Supplementary table 1).

DNA barcoding identification of leafminers has to date been commonly based on the 'non-universal' 3′ region of COI (e.g. Scheffer *et al.*, 2006). In the present study, both halves of COI (5′ and 3′) proved valuable for DNA sequence species identification (table 2); and it appears useful to examine both the 5′ and 3′ sections of COI when identifying *L sativae* in newly invaded geographic regions, given the large amount of reference material available for the 5′ region of COI on BOLD (for species identification) and for the 3′ region on GenBank (for haplotype identification).

Conclusions

The present study documents a significant range extension of *L. sativae* from multiple hosts (including both cultivated vegetable crops and some common weeds) and geographic locations (table 1, fig. 2). However, this species still appears to be unknown from certain sections of the Torres Strait (fig. 2b), and also from the Australian mainland; although it has now been found very close: i.e. Ngurupai (Horn) and Waiben (Thursday) Islands within the 'NAQS Special Quarantine Zone' (fig. 2b). Additional surveys of these regions would ideally also collect endemic species, which might potentially be confused with *Liriomyza*, as reference specimens, along with their endemic parasitoid fauna which may prove useful in controlling *L. sativae*. This surveillance should assist in detecting new incursions shortly after they occur to potentially aid in the control of the spread of this serious pest.

Supplementary material

To view supplementary material (table and figure) for this article, please visit http://dx.doi.org/S0007485315000383

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References

Andersen, A., Tran, T.T.A. & Nordhus, E. (2008) Distribution and importance of polyphagous *Liriomyza* species (Diptera, Agromyzidae) in vegetables in Vietnam. *Norwegian Journal of Entomology* **55**, 149–164.

Anderson, K.L. & Congdon, B.C. (2013) Population genetics suggest that multiple invasion processes need to be addressed in the management plan of a plant disease vector. *Evolutionary Applications* **6**, 660–672.

Anderson, K.L., Deveson, T.E., Sallam, N. & Congdon, B.C. (2010) Wind-assisted migration potential of the island sugarcane planthopper *Eumetopina flavipes* (Hemiptera: Delphacidae): implications for managing incursions across an Australian quarantine frontline. *Journal of Applied Ecology* **47**, 1310–1319.

Armstrong, K.F. & Ball, S.L. (2005) DNA barcodes for biosecurity: invasive species identification. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* **360**, 1813–1823.

Becker, S., Franco, J.R., Simarro, P.P., Stich, A., Abel, P.M. & Steverding, D. (2004) Real-time PCR for detection of *Trypanosoma brucei* in human blood samples. *Diagnostic Microbiology and Infectious Disease* **50**, 193–199.

Bhuiya, B.A., Amin, S. & Mazumdar, S. (2011) First report of vegetable leafminer *Liriomyza sativae* Blanchard (Diptera: Agromyzidae) through DNA barcoding from Bangladesh. *Journal of Taxonomy and Biodiversity Research* **5**, 15–17.

Bjorksten, T.A. & Hoffmann, A.A. (2005). Polymerase chain reaction-restriction fragment-length polymorphism method to distinguish polyphagous *Liriomyza spp.* (Diptera: Agromyzidae). Australian Centre for International Agricultural Research (ACIAR) CS/2000/090 Research Report for 2003 and 2004 (ed. P.M. Ridland).

Bjorksten, T.A., Robinson, M. & La Salle, J. (2005) Species composition and population dynamics of leafmining flies and their parasitoids in Victoria. *Australian Journal of Entomology* **44**, 186–191.

Blacket, M.J., Semeraro, L. & Malipatil, M.B. (2012) Barcoding Queensland Fruit Flies (*Bactrocera tryoni*): impediments and improvements. *Molecular Ecology Resources* **12**, 428–436.

Boykin, L.M., Armstrong, K., Kubatko, L. & De Barro, P. (2012) DNA barcoding invasive insects: database roadblocks. *Invertebrate Systematics* **26**, 506–514.

Burks, R.A., Heraty, J.M., Gebiola, M. & Hansson, C. (2011) Combined molecular and morphological phylogeny of Eulophidae (Hymenoptera: Chalcidoidea), with focus on the subfamily Entedoninae. *Cladistics* **27**, 581–605.

CABI (2013) *Liriomyza sativae (vegetable leaf miner) In: Crop Protection Compendium*. Wallingford, UK, CAB International. Available online at www.cabi.org/cpc (accessed February 2013).

