Phylogeographical structure in mitochondrial DNA of Diamondback Moth (*Plutella xylostella*) population in Southeast Asia

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ABSTRACT

Diamondback moth, *Plutella xylostella* L. (Plutellidae: Lepidoptera) is one of the most important insect pests, constraining brassica production worldwide. Since it has developed resistance to chemical and bio-pesticides, no single approach can provide satisfactory control of this notorious pest. Hence, integrated pest management strategies are warranted. In order to use the control options such as bio-control agents and sex pheromone lures, a thorough understanding of the population structure of the target pest is highly imperative. Hence, this study was undertaken to assess the genetic diversity of the *P. xylostella* population in Southeast Asia, especially in Cambodia, Lao PDR, Malaysia, Thailand, Taiwan and Vietnam. For comparison, a population from West Asia (Syria) was also included. The cytochrome c oxidase subunit 1 (*cox1*) gene was used to understand the phylogenetic relationship of geographically different *P. xylostella* population from Southeast Asia. Extensive sampling was done from different host plant species (broccoli, cabbage, cauliflower, Chinese cabbage, Chinese kale, green mustard, pak-choi, radish, and kohlrabi) in target countries. A total of 52 different populations containing 245 individuals were used in the study. An amplicon of 709 bp was produced by polymerase chain reaction, and editing resulted in a consensus sequence of 643 bp across all *P. xylostella* population. A total of 77 haplotypes were identified in 245 *P. xylostella* individuals. Phylogenetic analysis showed no difference among most of the *P. xylostella* population from different host plants, except few populations from Thailand and Vietnam, which formed a separate cluster. The high F_{ST} values (0.44-0.45) of *P. xylostella* population in Taiwan compared to Cambodia and Malaysia population seem to indicate the presence of genetically diverse populations, which are yet to be confirmed by additional sampling. The negative Tajima's *D* and Fu's F_S values showed the recent demographic expansion of the *P. xylostella* population in Cambodia, Lao PDR and Vietnam. Thus, this study showed the likely presence of genetically diverse *P. xylostella* in Southeast Asia, but it requires additional studies with more populations, especially from Cambodia, Lao PDR, Vietnam, Thailand and Taiwan.

Keywords

Plutella xylostella, mitochondrial cytochrome c oxidase I, phylogeny, population structure

INTRODUCTION

Vegetable brassicas are an important group of vegetables cultivated and consumed in Southeast Asia. Cabbages and other brassicas are cultivated in an area of over 123,000 ha, with an annual production of 2.89 million t in this region (FAO 2019). Cabbage (*Brassica oleracea* var. *capitata*), cauliflower (*B. oleracea* var. *botrytis*), broccoli (*B. oleracea* var. *italica*), Chinese cabbage (*Brassica rapa* var. *pekinensis*), pak-choi (*B. rapa* var. *chinensis*), choi sum (*B. rapa* var. *parachinensis*), Chinese kale (*B. oleracea* var. *alboglabra*), green mustard (*Brassica juncea*), radish (*Raphanus sativus*) and kohlrabi (*B. oleracea* var. *gongylodes*) are some of the most commonly cultivated brassica crops in Southeast Asia. These vegetables provide vitamins, minerals, and dietary fiber. They are also high value crops, since they provide quick (short-duration and repeat cycle crops) and higher incomes compared to staple crops.

Insect pests and plant diseases severely limit the productivity of brassicas and stimulate the overuse of pesticides in Southeast Asia. In the specific case of the Diamondback moth (*Plutella xylostella*), this is the dominant pest in brassicas, although *Spodoptera litura, Pieris rapae* and *Phyllotreta striolata* caused marketable yield losses of up to 100% in brassicas in Cambodia, Lao PDR and Vietnam (Ungsa and Vanharn 1995; Nhung et al. 2008; World Bank 2012; Srinivasan et al. 2019). In addition, the productivity of brassicas is comparatively lower in Southeast Asia (23.41 t/ha) than the world (28.68 t/ha) or East Asia

(35.04 t/ha) average (FAO 2019). The productivity significantly varies among the countries within Southeast Asia. For instance, the yield of green mustard is only 5.6 t/ha in Lao PDR, whereas it is 12.4 t/ha in Vietnam and 15 t/ha in Cambodia (Schreinemachers et al. 2017). Hence, the farmers predominantly rely on chemical pesticides to reduce the incidences of pests and diseases and thus increasing the yield and income. Unless these biotic constraints are addressed through a comprehensive integrated pest management (IPM) approach, it will not be possible to significantly reduce overall pesticide use on the brassica crops.

