# Diversity of cruciferous pests: genetic analysis of flea beetle and *Pieris rapae* populations from southeast asia based on mitochondrial *coxI* gene

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## ABSTRACT
Flea beetles and cabbage butterfly (*Pieris rapae*) are the most destructive and devastating pests of cruciferous crops worldwide. Individually, they may cause yield losses as much as 100% in brassicas and therefore cripple the economy of smallholder farmers especially in Southeast Asia. In order to understand the genetic diversity, phylogeography and intraspecific genetic variation among these pest populations, mitochondrial *cytochrome c oxidase 1* (mtcox1) gene was used. We first explored 89 individuals of *Pieris* and 187 individuals of flea beetle populations from five different geographically distinct regions including Taiwan, Cambodia, Vietnam, Thailand and Laos. Individual haplotype diversities based on *cox1* gene showed 10 and 35 different haplotypes of *Pieris* and flea beetles, respectively. Furthermore, Tajima's *D*, Fu's *Fs* and Analysis of Molecular Variance (AMOVA) of pairwise comparison ($F_{st}$) tests were performed across all *Pieris* and flea beetle populations in target countries. The results showed low nucleotide diversity, high genetic differentiation and gene flow, which suggest recent population expansion especially between Taiwan and Thailand populations of flea beetles. Similarly, isolated populations of *P. rapae* have higher haplotype diversity (h>28) and lower nucleotide diversity (0.00033) in Vietnam and Thailand. In addition, genetic differentiation results showed that the Taiwan population could be a separate species. Thus, our results provided better understanding on the genetic diversity of flea beetle and cabbage butterfly populations in Southeast Asia.

## Keywords
Mitochondrial *cytochrome c oxidase 1* (mtcox1), haplotype diversity, nucleotide diversity, population genetics, integrated pest management

## INTRODUCTION
Vegetable brassicas are an important crop in Southeast Asia, which is grown over 148,182 ha with an annual production of 3.25 million (FAO 2019). The productivity in Southeast Asia (19 t/ha) is lower than the Asian average of 25 t/ha. It also widely varies among the countries in the region. For instance, the marketable yield of leafy mustard is only 5.6 t/ha in Laos, whereas it is 15 t/ha in the neighboring Cambodia (Schreinemachers et al. 2017). Insect pests are one of the major limiting factors, causing up to 90% yield loss as reported in Vietnam (Nhung et al. 2008). Besides diamondback moth (*Plutella xylostella* L.), which is the dominant pest in brassicas, cabbage butterfly (*Pieris rapae* L.) and striped flea beetle (*Phyllotreta striolata* Fab.) cause marketable yield losses of up to 100% in leafy brassicas in Southeast Asia (Ungsa and Vanharn 1995; Nhung et al. 2008).

Brassica farmers heavily rely on indiscriminate, repeated application of chemical pesticides to prevent crop losses by pests. A study found that farmers in Cambodia, Laos, and Vietnam on average sprayed pesticides on brassicas weekly as a prophylactic measure, using about 1 kg/ha/week (Schreinemachers et al. 2017). About 75% of these growers mixed different pesticides together in a single spray. Indiscriminate pesticide use can adversely affect human and environmental health, besides creating high-risk perceptions among consumers due to high pesticide residues in the harvested vegetables (Praneetvatakul et al. 2013; Nguyen et al. 2020). In addition, pests experiencing tremendous pressure from pesticides increase the likelihood of developing resistance to the pesticides. Resistance of *P. striolata* to commonly used pesticides has been reported in Southeast Asia (Feng et al. 2000; Ulrichs et al. 2001).

Development of suitable pest control measures requires a thorough understanding of the population of the target pest species, because the population structure and dynamics usually vary from region to region and effective control strategies, such as pheromones and biological control have to be considered accordingly. DNA barcoding using the mitochondrial gene cytochrome c oxidase I (*coxI*) has been used to identify the species, since it is highly conserved across species (Hebert et al. 2003). Understanding the genetic diversity and a comparative phylogeographic studies using different types of markers has important economic implications (Hewitt 2001; Taberlet 1998) Molecular phylogeny using mitochondrial and/or nuclear gene sequences has been well established in thrips (Kadirvel et al. 2013), whiteflies (Ramasamy et al. 2013; Ram Kumar et al. 2017) and *Maruca vitrata* (Malini et al. 2019; Periasamy et al. 2015). Hence, the main objectives of this paper were to identify genetic differences among flea beetle and cabbage butterfly populations through partial mitochondrial *coxI* DNA sequences and to better understand phylogenetic

relationships among the pest populations predominantly occurring in selected Southeast Asian countries.

## MATERIALS AND METHODS

### Geographical location and sampling collection

The flea beetle and the *P. rapae* insects were collected from selected sites in Taiwan, Laos, Cambodia, Thailand and Vietnam (Tables 1 and 2). The collected insects were stored in 95% ethanol at a temperature of -20˚C before DNA extraction.

