Journal of Agricultural Technology 2011 Vol. 7(2): 349-368
Available online http://www.ijat-aatsea.com
ISSN 1686-9141

Identification of *Liriomyza* spp. (Diptera: Agromyzidae) on yardlong bean and cucumber in Songkhla province: II. Using Random Ampliflied Polymorphic DNA (RAPD) and male distiphallus technique

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Sappanukhro, P., Petcharat, J., Nualsri, J., and Permkam, S. (2011). Identification of *Liriomyza* spp. (Diptera: Agromyzidae) on yardlong bean and cucumber in Songkhla province: II. Using Random Ampliflied Polymorphic DNA (RAPD) and male distiphallus technique. Journal of Agricultural Technology 7(2): 349-368.

DNA polymorphism of 5 sets of 5 adult flies of both sexs, each set derived from the same mother of leafminer infested yardlong bean and cucumber fields in Songkhla Province were done using Random Ampliflied Polymorphic DNA (RAPD) technique. Because all sets of adult flies showed the same DNA pattern and from male distipallas characters, it was concluded that there was at least a species of *L. trifolii* infested all yardlong bean and cucumber fields of studied.

Key words: *Liriomyza* spp., *Liriomyza trifolii, Liriomyza sativae,* agromyzid leafminer, identification

Introduction

In this decade, molecular biological techniques have been proven as reliable tools for identification of both sexs and all life stage of *Liriomyza* species. In the year 2000, Chiu *et al.* (2000) used Random Amplified Polymorphic DNA (RAPD) and Polymerase Chain Reaction (PCR) in identification of *L. asterivora, L. bryoniae, L. chinensis, L.huidobrensis, L. sativae and L. trifolii.* Scheffer and coworkers used Polymerase Chain Reaction-Restriction Fragment Length Polymerase (PCR-RFLP) to identified *L. langei* from its cryptic species, *L.huidobrensis* (Scheffer *et al.,* 2001). Kox *et al.* (2005) used male genitalia of adult male flies to identified *L. bryoniae, L. huidobrensis, L. sativae and L. trifolii* and by PCR-RFLP techniques they were abled to identified adult females, pupae and larvae of *Liriomyza* species.

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The present study was performed to identify the agromyzid leafminer *Liriomyza* spp. attacked yardlong bean *Vigna sesquipedalis* (L.) and cucumber *Cucumis sativus* L. in Songkhla province, southern Thailand using male genitalia and PCR-RAPD techniques.

Materials and methods

Field sampling of leafminer

Adults *Liriomyza* leafminer were collected from four yardlong bean fields in Bangklam, Rattapum and Had Yai districs and one cucumber field in Rattapum distric. In laboratory each fly was separated in a cage (30×30×30 cm.) provided with host plant (yardlong bean or cucumber) in according to its field host plant. After a period of time feeding tunnels of young leafminer produced by female flies collected from field were appeared on plant leaves. Only one set (or cage)of the first generation leafminer maggot produced by a female from each field was choosed. The young leafminers were allowed to feed until they became fullgrown. The infested leaves from each cage were cut and each leaf sample was wraped at base with cotton soaked with water to keep the leaf fresh. The leaves then kept in an insect rearing plastic box of 19x25x10 cm. until the adult flies emerged. The flies from the same insect box were assumed to be the same species because they were produced by the same mother. Newly emerged flies were fed with 15% honey solution and bean or cucumber leaf in according to their mother's food plant. After 2 days, the fullgrown leafminer flies with complety developed color and setae were killed and kept in 95% ethyl alchohol for morphological and DNA polymorphism study.

Identification of adult leafminers

1. Identification to species using adult flies external morphology

Adult flies (from 1.1) were preserved in 95% ethyl alcohol and examine under stereomicroscope. The fly morphology especially the following characters were observed and photographed in according to Spencer (1973).