In order to use the most effective pest control options especially bio-control agents and sex pheromone lures, a thorough understanding of the population structure of the target pest is highly imperative. Studies in Australia have revealed the presence of a cryptic species *Plutella australiana*, besides *P. xylostella* (Landry and Hebert 2013; Perry et al. 2018). However, a recent study showed that there was no genetic differentiation among *P. xylostella* populations in Australia irrespective of geographic location, host plant or sampling year, and no evidence for isolation-by-distance (Perry et al. 2020). Little genetic differentiation was found among the *P. xylostella* populations from China and Korea (Li et al. 2006). Similarly, there was no genetic differentiation found among the *P. xylostella* populations and no correlation between genetic and geographical distance was found. However, pairwise analysis of the mitochondrial genes indicated that *P. xylostella* populations from the southern region of China were more differentiated than those from the northern region (Wei et al. 2013). A recent study on spatial genetic structure analysis in China also revealed three genetic clusters of *P. xylostella* in the southern provinces (Chen et al. 2021). However, there was no study assessing the populations of *P. xylostella* in Southeast Asia. Therefore, this study was undertaken to assess the genetic diversity of the *P. xylostella* population in Southeast Asia, especially in Cambodia, Lao PDR, Malaysia, Thailand, Taiwan and Vietnam.

MATERIALS AND METHODS

Insect sampling

For this study, a total of 52 different *P. xylostella* populations from nine host plants (cabbage, cauliflower, broccoli, Chinese cabbage, Chinese kale, green mustard, pak-choi, radish and kohlrabi) in six countries, *viz*., Cambodia (11°50'N 105°01'E), Lao PDR (19°05'N 102°24'E to 19°51'N 102°06'E, and 17°49'N 102°41'E to 18°07'N 102°42'E), Malaysia (5°59'N 116°34'E), Taiwan (23°12N 120°30'E), Thailand (14°01'N 99°57E) and Vietnam (21°07'N 105°49"E to 21°16'N 105°76"E, 20°51'N 109°36"E, 19°09'N 105°43'E, 19°12'N 105°30'E, and 14°50'N 105°49"E) were collected from the field, and preserved in 95% ethanol. For comparison, a population from West Asia (Syria) was also included.

DNA extraction

The whole insect was placed on Whatman filter paper, washed with double distilled water, and allowed to dry for 10 min. A small part of the larva was cut and transferred to eppendorf tubes containing 50 µl UniversAll™ extraction solution. The samples were vortexed for 15 s, incubated in a dry heater for 23-25 min at 98°C. The samples were left to cool down in room temperature and then centrifuged at 3000 rpm. The DNA solution was treated with RNase and stored in aliquots at -20°C.

Polymerase chain reaction (PCR) and DNA sequencing

Cytochrome c oxidase subunit 1 (*cox1*) gene was selected for this study. The universal *cox1* primer pair (HC02198 5Rev'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3' and LCO1490: 5'For-GGT CAA CAA ATC ATA AAG ATA TTG G-3') reported by Folmer et al. (1994) was used to amplify the partial sequence of the *cox1* gene of *P. xylostella* larvae. PCR was performed in a total reaction volume of 25 µl containing 2.0 µl 10x PCR buffer, 1.5 µl 0.15 µM dNTPs, 2.0 µl 5 µM of each forward and reverse primer, 0.25 µl 0.015 unit/µl *Taq* polymerase, 16.45 µl of double distilled water (ddH_2O), and 0.8 µl of DNA template. PCR was performed in a MJ Research Thermocycler (PTC200 DNA Engine Cycler, Bio-Rad Laboratories, Inc.) following the profile: 95°C for 10 min followed by 4 cycles of 95°C for 30 s, 50°C for 45 s, 72°C for 1 min, followed by 30 cycles of 95°C for 30 s, 65°C for 45 s, 72°C for 45 s with the final extension at 72°C for 8 min. After amplification, 5 µl of the PCR product of each sample was analyzed by electrophoresis on 1.5% agarose gel containing ethidium bromide. Bands were revealed and photographed under ultraviolet light. After electrophoresis, the remaining PCR products were used for sequencing with the previously mentioned forward and reverse primers, using ABI 3730XL systems at Genomics Bioscience and Technology Company Limited, Taiwan.