**Table 1. List of *P. rapae* samples with their host plants and geographical origin**

| *Sample and Sequence id* | *Host Plant* | *Geographical Locations* |
|---|---|---|
| *Pieris rapae* | | |
| Taiwan | | |
| *PR-BRC_Tw-1 to PR-BRC_Tw-5* | Broccoli | WorldVeg HQ, Shanhua, Tainan |
| Lao PDR | | |
| *PR-Lao-Cb8a-1 to PR-Lao-Cb8a-6* | Cabbage | Nong, Kasi district, Vientiane province |
| *PR-Lao-Cb9a-1 to PR-Lao-Cb9a-5* | Cabbage | Ban Pha Tang, Vang Vieng, Vientiane province |
| *PR-Lao-Cb46-1 to PR-Lao-Cb46-4* | Cabbage | Phaxang village, Kasi district, Vientiane province |
| *PR-Lao-pk37-1 to PR-Lao-pk37-5* | Pakchoy | Nadao village, Vang Vieng, Vientiane province |
| Vietnam | | |
| *PR-SVT-CC-2-7* | Common cabbage | Mai Son, Son la province |
| *PR-SVT-FR-20-1 to PR-SVT-FR-20-4* | Forage radish | Moc chau district, Son la province |
| *PR-VT-C3C-1 to PR-VT-C3C-5* | Cauliflower | Khuyen Luong, Thanh tri, Hanoi |
| *PR-VT-Cb-2a-1 to PR-VT-Cb-2a-5* | Cabbage | Quynh Luong, Quynh Luu, Nghe An |
| *PR-VT-Cb5B-1 to PR-VT-Cb5B-5* | Cabbage | Co do Moc chau district, Son la province |
| *PR-VT-CC-1-1 to PR-VT-CC-1-5* | Common cabbage | Giam Lam district, Hanoi |
| *PR-VT-CC2F-1 to PR-VT-CC2F-5* | Cauliflower, Cabbage | Song Phuong, Hoai Duc, Hanoi |
| *PR-VT-CC-10-1, PR-VT-CC-10-2, PR-VT-CC-10-4, PR-VT-CC-10-5* | Common cabbage | Thanh tri district, Hanoi |
| *PR-VT-Cf6a-1 to PR-VT-Cf6a-5* | Cauliflower | Da Mai, Bac Giang city |
| *PR-VT-Lb9B-1 to PR-VT-Lb9B-5* | Leafy Brassica | Mai Son, Son La province |
| *PR-VT-LM4B-1 to PR-VT-LM4B-5* | Leafy mustard | Tay Tuu, Tu Liem district, Hanoi |
| Thailand | | |
| *PR-Th-1 to PR-Th-5* | Cabbage | WorldVeg, Kamphaeng Saen, Nakhon Pathom |

**Table 2. List of flea beetle samples with their host plants and geographical origin**

| Sample and Sequence id | Host Plant | Geographical Locations |
|---|---|---|
| Taiwan | | |
| *SFB-M_Tw1-2 to SFB-M_Tw1-5* | Mustard | WorldVeg HQ, Shanhua, Tainan |
| *SFB-R_Tw2-1 to SFB-R_Tw2-5* | Radish | WorldVeg HQ, Shanhua, Tainan |
| Cambodia | | |