1.1 Colour of the ground where inner and outer vertical bristles were situated

1.2 Proportion of the last (a) to the penultimate (b) section of vein M_{3+4} of front wing

1.3 Colour, patch size and colour, number and location of setae on mesopleura

2. Identification to species using the male distiphallus

2.1 Only male of adult leafminers were put in a beaker contained 3 ml of 10 % Potassium hydroxide and warm over alcohol lamp for 10 minutes to reduce the colour and hardening of their exoskeleton.

2.2. Under compound microscope the fly sample which exoskeleton became solfer and clearer was dissected. The fly phallus (aedeagus) was removed, examined and compared with those in Spencer (1973), Collins, (1996) and OEPP/EPPO (2005) for species identification (Figure 4 and 5). The distiphallus sample was photographed and the fly cadaver was kept together with female flies of the same mother for DNA Extraction.

3. Identification to species using PCR-RAPD technique

After the investigation on male ditiphallus, the fly DNA polymorphic was studied using the male insect corpse and the female flies of the same mother.

3.1 DNA Extraction: Preliminary DNA extraction using methods of Miura *et al.* (2004), Doyle and Doyle (1990), Morgan *et al.* (2000) showed no DNA or only small amount of DNA. Modification of Morgan method using 10 % chelex-100 and incubated at 56°c for 2 hours then boiled 3 minutes, instead of using 5 % chelex-100 and incubated at 56°c over night then boiled 5 minutes, were used for leafminer DNA Extraction.

3.2 PCR-RAPD technique: Fifteen PCR primers (Morgan *et al.*, 2000) as shown in Table 1 were tried with *Liriomyza* sp. DNA.

Table 1. Primers and their base sequences used in PCR of *Liriomyza* DNA.

Primers	Base sequences
OPA-05	5'AGGGGTCTTG3'
OPA-07	5'GAAACGGGTG3'
OPA-08	5'GTGACGTAGG3'
OPA-09	5'GGGTAACGCC3'
OPA-10	5'GTGATCGCAG3'
OPB-01	5'GTTTCGCTCC3'
OPB-05	5'TGCGCCCTTC3'
OPB-14	5'TCCGCTCTGG3'
OPB-17	5'AGGGAACGAG3'
OPC-01	5'TTCGAGCCAG3'
OPC-02	5'GTGAGGCGTC3'
OPC-03	5'GGGGGTCTTT3'
OPC-05	5'GATGACCGCC3'
OPC-09	5'CTCACCGTCC3'
OPC-12	5'TGTCATCCCC3'

The reaction mixture composed of dNTP mixture (10 mM) 2µl, Primer set 1.5 µl, Taq polymerase (5 U/ µl) 0.2 µl, PCR polymerase buffer 2.5 µl, PCR water 16.6 µl, DNA 1 µl. The PCR was performed with the following parameters: 1 cycle of 2 minutes at 94°c, 30 seconds at 94°c, 1 minutes at 37°c, 2 cycles of 2 minutes at 72°c, 30 seconds at 94°c, 1 minute at 35°c, 2 cycles of 2 minutes at 72°c, 30 seconds at 93°c, 1minute at 35°c, 41 cycles of 2 minutes at 72°c, and 1 cycle of 5 minutes at 72°c and cooling to room temperature.

Electrophoresis: After PCR, the DNA fragments were electrophoresed on 1.5% agarose at 100 volts for 30-60 minutes. Gel was strained for 20 µl of 5 mg/ml ethidium bromine in 300 ml. water for 30 minutes and washed with steriled distilled water for 30 minutes. DNA fragments were viewed and photographed under UV Interpreted of band patterns.

Results and discussion

Identification to species using adult flies external morphology and male distiphallus

In laboratory, the adult female fly collected from each field produced its first generation fly as showed in Table 2.

Adult flies of both sexes were identified in according to their external morphology and male distiphallus. Again external morphology were varied, the same fly was assigned to different species when different characters were used (Table 3-7).

Table 2. Numbers and sexes of the first generation *Liriomyza* spp. produced bya female fly collected from yardlong bean and cucumber fields in Songkhla province.