Molecular divergence and population genetic analyses

The *cox1* sequences were aligned and edited by using BioEdit version 7.0 (Hall 1999). The obtained sequences were aligned with mitochondrion genome reference sequences from National Center for Biotechnology Information (NCBI) GenBank (GenBank accession number NC_025322.1) to confirm that the amplified gene region is located in the

mitochondria only. The sequences were also examined for polymorphism among the *P. xylostella* population collected from different locations or host plants. Reference sequences were obtained from the NCBI GenBank database. The number of variable nucleotide sites, number of haplotypes, nucleotide diversity and haplotype diversity were calculated for investigating the *cox1* sequence diversity using DnaSP 5.10 software (Librado and Rozas 2009). Statistical tests of Tajima's *D* and Fu's F_S values were used to detect the deviation from the neutral model of evolution using DnaSP 5.10. Pairwise F_{ST} values used to appraise the genetic structure among population were obtained with 1000 permutations and at the significance level of 0.05 using the Kimura 2-parameter (K2P) model (Kimura 1980).

Phylogenetic analysis

The FASTA formatted *cox1* sequences were imported into MEGA X software package sequence alignment application and a multiple sequence alignment was performed with ClustalW algorithm using default parameters (Tamura et al. 2011). The insects that showed 100% nucleotide similarities were designated as a single *cox1* haplotype and the others showing different sequence polymorphism were designated as different *cox1* haplotype. *cox1* sequence of *P. xylostella* from China obtained from NCBI GenBank was used as the reference sequence. The aligned sequences were used for phylogenetic analysis. Maximum Likelihood (ML) phylogenetic analysis was used to identify major clades and to evaluate the relationship among the haplotypes of the *P. xylostella cox1* sequences. The appropriate model of sequence evolution, including model parameters, was calculated using corrected Akaike Information Criterion (AICc value) with MEGA X, and resulted in T92+G+I as the best model (Tamura et al. 2011). The model was also selected based on partitioning by codon position. With those settings, a heuristic search was performed (nearest neighbor interchange algorithm starting tree obtained via neighbor joining). Non-uniformity of evolutionary rates among sites was modeled by using a discrete Gamma distribution (+G) with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+I). Whenever applicable, estimates of gamma shape parameters were included. The clustering probabilities of each resulting phylogenetic tree node were statistically tested by a bootstrap method consisting of 1000 replicates. The tree was rooted by the outgroup *Plutella australiana* (KF370868).

RESULTS AND DISCUSSION

The universal *cox1* primer pair (HC02198 and LCO1490) successfully amplified PCR products of 709 bp size in different *P. xylostella* population. Although the sequence alignment and editing resulted in a consensus sequence of 643 bp across all *P. xylostella* population, we used 632 bp consensus sequences to include the reference sequence in this study.

A total of 77 *cox1* haplotypes were identified in 245 *P. xylostella* individuals (Table 1). The largest haplotype (Haplotype 9) contained 44 *P. xylostella* individuals, which were collected from Cambodia, Lao PDR, Vietnam and Syria. Haplotype 6 with 43 *P. xylostella* individuals from Cambodia, Lao PDR, Malaysia, Vietnam and Syria formed the second largest haplotype group. Haplotype 13 contained 20 *P. xylostella* from Lao PDR and Vietnam. A total of 42 haplotypes occurred within the populations from Vietnam, followed by 37 in Lao PDR, 8 in Cambodia and 4 each in Thailand and Malaysia.

Table 1. List of identified *Plutella xylostella* haplotypes with their geographical origin

Country	No. of samples	No. of haplotypes	Haplotypes
Cambodia	31	8	H6, H9, H44 to H49
Lao PDR	84	37	H1, H6, H9 to H43
Malaysia	10	4	H3, H4, H5, H6
Taiwan	5	2	H1, H2
Thailand	13	4	H1, H6, H7, H8
Vietnam	99	42	H1, H2, H6, H9, H11, H13, H14, H16, H18, H21, H25, H32, H37, H43, H46, H50 to H76
Syria	3	3	H6, H9, H77
All countries	245	77	

Nucleotide diversity is used to measure the degree of polymorphism within a population (Nei and Li 1979). The nucleotide diversity of *P. xylostella* population from Syria was the lowest (0.00181) with Malaysia being the highest (0.00995), followed by Thailand, Vietnam, Lao PDR, Taiwan and Cambodia (Table 2). The total nucleotide diversity of

all *P. xylostella* population from sampled countries was 0.00637. The haplotype diversity is a measure of the uniqueness of a particular haplotype in a given population (Nei and Tajima 1981). The haplotype diversity value was lowest in Taiwan, followed by Cambodia; it was highest in Lao PDR, followed by Vietnam. The total haplotype diversity value of all *P. xylostella* population from sampled countries was 0.926 (Table 2).