| | | |
|---|---|---|
| *CaM_SFB-5-2, CaM_SFB-5-10* | Pak choi | Krong Stung Treng, Stung Treng province |
| *CaM_SFB-10-1 to CaM_SFB-10-10* | Leafy mustard | Anlong veng, Oddar Meanchey province |
| *CamJ06_2-1_MV to CamJ06_2-10* | Leafy mustard | Samkhuoy, Stung Treng province |
| *CaMM06_SFB-16-1, CaMM06_SFB-16-2, CaMMJ06_SFB-5-1* | Radish | Stueng village, Kandal province |
| *SFB-13-1 to SFB-13-5* | Pak-choi | Preah Sdach district, Prey Veng province |
| Thailand | | |
| SFB-Th_pk1-1 to SFB-Th_pk1-5 | Pak-choi | Kamphaeng Phet province |
| Lao PDR | | |
| *Lao_SFB8c-1, Lao_SFB8c-2, Lao_SFB8c-4, Lao_SFB8c-5* | Cabbage | Nong, Kasi district, Vientiane province |
| *Lao_SFB9c-1, Lao_SFB9c-2, Lao_SFB9c-4, Lao_SFB9c-5* | Cabbage | Ban Pha Tang, Vang Vieng, Vientiane province |
| *SFB-6a-1, SFB-6a-3, SFB-6a-4, SFB-6a-5* | Cabbage | Na Then, Kasi district, Vientiane province |
| *Fb-4_1 to Fb-4_14* | Leafy mustard | Homtai Village, Hatsayphong district, Vientiane Capital |
| *Fb13_1 to Fb-13_10* | Cauliflower | Koksay village, Hatsayphone district, Vientiane Capital |
| *Fb-14_1 to Fb-14_10* | Green mustard | Don village, Hatsayphone district, Vientiane Capital |
| *Fb-15_1, Fb-15_2, Fb-15_6 to Fb-15_10* | Cabbage | Nonetea village, Xaythany district, Vientiane Prefecture |
| *Fb-16_1 to Fb-16_10* | Cauliflower | Sitantai village, Hatsayphone district, Vientiane Capital |
| *FB-2a-1, FB-2a-2* | Chinese cabbage | Luang Prabang |
| Vietnam | | |
| *VT_FB-1c-1, VT_FB-1c-3* | Chinese cabbage | Quynh Luong, Quynh Luu, Nghe An |
| *VT_FBCM-10-3, VT_FBCM-10-4* | Chinese mustard | Mai Son, Son La province |
| *VT_SFB1b-1, VT_SFB1b-5* | Mustard | Quynh Luong, Quynh Luu, Nghe An |
| *VT_SFBCC-7B-2* | Chinese cabbage | Van Duc, Gia Lam district, Hanoi |
| *VT_SFBLB-7A-1 to VT_SFBLB-7A-5* | Leafy brassica | Thuan Chau district, Son La Province |
| *VT_SFBLB-8B-1 to VT_SFBLB-8B-5* | Leafy brassica | Mai Son, Son La province |
| *VT_SFBLB-10B-1 to VT_SFBLB-10B-5* | Leaf brassica | Ham Duc, Ham Thuan Bac district, Bình Thuận Province |
| *VT_SFBLB-11B-1 to VT_SFBLB-11B-5* | Leaf brassica | Xuan Loc district, Đồng Nai Province |
| *VT_SFBLB-12A-1 to VT_SFBLB-12A-5* | Leaf brassica | Buon Me Thuot city, Dak Lak province |
| *VT-_SFBPLM-6B-4, VT-_SFBPLM-6B-5* | Pak-choy | Da Mai, Bac Giang city, Bac Giang Province |
| *VT-FB-1c-4, VT-FB-1c-5* | Chinese cabbage | Quynh Luong, Quynh Luu, Nghe An |
| *VT-FBCM-10-1* | Chinese mustard | Mai Son, Son la province |
| *VT-SFBLB-7A-5* | Leaf brassica | Mai Son, Son la province |
| *VT-SFBLF-1B-1 to VT-SFBLF-1B-5* | Pak- choy | Tien Phong Me Linh district, Hanoi |
| *VT-SFBLM-4C-1 to VT-SFBLM-4C-5* | Leafy mustard | Tay Tuu, Tu Liem district, Hanoi |
| *VT-SFBLM-5B-1 to VT-SFBLM-5B-5* | Leafy mustard | Co do, Moc Chau district, Son la province |

| | | |
|---|---|---|
| *Vt_SFBCC-3-1 to Vt _SFBCC-3-5* | Common cabbage | Co Noi, Mai son, Son la province |
| *Vt _SFBLM-4-2, Vt _SFBLM-4-3, Vt _SFBLM-4-5* | Leafy mustard | Co Noi, Mai son, Son la province |
| *Vt -SFBCC-5-1 to Vt -SFBCC-5-3* | Chinese cabbage | Co Noi, Mai son, Son la province |
| *Vt -SFBR-15-1, Vt -SFBR-15-2, Vt -SFBR-15-5* | Radish | Muong Bon, Mai Son, Son la province |

## DNA Extraction and Sequencing

Extraction of genomic DNA was done using the individual larva or adult tissues of *P. rapae* and flea beetle using Geneaid genomic DNA mini kit (Taipei, Taiwan) following the procedure outlined by (Liu et al. 2011). The mitochondrial *coxI* gene fragment was amplified using universal *cox1* primers (HCO2198, 5'-TAAACTTCAGGGTGACCAAAAAATCA-3', and LCO1490, 5'-GGTCAACAAATCATAAAGATATTGG-3') (Folmer et al. 1994) (Folmer et al. 1994), following PCR protocol: 95°C for 10 min followed by 4 cycles of 95°C for 30s, 55°C for 45s and 72°C for 1.30 min, followed by 30 cycles of 95°C for 30s, 50°C for 45s, with the final extension at 72°C for 8 min. The PCR products were sequenced at Genomics Bioscience and Technology Company Limited, Taiwan.

## Molecular divergence and population genetic analyses

The *coxI* sequences were aligned and edited using BioEdit version 7.0 (Hall 1999). c*ox1* sequences of *P. rapae* from Canada (KT140090.1, KM547147.1), Australia (KF404991.1), Korea EU105213.1, Japan (LC090567.1), Spain (JN827888.1), Romania (HQ004953.1), South Korea (EU105296.1), Kazakhstan (FJ663942.1), Germany (JF415723.1), China (JQ996397.1), USA (JF283398.1), France (KX041897.1), Norway (KX048703.1), Finland (KM573502.1), and flea beetle from Spain (KF656309), Finland (KJ966332), Germany (KM450461), Spain (KF654007), Canada (MG054786), Italy (MH323316.1), Sweden (JX243022), and Finland (KJ962687) were obtained from National Center for Biotechnology Information (NCBI) GenBank. The number of haplotypes, nucleotide diversity and haplotype diversity were calculated for investigating the *coxI* sequence diversity using DnaSP 5.10 software (Librado and Rozas 2009). Statistical tests of Tajima's *D* and Fu's *Fs* values were also conducted using DnaSP 5.10.