Dowton, M., Cameron, S.L., Dowavic, J.I., Austin, A.D. & Whiting, M.F. (2009) Characterization of 67 mitochondrial tRNA gene rearrangements in the Hymenoptera suggests that mitochondrial tRNA gene position is selectively neutral. *Molecular Biology and Evolution* **26**, 1607–1617.

Fisher, N., Ubaidillah, R., Reina, P. & La Salle, J. (2005) *Liriomyza* parasitoids in Southeast Asia. Lucid key. Available online at http://www.ces.csiro.au/science/Liriomyza_ver3/key/Liriomyza_Parasitoids_Key/Media/Html/home.html

Floyd, R., Lima, J., de Waard, J., Humble, L. & Hanner, R. (2010) Common goals: policy implications of DNA barcoding as a protocol for identification of arthropod pests. *Biological Invasions* **12**, 2947–2954.

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* **3**, 294–299.

Gao, Y., Reitz, S.R., Wei, Q., Yu, W. & Lei, Z. (2012) Insecticide-mediated apparent displacement between two invasive species of leafminer fly. *PLoS ONE* **7**, e36622.

Gómez, P.L.M. & Uribe, S.I. (2007) Preservation and storage of butterfly DNA using filter paper. *Revista Colombiana de Entomologia* **33**, 197–199.

Harvey, M.L. (2005) An alternative for the extraction and storage of DNA from insects in forensic entomology. *Journal of Forensic Sciences* **50**, 627–629.

He, L., Zhang, Y., Xiao, N., Wei, J. & Kuang, R. (2002) *Liriomyza huidobrensis* in Yunnan, China: current distribution and genetic structure of a recently established population. *Entomologia Experimentalis et Applicata* **102**, 213–219.

Huang, L.H. & Kang, L. (2007) Cloning and interspecific altered expression of heat shock protein genes in two leafminer species in response to thermal stress. *Insect Molecular Biology* **16**, 491–500.

IPPC (2008) *Detection of Vegetable Leafminer in Torres Strait*. IPPC Official Pest Report, No. AU-13/1. Rome, Italy, FAO. Available online at https://www.ippc.int/IPP/En/default

Karimian, F., Sedaghat, M.M., Oshaghi, M.A., Mohtarami, F., Sanei, D.A., Koosha, M., Akbari, S. & Hashemi-Aghdam, S. S. (2011) Utility of filter paper for preserving insects, bacteria, and host reservoir DNA for molecular testing. *Iranian Journal of Arthropod-Borne Diseases* **5**, 42–50.

Kox, L.F.F., van den Beld, H.E., Lindhout, B.I. & de Goffau, L.J. W. (2005) Identification of economically important *Liriomyza* species by PCR-RFLP analysis. *OEPP/EPPO Bulletin* **35**, 79–85.

Lambkin, C.L., Fayed, S.A., Manchester, C., La Salle, J., Scheffer, S. J. & Yeates, D.K. (2008) Plant hosts and parasitoid associations of leaf mining flies (Diptera: Agromyzidae) in the Canberra region of Australia. *Australian Journal of Entomology* **47**, 13–19.

Maharjan, R., Oh, H.W. & Jung, C. (2014) Morphological and genetic characteristics of *Liriomyza huidobrensis* (Blanchard) (Diptera: Agromyzidae) infesting potato crops in Korea. *Journal of Asia-Pacific Entomology* **17**, 281–286.

Malipatil, M. & Ridland, P. (2008) Polyphagous Agromyzid Leafminers: Identifying polyphagous agromyzid leafminers (Diptera: Agromyzidae) threatening Australian primary industries. The Department of Agriculture, Fisheries and Forestry. Available online at http://www.lucidcentral.org/keys/v3/leafminers, CD-Rom.

Martin, N.A. (2004) History of an invader, *Scaptomyza flava* (Fallen, 1823) (Diptera: Drosophilidae). *New Zealand Journal of Zoology* **31**, 27–32.

Maynard, G.V., Hamilton, J.G. & Grimshaw, J.F. (2004) Quarantine – Phytosanitary, sanitary and incursion management: an Australian entomological perspective. *Australian Journal of Entomology* **43**, 318–328.

https://doi.org/10.1017/S0007485315000383 Published online by Cambridge University Press

Miura, K., Tagami, Y., Ohtaishi, M. & Iwasaki, A. (2004) Application of molecular techniques to distinguish *Liriomyza trifolii* from *L.* sativae (Diptera: Agromyzidae) on tomato cultivation in Japan. *Journal of Economic Entomology* **97**, 964–969.