Field*	Male	Female	Total
T1	5	5	10
T2	10	7	17
T3	6	7	13
T4	8	4	12
T5	30	19	49
Total	59	42	101

Field*
T1= Tumbon Kor-hong, Had Yai district (yardlong bean)
T2= Tumbon Thakam, Had Yai district (yardlong bean)
T3= Tumbon Thachamuang, Rattapum district (yardlong bean)
T4= Tumbon Bangreang, Kuanneang district (yardlong bean)
T5= Tumbon Thachamaung, Rattapum district (cucumber)

Table 3. Identification of first generation progeny of a female fly collected from yardlong bean field in tumbon Kor-hong, Had Yai district, T1).

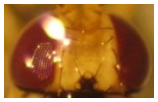
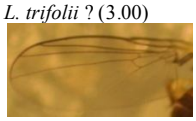
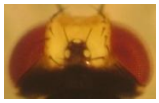
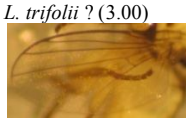
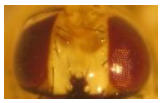
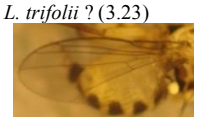
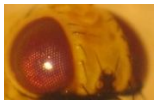
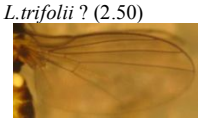
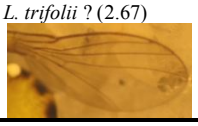
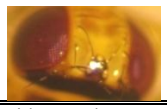
Sample number	Vertical bristles (40X)	Mesopleuron (40X)	a/b of vein M_{3+4}	Male distiphallus (200X)	Species inconclusion
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (3.00)		
T1.1				*	*L. trifolii*
	L. sativae	*L. sativa* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (3.00)	*L. trifolii*	
T1.2					*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (3.00)		
T1.3				-	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (3.23)		
T1.4				-	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L.trifolii* ? (2.50)		
T1.5				*	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (2.92)		
T1.6				-	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (3.17)	*L. trifolii*	
T1.7					*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (3.60)		
T18.				*	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (2.67)		
T1.9				*	*L. trifolii*

Table 3. (Continue)

Sample number	*Vertical bristles (40X)*	*Mesopleuron (40X)*	*a/b of vein M3+4*	*Male distiphallus (200X)*	*Species inconclusion*
T1.10	*L. sativae* 	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (3.00)	*	*L. trifolii*

*Female fly without aedeagus
- Male undisected

Table 4. Identification of first generation progeny of a female fly collected from yardlong bean field in tumbon Thakam, Had Yai district, T2).

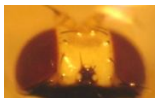
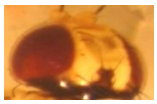
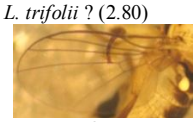
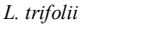
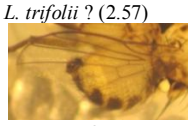
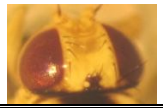
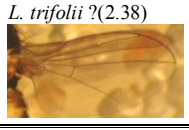
Sample number	Vertical bristles (40X)	Mesopleuron (40X)	a/b of vein M_{3+4}	Male distiphallus (200X)	Species in conclusion
T2.1	*L. sativae* 	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (2.50)	*	*L. trifolii*
T2.2	*L. sativae* 	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (2.71)	-	*L. trifolii*
T2.3	*L. sativae*	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (2.86)	*L. sativae*	*L. trifolii*
T2.4	*L. sativae*	*L. sativae* ? *L. trifolii*	*L. sativae* ? *L. trifolii* ? (2.57) 	*  *L. trifolii*	*L. trifolii*
T2.5	*L. sativae*	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ?(2.38) 		*L. trifolii*
T2.6				-	*L. trifolii*

Table 7. (Continue)