The genetic structure of flea beetle and *P. rapae* populations based on *coxI* sequences was examined by Analysis of Molecular Variance (AMOVA) using Arlequin 3.5 software (Excoffier and Lischer 2010). Maximum-likelihood (ML) phylogenetic analysis was used to identify major clades and to evaluate the relationships among the haplotypes of the *coxI* sequences. The appropriate model of sequence evolution, including model parameters, were calculated using corrected Akaike Information Criterion (AICc value) with MEGA 7 (Tamura et al. 2011). The clustering probabilities of each resulting phylogenetic tree node were statistically tested by a bootstrap method consisting of 1000 replicates. *Pieris melete* (Syn: *Artogeia melete*) (Lepidoptera: Pieridae) was used as an outgroup for cabbage butterfly. *Chrysolina fastuosa* (Coleoptera: Chrysomelidae) was used as an outgroup for flea beetles.

## Sequence data

Sequence data that support the findings of this study has been deposited in NCBI GenBank with accession numbers MZ032867- MZ032876 for *Pieris rapae* and MZ033002 – MZ033036 for flea beetle.

## RESULTS AND DISCUSSION

We examined the genetic diversity and structure of *P. rapae* and flea beetles based on the c*oxI* gene. A total of 89 individuals of *P. rapae* and 187 individuals of flea beetles from five countries (Taiwan, Cambodia, Vietnam, Thailand and Laos) were examined. We successfully amplified 690 bp utilizing *coxI* universal primers. After trimming and editing, the final length consisted of approximately 582 bp for *P. rapae* and 584 bp for flea beetles. A total of 10 *coxI* haplotypes were identified in 83 *P. rapae* samples based on sequence similarity (Table 3). The largest haplotype contained nine individuals from Vietnam. This was followed by four haplotypes from Laos.

**Table 3. List of number of samples studied, number of haplotypes, haplotype diversity (h), nucleotide diversity (π), Tajima's *D* and Fu's *Fs* tests for *Pieris rapae* populations from Southeast Asia**

| Country | No. of Samples | No. of haplotypes | Haplotype diversity (h) | Nucleotide diversity (π) | Tajima's *D* | Fu's *Fs* |
|---|---|---|---|---|---|---|
| Taiwan | 5 | 1 | - | - | - | - |
| Laos | 24 | 4 | 0.5688* | 0.02623* | 2.95875 * | 2.2939* |
| Vietnam | 49 | 9 | 0.4804 | 0.00891 | - 1.21535* | 0.2245 |
| Thailand | 5 | 2 | 0.3333* | 0.00057* | -0.93302* | -0.9647* |
| All countries | 83 | 10 | 0.5631 | 0.0149 | 0.5408 | 0.9384 |

The haplotype diversity is a measure of the uniqueness of a particular haplotype in a given population (Nei and Tajima 1981), whereas nucleotide diversity is used to measure the degree of polymorphism within a population (Nei and Li 1979). The total haplotype diversity value of all *P. rapae* population from sampled countries was 0.5631, whereas the total nucleotide diversity of all *P. rapae* population from sampled countries was 0.0149 (Table 3). The highest haplotype and nucleotide diversity values were recorded in Laos, whereas the lowest values were recorded in Thailand. However, no nucleotide and haplotype diversity values of *P. rapae* were recorded for the Taiwan population. This can be due to the absence of haplotypes.

Tajima's *D* and Fu's *Fs* tests were used to calculate neutrality indices (Fu 1995; Fu 1997; Tajima 1989). Negative Tajima's *D* values were recorded for the Thailand and Vietnam populations (Table 3). The negative Tajima's *D* values in these countries indicated that the *P. rapae* population began to expand recently. However, the significantly positive Tajima's *D* value for Laos indicated that the *P. rapae* population may have suffered a recent sharp decline in its size (bottleneck). Such studies are lacking for the brassica pests in Southeast Asia. However, Tajima's *D* test was used to study the population structure of legume pod borer (*Maruca vitrata*) in Southeast Asian countries (Periasamy et al. 2015). A significantly positive value of Fu's *Fs* for Laos's population is evidence for the deficiency of alleles due to a recent population decrease. Thus, both Tajima's *D* and Fu's $F_S$ values for *P. rapae* population in Laos indicated the population bottleneck events. Although the positive value of Fu's *Fs* was recorded for the P. rapae population in Vietnam, it was not significant. Besides Tajima's *D*, a negative value of Fu's *Fs* for Thailand *P. rapae* population is evidence for a possible recent population expansion or genetic drift due to random sampling.