Tajima's *D* test showed positive values but not significant for Syria, Thailand and Taiwan populations (Table 2). However, Tajima's *D* test showed negative values for all other samples including the overall population. The negative Tajima's *D* values indicated that the *P. xylostella* population in Cambodia, Lao PDR, Malaysia and Vietnam began to expand recently, and they provide evidence for purifying selection at this locus. However, positive Tajima's *D* value for Syria, Thailand and Taiwan indicated that the *P. xylostella* population may have suffered a recent sharp decline in its size (bottleneck). Besides Tajima's *D*, a significant negative value of Fu's F_S especially for Cambodia, Lao PDR, Syria and Vietnam population is evidence for a possible recent population expansion or genetic drift due to random sampling. A positive value of Fu's F_S for Taiwan and Thailand population is evidence for the deficiency of alleles due to a recent population decrease.

Table 2. List of number of samples studied, number of haplotypes, haplotype diversity (*h*), nucleotide diversity (π), Tajima's *D* and Fu's F_S tests for *Plutella xylostella* populations from 7 countries in Southeast Asia, and West Asia

Country	Haplotype diversity (h)	Nucleotide diversity (π)	Tajima's *D*	Fu's F_S
Cambodia	0.686	0.00256	-1.87799*	-5.239**
Lao PDR	0.946	0.00604	-1.16204	-25.774**
Malaysia	0.889	0.00995	-0.45258	0.466
Thailand	0.744	0.00817	0.08186	4.149
Vietnam	0.914	0.00721	-0.97574	-25.584**
Taiwan	0.600	0.00373	1.64070	3.022
Syria	0.833	0.00181	0.00000	-1.216*
All countries	0.926	0.00637	-1.55611	-32.696

The F_{ST} values of all population pairwise comparisons were ranged from -0.07 to 0.45 (Table 3). 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). The genetic difference of Taiwan population from Cambodia or Malaysia population was highly significant based on pairwise F_{ST} values (0.44–0.45; $p<0.01$). Surprisingly, Syrian *P. xylostella* population has little genetic differentiation from Southeast Asian population, except Malaysia and Taiwan.

The intraspecific phylogenetic relationships based on the *cox1* sequences of *P. xylostella* are shown in Fig 1. Phylogenetic analysis based on partial *cox1* sequences was used to classify *P. xylostella* collected from different crops in different locations of selected countries in Southeast Asia. According to the maximum likelihood phylogenetic tree in the current study, there was no difference among most of the *P. xylostella* population from different host plants, except a few populations from Thailand and Vietnam, which formed a separate cluster. Population genetic structure of *P. xylostella* has been assessed in various countries including Australia, China, Korea and India. Another study assessed genetic differentiation among 14 *P. xylostella* populations from the USA, Brazil, Japan, the Philippines, Uzbekistan, France, Benin, South Africa, Reunion Island, and Australia (Pichon et al. 2006).

Table 3. Pairwise F_{ST} values (below diagonal) and the statistical significance (above diagonal) comparing populations of *Plutella xylostella* based on *coxI*

Population	Cambodia	Lao PDR	Malaysia	Thailand	Vietnam	Taiwan	Syria
Cambodia	0.000	**	**	**	**	**	ns
Lao PDR	0.093	0.000	**	**	*	ns	ns
Malaysia	0.222	0.190	0.000	**	**	**	*
Thailand	0.358	0.148	0.257	0.000	**	ns	ns
Vietnam	0.085	0.010	0.145	0.106	0.000	**	ns
Taiwan	0.445	0.173	0.448	0.013	0.147	0.000	*
Syria	-0.021	-0.070	0.277	0.135	-0.067	0.310	0.000