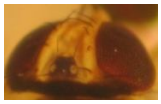
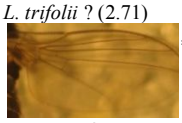
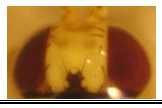
Sample number	Vertical bristles (40X)	Mesopleuron (40X)	a/b of vein M_{3+4}	Male distiphallus (200X)	Species in conclusion
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (3.17)		
T5.13				-	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (3.33)		
T5.14				-	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (3.33)		
T5.15				*	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (2.38)	*L. trifolii*	
T5.16					*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ?(2.50)		
T5.17				*	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (2.38)		
T5.18				-	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (3.17)		
T5.19				*	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (2.71)		
T5.20				*	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (2.50)		
T5.21				*	*L. trifolii*

Table 7. (Continue)

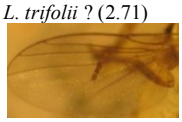
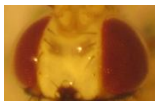
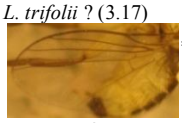
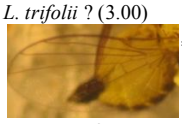
Sample number	Vertical bristles (40X)	Mesopleuron (40X)	a/b of vein M_{3+4}	Male distiphallus (200X)	Species in conclusion
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (2.71)	*L. trifolii*	
T5.22					*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (2.83)		
T5.23				*	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (3.17)		
T5.24				-	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (3.17)		
T5.25				*	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (3.00)		
T5.26				*	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (2.71)		
T5.27				*	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (2.13)		
T5.28				-	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (2.86)		
T5.29				-	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (3.17)		
T5.30				-	*L. trifolii*

Table 7. (Continue)

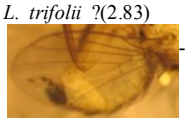
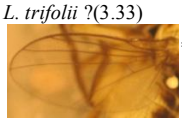
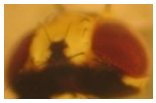
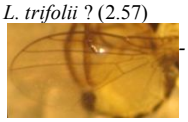
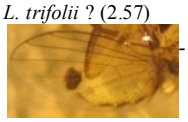
Sample number	Vertical bristles (40X)	Mesopleuron (40X)	a/b of vein M_{3+4}	Male distiphallus (200X)	Species in conclusion
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ?(2.83)		
T5.31				-	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ?(3.33)		
T5.32				-	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ?(3.33)		
T5.33				*	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (2.57)		
T5.34				-	*L. trifolii*
	L. sativae	*L. sativae* ? *. trifolii* ?	*L. sativae* ? *L. trifolii* ? (2.57)		
T5.35				-	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (2.71)		
T5.36				-	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (2.86)		
T5.37				*	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (3.33)		
T5.38				-	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (3.33)		
T5.39				-	*L. trifolii*

Table 7. (Continue)

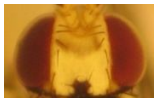
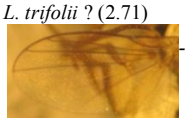
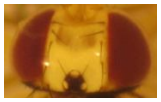
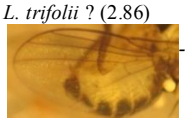
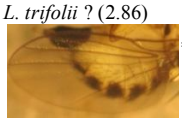
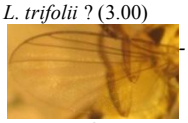
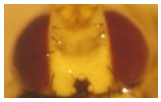
Sample number	Vertical bristles (40X)	Mesopleuron (40X)	a/b of vein M_{3+4}	Male distiphallus (200X)	Species in conclusion
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (2.86)		
T5.40				-	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (2.71)		
T5.41				-	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (2.86)		
T5.42				-	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (3.67)		
T5.43				*	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (2.86)		
T5.44				*	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (3.00)		
T5.45				-	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (3.50)		
T5.46				*	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (3.17)		
T5.47				*	*L. trifolii*
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (2.25)		
T5.48				-	*L. trifolii*