The $F_{ST}$ values of all population pairwise comparisons ranged from -0.06 to 1.00 (Table 4). Negative $F_{ST}$ values can be interpreted as no genetic differences between the two populations compared, due to imprecision of the algorithm used (Jaramillo et al. 2001), as shown between Taiwan and Vietnam *P. rapae* populations. The low and non-significant $F_{ST}$ values (0.13-0.24) among Taiwan vs Laos and Vietnam, Laos vs Vietnam and Thailand, and Vietnam vs Thailand populations showed less genetic differences among them. However, the Thailand population recorded a higher $F_{ST}$ value (1.00) when compared with Taiwan population and hence making it unique and maybe a separate species (Roderick 1996), which warrants further studies including morphological characterization. This can be also explained by the fact that absence of heterozygote in the sub-population relative to the target populations could be the major factor for the complete genetic differentiation in Taiwan (Nei et al. 1975; Roderick 1996). Such genetically less diverse populations were recorded in previous studies with *P. xylostella* and *Xyleutes ceramicus* (Li et al. 2006; Panyamang et al. 2018).

**Table 4. Pairwise $F_{ST}$ values comparing populations of *Pieris rapae***

| Population | Taiwan | Laos | Vietnam | Thailand |
|---|---|---|---|---|
| **Taiwan** | 0 | | | |
| **Laos** | 0.19 | 0 | | |
| **Vietnam** | -0.06 | 0.24 | 0 | |
| **Thailand** | **1.00 *** | 0.24 | 0.13 | 0 |

The phylogenetic pattern of *P. rapae* was developed utilizing all haplotypes obtained in the present study. According to the Maximum likelihood (ML) phylogenetic tree, two different clades were formed for *P. rapae* (Figure 1). Clade I contained the majority of the samples from Laos, Taiwan and Vietnam; interestingly, Thailand population and one sample from the highlands of Vietnam (Son la province) were assembled as a subclade with 61% bootstrap value within this clade I. Similarly, two samples

from lowlands of Vietnam also formed another subclade with 64% bootstrap value within clade I. It should be noted that all the reference samples of *P. rapae* from NCBI aligned with clade I. Clade II contained two populations from Laos (Kasi and Vangvieng districts of Vientiane province) and two populations from Vietnam highlands (Moc Chau and Mai Son of Son la province). These results indicated the genetic dissimilarity of *P. rapae* populations originating, especially from Laos and Vietnam. This could be possibly due to speciation events occurring in those regions (Coyne 1992; Avise 2000), although it may not be so obvious in the adult butterflies. Hence, additional samples from these regions should be obtained to validate the findings of this study, especially using the nuclear regions, since mitochondrial DNA is often unable to identify recently emerged species because of the time required to separate the intraspecific variation from interspecific divergence (barcoding gap). In addition, adult samples should also be collected for morphological characterization.

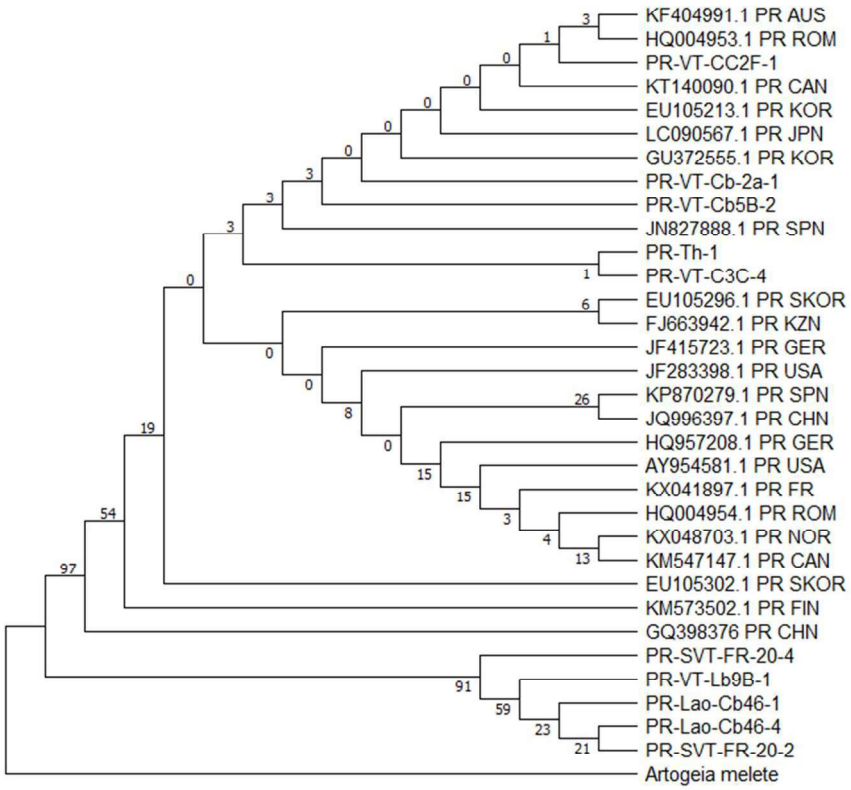


**Figure 1. Phylogenetic relationship based on partial mtcox1 sequence data of *Pieris rapae* populations from South-East Asia.**

The evolutionary history was inferred using the maximum likelihood method employing the best fit Tamura 3-parameter model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown. This analysis involved 33 nucleotide sequences. Evolutionary analyses were conducted in MEGA 7 (Tamura et al. 2011)

A total of 36 *coxI* haplotypes were identified in 187 flea beetle samples based on sequence similarity (Table 5). The largest number of haplotypes occurred in Vietnam, followed by 11 haplotypes in Laos. The total haplotype diversity value of all flea beetle population from sampled countries was 0.82, whereas the total nucleotide diversity of all flea beetle population from sampled countries was 0.00601 (Table 5). The haplotype diversity values were almost the same for both Laos (0.855) and Vietnam (0.858), and they were significant. Similarly, Vietnam recorded the significantly highest nucleotide diversity value (0.00729). The lowest haplotype and nucleotide diversity values were recorded in Taiwan.