Moreau, C.S., Wray, B.D., Czekanski-Moir, J.E. & Rubin, B.E.R. (2013) DNA preservation: a test of commonly used preservatives for insects. *Invertebrate Systematics* **27**, 81–86.

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.

O'Grady, P.M., Lapoint, R.T., Bonacum, J., Lasola, J., Owen, E., Wu, Y. & Desalle, R. (2011) Phylogenetic and ecological relationships of the Hawaiian *Drosophila* inferred by mitochondrial DNA analysis. *Molecular Phylogenetics and Evolution* **58**, 244–256.

Oliveira, D.C.S.G., Raychoudhury, R., Lavrov, D.V. & Werren, J. H. (2008) Rapidly evolving mitochondrial genome and directional selection in mitochondrial genes in the parasitic wasp *Nasonia* (Hymenoptera: Pteromalidae). *Molecular Biology and Evolution* **25**, 2167–2180.

Parrella, M.P. & Keil, C.B. (1984) Insect pest management: the lesson of *Liriomyza*. *Bulletin of the Entomological Society of America* **30**, 22–25.

Prijono, D., Robinson, M., Rauf, A., Bjorksten, T. & Hoffmann, A.A. (2004) Toxicity of chemicals commonly used in Indonesian vegetable crops to *Liriomyza huidobrensis* populations and the Indonesian parasitoids *Hemiptarsenus varicornis, Opius sp.*, and *Gronotoma micromorpha*, as well as the Australian parasitoids *Hemiptarsenus varicornis* and *Diglyphus isaea*. *Journal of Economic Entomology* **97**, 1191–1197.

Qiagen (2010) QIAamp DNA Investigator Handbook 04/2010, pp. 18–19.

Qu, W., Zhou, Y., Zhang, Y., Lu, Y., Wang, X., Zhao, D., Yang, Y. & Zhang, C. (2012) MFEprimer-2.0: a fast thermodynamics-based program for checking PCR primer specificity. *Nucleic Acids Research* **40**, W205–W208.

Ratnasingham, S. & Hebert, P.D.N. (2007) BOLD: the barcode of life data system. *Molecular Ecology Notes* **7**, 355–364. Available online at www.barcodinglife.org

Rauf, A., Shepard, B.M. & Johnson, M.W. (2000) Leafminers in vegetables, ornamental plants and weeds in Indonesia: surveys of host crops, species composition and parasitoids. *International Journal of Pest Management* **46**, 257–266.

Régnier, C., Gargominy, O., Falkner, G. & Puillandre, N. (2011) Foot mucus stored on FTA cards is a reliable and non-invasive source of DNA for genetics studies in molluscs. *Conservation Genetics Resources* **3**, 377–382.

Rozen, S. & Skaletsky, H. (2000) PRIMER 3 on the WWW for general users and for biologist programmers. pp. 365–386 *in* Krawetz, S. & Misener, S. (*Eds*) *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Totowa, New Jersey, Humana Press.

Scheffer, S.J. (2000) Molecular evidence of cryptic species within the *Liriomyza huidobrensis* (Diptera: Agromyzidae). *Journal of Economic Entomology* **93**, 1146–1151.

Scheffer, S.J. & Lewis, M.L. (2005) Mitochondrial phylogeography of vegetable pest *Liriomyza sativae* (Diptera: Agromyzidae): divergent clades and invasive populations. *Annals of the Entomological Society of America* **98**, 181–186.

Scheffer, S.J., Wijesekara, A., Visser, D. & Hallett, R.H. (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.

Scheffer, S.J., Lewis, M.L. & Joshi, R.C. (2006) DNA Barcoding Applied to Invasive Leafminers (Diptera: Agromyzidae) in the Philippines. *Annals of the Entomological Society of America* **99**, 204–210.

Scheffer, S.J., Winkler, I.S. & Wiegmann, B.M. (2007) Phylogenetic relationships within the leaf-mining flies (Diptera: Agromyzidae) inferred from sequence data from multiple genes. *Molecular Phylogenetics and Evolution* **42**, 756–775.

Semeraro, L. & Malipatil, M. (2007) Molecular diagnostic test for three exotic species of leafminers, *Liriomyza* (Agromyzidae) flies. O.R.L. 3.1, Our Rural Landscape. Project Report to Department of Primary Industries, Knoxfield Victoria.