Table 7. (Continue)

Sample number	Vertical bristles (40X)	Mesopleuron (40X)	a/b of vein M_{3+4}	Male distiphallus (200X)	Species in conclusion
	L. sativae	*L. sativae* ? *L. trifolii* ?	*L. sativae* ? *L. trifolii* ? (3.50)		
T5.49				-	*L. trifolii*

*Female fly without aedeagus
- Male undisected

Identification to species using PCR-RAPD technique

DNA extraction

Modification of Morgan method (Morgan *et al*., 2000) using 10 % chelex-100 and incubated at 56°c for 2 hours then boiled 3 minutes, instead of using 5 % chelex-100 and incubated at 56°c over night then boiled 5 minutes, gave a satisfied amount of DNA. The extracted DNA was then ampliflied by PCR RAPD technigue. Very small amount of DNA was obtained because of the small size of leafminer (1-2 mm.). The amount was even less not enough for PCR after DNA sediment was dissolved in distilled water or TE.

PCR-RAPD technique

Only 5 primers, OPA-09, OPC-02, OPC-05, OPC-12 and OPB-14, produced quite clear DNA patterns as shown in figures 1- 5. Observation on DNA fragments of *Liriomyza* from 5 yardlong bean and 1 cucumber fields using OPA-09 (Figure 1), OPC-02 (Figure 3), and OPC- 05 (Figure 4) found no diference. However when OPB-14 was applied, DNA fragment of T1 sample showed 140 and 220 while those of T2, T3, T4 and T5 were of 160 and 250. DNA patterns of T2, T3, and T4 were the same when primer OPC-12 was applied (Figure 5) while those of T1 was same as T5. All primers gave about the same DNA patterns when they were applied to DNA fragments of *Liriomyza* from all fields.

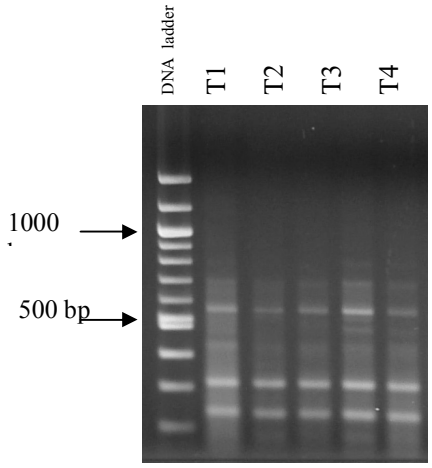


Fig. 1. DNA pattern of *Liriomyza* leafminer using RAPD technique, primer OPA 09. Column 1 is 100 bp DNA ladder, column 6 -2 are DNA of leafminers

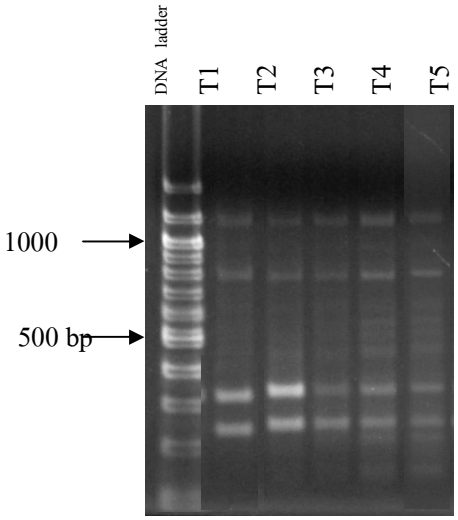


Fig. 2. DNA pattern of *Liriomyza* leafminer using RAPD technique, primer OPB14. Column 1 is 100 bp DNA ladder, column 6 -2are DNA of leafminers.