**Table 5. List of number of samples studied, number of haplotypes, haplotype diversity (h), nucleotide diversity (π), Tajima's *D* and Fu's *Fs* tests for flea beetle populations from Southeast Asia**

| Country | No. of Samples | No. of haplotypes | Haplotype diversity (h) | Nucleotide diversity (π) | Tajima's *D* | Fu's *Fs* |
|---|---|---|---|---|---|---|
| Taiwan | 10 | 2 | 0.2 | 0.00034 * | -1.11173 * | -0.339 * |
| Laos | 26 | 11 | 0.855 * | 0.004 | -1.40894 * | -3.981 * |
| Vietnam | 109 | 28 | 0.858 * | 0.00729 * | -2.70033 * | -9.497 * |
| Thailand | 5 | 2 | 0.4 | 0.00205 | -1.04849 * | 1.688 |
| Cambodia | 37 | 7 | 0.643 | 0.00333 | -0.28919 * | -0.201 * |
| All countries | 187 | 35 | 0.82 | 0.00601 | -2.65988 | -18.104 |

The negative Tajima's *D* values for all the countries indicated that the flea beetle population began to expand recently. Besides Tajima's *D*, significant and negative values of Fu's *Fs* for all the countries except Thailand are also an evidence for a possible recent population expansion or genetic drift due to random sampling.

The $F_{ST}$ values of all flea beetle population pairwise comparisons ranged from -0.079 to 0.78502 (Table 6). Negative $F_{ST}$ values between Cambodia and Thailand flea beetle populations confirmed the absence of genetic differences. The low and non-significant $F_{ST}$ values among Laos and Vietnam (0.01407), Vietnam and Thailand (0.06434), and Taiwan and Vietnam (0.0026) populations showed less or negligible genetic differences among them. However, significantly higher $F_{ST}$ values among Taiwan and Cambodia (0.46616), and Taiwan and Thailand (0.78502) populations can be considered as a different subspecies or species, which warrant further molecular studies using nuclear regions and morphological characterization. It should be noted that migration by means of anthropogenic activities played a critical role in shaping the flea beetle population structure in Taiwan (Lee et al. 2011). The maximum likelihood (ML) tree was constructed for *cox1* gene using *Phyllotreta* reference sequences from Europe (Sweden, Italy, Germany, Finland, and Spain), North America (the USA and Canada) and Oceania (Australia). According to the phylogenetic analysis (Figure 2), all populations are more related to *P. striolata* suggesting the presence of close genetic relationship and are not distinct with bootstrap value 1000.

**Table 6. Pairwise $F_{ST}$ values comparing populations of flea beetle**

| Population | Taiwan | Cambodia | Laos | Vietnam | Thailand |
|---|---|---|---|---|---|
| Taiwan | 0 | | | | |
| Cambodia | 0.46616 * | 0 | | | |
| Laos | 0.2983 | 0.2817 | 0 | | |
| Vietnam | 0.0026 | 0.1086 | 0.01407 | 0 | |
| Thailand | 0.78502 * | -0.079 | 0.31787 | 0.06434 | 0 |