Spencer, K.A. (1973) *Agromyzidae (Diptera) of Economic Importance*. The Hague, Dr. W. Junk B.V., 418 pp.

Spencer, K.A. (1989). Leafminers. pp. 77–98 *in* Kahn, R.P. (*Ed.*) *Plant Protection and Quarantine, Vol. 2, Selected Pests and Pathogens of Quarantine Significance*. Boca Raton, CRC Press.

Spencer, K.A. (1990) *Host Specialization in the World Agromyzidae (Diptera)*. Dordrecht, Kluwer Academic Publishers.

Strutzenberger, P., Brehm, G. & Fiedler, K. (2012). DNA barcode sequencing from old type specimens as a tool in taxonomy: a case study in the diverse genus *Eois* (Lepidoptera: Geometridae). *PLoS ONE* **7**, e49710.

Takano, S.-I., Iwaizumi, R., Nakanishi, Y. & Someya, H. (2008) Laboratory hybridization between the two clades of *Liriomyza huidobrensis* (Diptera: Agromyzidae). *Applied Entomology and Zoology* **43**, 397–402.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**, 2731–2739.

Tantowijoyo, W. & Hoffmann, A.A. (2010) Identifying factors determining the altitudinal distribution of the invasive pest leafminers *Liriomyza huidobrensis* and *Liriomyza sativae*. *Entomologia Experimentalis et Applicata* **135**, 141–153.

Tantowijoyo, W. & Hoffmann, A.A. (2011) Variation in morphological characters of two invasive leafminers, *Liriomyza huidobrensis* and *L. sativae*, across a tropical elevation gradient. *Journal of Insect Science* **11**, 1–16.

Tran, H.D. (2009) Agromyzid leafminers and their parasitoids on vegetables in central Vietnam. *Journal of ISSAAS* **15**, 21–33.

Wang, H., Reitz, S.R., Xiang, J., Smagghe, G. & Lei, Z. (2014) Does temperature-mediated reproductive success drive the direction of species displacement in two invasive species of leafminer fly? *PLoS ONE* **9**, e98761.

Wang, L.-P., Du, Y.-Z., He, Y.-T., Zheng, F.-S. & Lu, Z.-Q. (2008) Genetic variation of host populations of *Liriomyza sativae* Blanchard. *Agricultural Sciences in China* **7**, 585–590.

Wang, S., Lei, Z., Wang, H., Dong, B. & Ren, B. (2011) The complete mitochondrial genome of the leafminer *Liriomyza trifolii* (Diptera: Agromyzidae). *Molecular Biology Reports* **38**, 687–692.

Whiteman, N.K., Groen, S.C., Chevasco, D., Bear, A., Beckwith, N., Gregory, T.R., Denoux, C., Mammarella, N., Ausubel, F. M. & Pierce, N.E. (2011) Mining the plant–herbivore interface with a leafmining *Drosophila* of *Arabidopsis*. *Molecular Ecology* **20**, 995–1014.

Winkler, I.S., Scheffer, S.J. & Mitter, C. (2009) Molecular phylogeny and systematics of leaf-mining flies (Diptera:

https://doi.org/10.1017/S0007485315000383 Published online by Cambridge University Press

Agromyzidae): delimitation of *Phytomyza* Fallén *sensu lato* and included species groups, with new insights on morphological and host-use evolution. *Systematic Entomology* **34**, 260–292.

Xiang, J., Wang, H. & Gao, Y. (2012) Interspecific competition among three invasive *Liriomyza* species. *Acta Ecologica Sinica* **32**, 1616–1622.

Xiao, J.-H., Jia, J.-G., Murphy, R.W. & Huang, D.-W. (2011) Rapid evolution of the mitochondrial genome in Chalcidoid wasps (Hymenoptera: Chalcidoidea) driven by parasitic lifestyles. *PLoS ONE* **6**, e26645.

Yang, F., Du, Y.Z., Wang, L.P., Cao, J.M. & Yu, W.W. (2011) The complete mitochondrial genome of the leafminer *Liriomyza sativae* (Diptera: Agromyzidae): great difference in the A+T-rich region compared to *Liriomyza trifolii*. *Gene* **485**, 7–15.

Yang, F., Du, Y., Cao, J. & Huang, F. (2013) Analysis of three leafminers' complete mitochondrial genomes. *Gene* **529**, 1–6.

https://doi.org/10.1017/S0007485315000383 Published online by Cambridge University Press