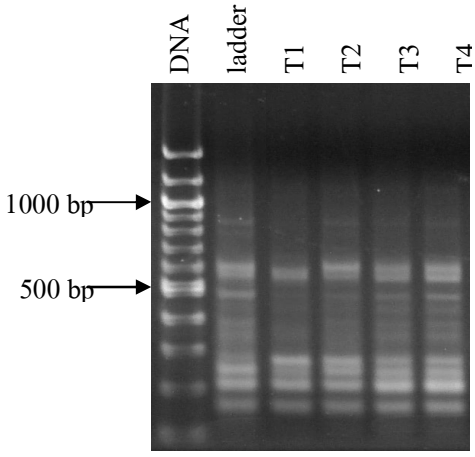


Fig. 3. DNA pattern of *Liriomyza* leafminer using RAPD technique, primer OPC- 02. Column 1 is100 bp DNA ladder, column 6 -2are DNA of leafminers.

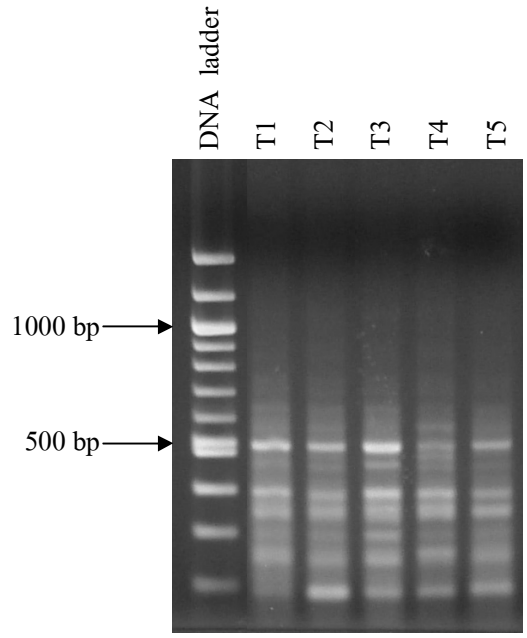


Fig. 4. DNA pattern of *Liriomyza* leafminer using RAPD technique, primer OPC- 05. Column 1 is 100 bp DNA ladder, column 6 -2are DNA of leafminers.

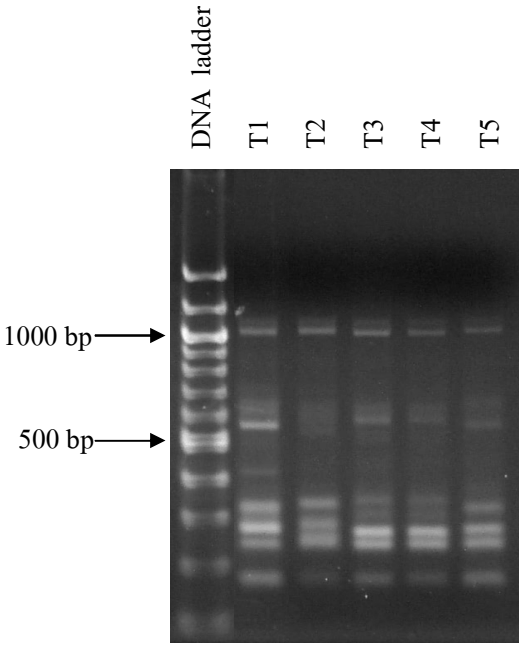


Fig. 5. DNA pattern of *Liriomyza* leafminer using RAPD technique, primer OPC 12. Column 1 is 100 bp DNA ladder, column -2 6are DNA of leafminers.

Using PCR-RAPD technique revealed that all *Liriomyza* leafminer from all yardlong bean fields (Tumbon Korhong (T1), TumbonThakam (T2), Had Yai district; Tumbon Thachamuang (T3), Rattapum district and Tumbon Bangreang (T4), Kuanneang district) and cucumber field (Tumbon Thachamaung (T5), Rattapum district) had the same DNA pattern which means they are in the same species. When using male distipullas and DNA polymorphism together we concluded that the *Liriomyza* leafminer infested yardlong bean and cucumber in Songkhla province included at least a species of *Liriomyza trifolii.*

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(Received 22 January 2010; accepted 4 March 2011)