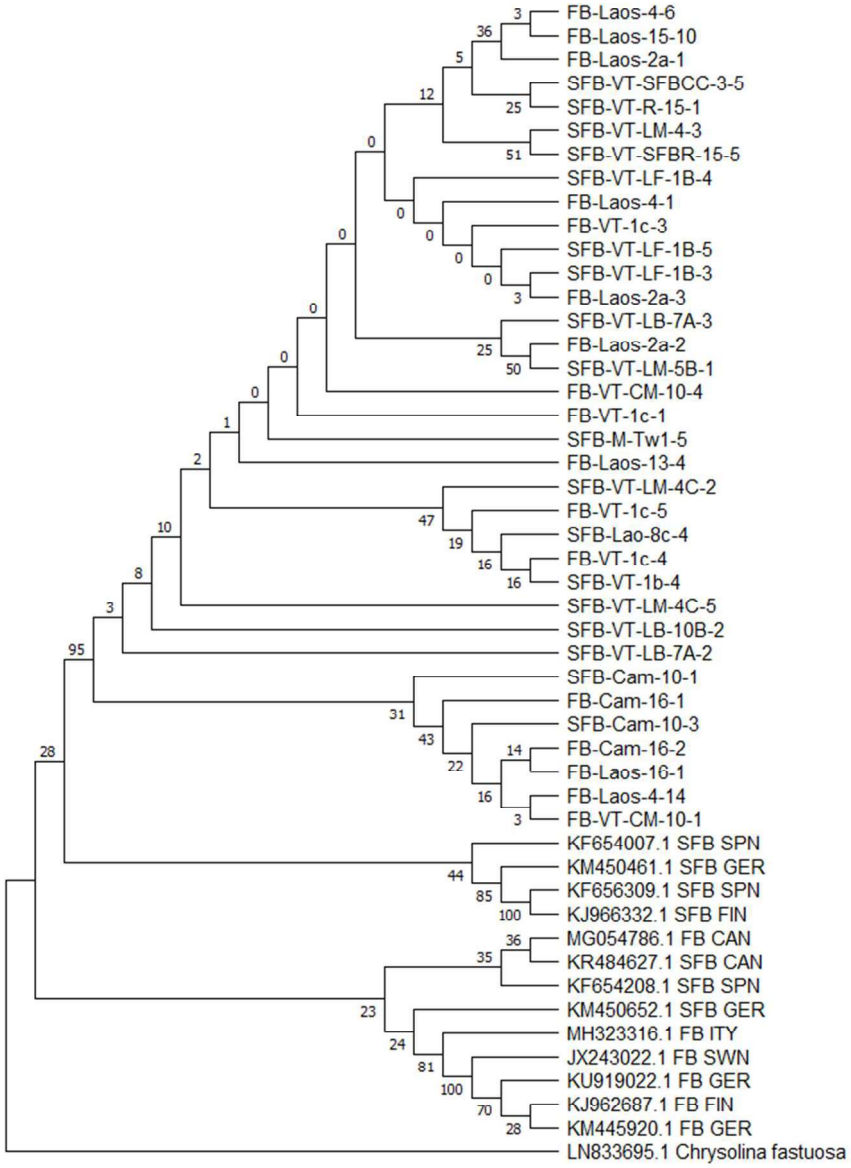


**Figure 2. Phylogenetic relationship based on partial mtcox1 sequence data of Flea beetle populations from South-East Asia.**

The evolutionary history was inferred using the maximum likelihood method employing best fit Hasegawa-Kishino-Yano model (Hasegawa et al. 1985). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown. This analysis involved 49 nucleotide sequences. Evolutionary analyses were conducted in MEGA 7 (Tamura et al. 2011)

## CONCLUSIONS

The study was carried out to understand the phylogenetic relationship among geographically different flea beetles and *P. rapae* populations in Southeast Asia using the *cox1* gene. No such studies were carried out in Southeast Asia in the past. The results showed that both *Phyllotreta* flea beetles and *Pieris rapae* had more haplotypes in Laos and Vietnam. A part of the *P. rapae* population from Vientiane province in Laos and the Son la highlands in Vietnam formed a separate clade in the phylogenetic tree. The majority of the *Phyllotreta* flea beetles in target countries were identified as *P. striolata*. Additional studies are suggested to validate the findings of this study, especially using the nuclear regions, since mitochondrial DNA is often unable to identify recently emerged species. It is imperative to understand the genetic differences or similarities existing among the flea beetle and *P. rapae* populations in Southeast Asia in order to introduce effective pheromone-based and biological control options to manage these pests, and thus reducing the indiscriminate use of hazardous pesticides.

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## References

Avise JC. 2000. Phylogeography: the history and formation of species. Harvard University Press.

Coyne JA. 1992. Genetics and speciation. Nature 355:511-515.

Excoffier L, Lischer HE. 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources 10:564-567.

FAO. 2019. Crops. FAOSTAT. http://www.fao.org/faostat/en/#data/QC

Feng H, Huang YJ, Hsu J. 2000. Insecticide susceptibility of cabbage flea beetle (*Phyllotreta striolata* (Fab.)) in Taiwan. Plant Protection Bulletin (Taipei), 42(1): 67-72.

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.

Fu YX. 1995. Statistical properties of segregating sites. Theoretical Population Biology 48:172-197.

Fu YX. 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 147:915-925.

Hasegawa M, Kishino H, Yano T. 1985. Dating the human-ape split by a molecular clock of mitochondrial DNA. Journal of Molecular Evolution 22:160-174.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NTNucleic acids symposium series: [London]: Information Retrieval Ltd., c1979-c2000. pp 95-98.

Hebert PD, Cywinska A, Ball SL, deWaard JR. 2003. Biological identifications through DNA barcodes. Proceedings. Biological Sciences 270:313-321.

Hewitt GM. 2001. Speciation, hybrid zones and phylogeography - or seeing genes in space and time. Molecular Ecology 10:537-549.

Jaramillo C, Montana MF, Castro LR, Vallejo GA, Guhl F. 2001. Differentiation and genetic analysis of *Rhodnius prolixus* and *Rhodnius colombiensis* by rDNA and RAPD amplification. Memorias do Instituto Oswaldo Cruz 96:1043-1048.

Kadirvel P, Srinivasan R, Hsu YC, Su FC, De La Pena R. 2013. Application of cytochrome oxidase I sequences for phylogenetic analysis and identification of thrips species occurring on vegetable crops. Journal of Economic Entomology 106:408-418.

Lee C, Chang H, Wang CL, Chen WS. 2011. A review of *Phyllotreta* Chevrolat in Taiwan (Coleoptera: Chrysomelidae: Galerucinae: Alticini). Zoological Studies 50:525-533.

Li J, Zhao F, Choi YS, Kim I, Sohn HD, Jin BR. 2006. Genetic variation in the diamondback moth, *Plutella xylostella* (Lepidoptera: Yponomeutidae) in China inferred from mitochondrial COI gene sequence. European Journal of Entomology 103:605-611.

Librado P, Rozas J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451-1452.

Liu L, Liu J, Wang Q, Ndayiragije P, Ntahimpera A, Nkubaye E, Yang Q, Li Z. 2011. Identification of *Bactrocera invadens* (Diptera: Tephritidae) from Burundi, based on morphological characteristics and DNA barcode. African Journal of Biotechnology 10(62): 13623-13630.

Malini P, Ramasamy S, Schafleitner R, Muthukalingan K. 2019. Pheromone-binding proteins based phylogenetics and phylogeography of *Maruca* spp. from Asia, Africa, Oceania, and South America. Ecology and Evolution 9:9239-9272.

Nei M, Li WH. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proceedings of the National Academy of Sciences of the United States of America 76: 5269-5273.

Nei M, Tajima F. 1981. DNA polymorphism detectable by restriction endonucleases. Genetics. 97(1): 145–163.

Nei M, Maruyama T, Chakraborty R. 1975. The Bottleneck Effect and Genetic Variability in Populations. Evolution. International Journal of Organic Evolution 29:1-10.

Nguyen T, Mai H, Boulom S, Voe P, Heang C, Ha D, Chialue L, Bor B, Phommaluesa S, Pham D. 2020. Consumers' risk perception of vegetables in Southeast Asia: Evidence from Laos, Cambodia, and Viet Nam. APN Science Bulletin, 10(1); doi:10.30852/sb.2020.1130.

Nhung NT, Viet TQ, Nghiep NT. 2008. Insect pests on Crucifers and their control by chemicals at Vo cuong, Bac Ninh Province, *Proceeding of the 6th Vietnam National Conference on Entomology, May -10, 2008*. Agricultural Publishing House, Hanoi, Vietnam.

Panyamang A, Duangpakdee O, Rattanawannee A. 2018. Genetic structure of teak beehole borer, *Xyleutes ceramicus* (Lepidoptera: Cossidae), in northern Thailand. Agriculture and Natural Resources 52:66-74.

Periasamy M, Schafleitner R, Muthukalingan K, Ramasamy S. 2015. Phylogeographical Structure in Mitochondrial DNA of Legume Pod Borer (*Maruca vitrata*) Population in Tropical Asia and Sub-Saharan Africa. PloS One 10:e0124057.

Praneetvatakul S, Schreinemachers P, Pananurak P, Tipraqsa P. 2013. Pesticides, external costs and policy options for Thai agriculture. Environmental Science & Policy 27:103-113.

Ram Kumar N, Chang JC, Narayanan MB, Ramasamy S. 2017. Phylogeographical structure in mitochondrial DNA of whitefly, *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) in southern India and Southeast Asia. Mitochondrial DNA. Part A, DNA Mapping, Sequencing, and Analysis 28:621-631.

Ramasamy S, Hsu YC, Palchamy K, Lin MY. 2013. Analysis of *Bemisia tabaci* (Hemiptera: Aleyrodidae) species complex in Java Island, Indonesia based on mitochondrial cytochrome oxidase I sequences. The Philippine Agriculturist 96:290-295.

Roderick GK. 1996. Geographic structure of insect populations: gene flow, phylogeography, and their uses. Annual Review of Entomology 41:325-352.

Schreinemachers P, Chen HP, Nguyen TTL, Buntong B, Bouapao L, Gautam S, Le NT, Pinn T, Vilaysone P, Srinivasan R. 2017. Too much to handle? Pesticide dependence of smallholder vegetable farmers in Southeast Asia. The Science of the Total Environment 593-594:470-477.

Taberlet P. 1998. Biodiversity at the intraspecific level: The comparative phylogeographic approach. Journal of Biotechnology 64:91-100.

Tajima F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585-595.

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.

Ulrichs C, Mewis I, Schnitzler WH, Burleigh JR. 2001. Effectivity of synthetic insecticides against *Maruca vitrata* F. and the parasitoid *Bassus asper* Chou & Sharkey in the Philippines. Mitteilungen der Deutschen Gesellschaft fur Allgemeine und Angewandte Entomologie. 13(1-6): 279–282.

Ungsa M, Vanharn H. 1995. Vegetable Production, Research and Policy in Cambodia. *In*: AVRDC.1995. Vegetable research and development in Cambodia, Lao PDR and Vietnam; Proceedings of a workshop. Asian Vegetable Research and Development Center. Shanhua, Tainan, Taiwan. Publication no. 94-431.130 p.