However, there was no study assessing the populations of *P. xylostella* in Southeast Asia, especially in Cambodia, Lao PDR, Malaysia, Thailand, Taiwan and Vietnam. Hence, the current study was conducted using *P. xylostella* populations from these countries. Our results from phylogenetic analysis showed that the few *P. xylostella* populations from Thailand and Vietnam formed a separate clade in the ML phylogenetic tree. Otherwise, the majority of the populations formed a single clade, without showing significant genetic variations. Earlier results from Australia also showed that there was no genetic differentiation among *P. xylostella* populations irrespective of geographic location or distance, host plant or sampling year (Perry et al. 2020), although a cryptic species *P. australiana*, was known to occur in Australia (Landry and Hebert 2013; Perry et al. 2018). Similarly, little genetic differentiation was found among the *P. xylostella* populations from China and Korea (Li et al. 2006; Wei et al. 2013). Another study from India, which involved *P. xylostella* populations from 13 provinces demonstrated that all the populations were highly interrelated based on *cox1* gene (Ojha et al. 2016). However, a recent study on spatial genetic structure analysis revealed three genetic clusters of *P. xylostella* in the southern provinces of China (Chen et al. 2021).

Based on Random Amplified Polymorphic DNA (RAPD) markers, considerable genetic variation was found among *P. xylostella* populations from hilly regions of Himachal Pradesh and the Indo-Gangetic plains in India (Arvind Kumar et al. 2018). Therefore, the *P. xylostella* populations from Thailand and Vietnam that showed some genetic variations should be studied further to understand if the degree of differentiation varies within a population of *P. xylostella* due to migrations or reduction of size due to environmental conditions.

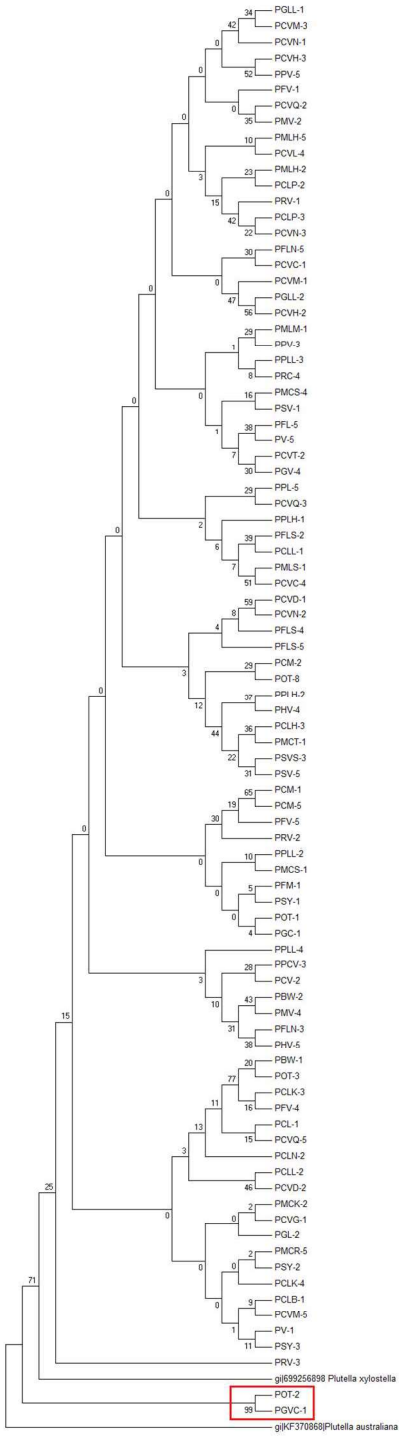

Fig. 1. Phylogenetic relationship among *Plutella xylostella* populations based upon a 643 bp mitochondrial *coxI* gene fragments using maximum likelihood (ML) algorithm

In our study, the *P. xylostella* population may have experienced a recent population expansion, indicated by the negative Tajima's *D* and Fu's F_S values for Cambodia, Lao PDR and Vietnam populations. Negative values of Tajima's *D* are associated with selective sweeps or population expansion after a recent sharp decline (Tajima 1989). Similarly, negative values of Fu's F_S are usually caused by an excess of singletons in a population expansion event (Fu 1995; Fu 1997). In consequence, *P. xylostella* population in Cambodia, Lao PDR and Vietnam could have experienced recent demographic expansion events. The statistically non-significant numbers indicating recent population growth could have been confined mostly by local geographical regions, except in Cambodia (Liao et al. 2010). It should be noted that even subspecies could be sharply genetically differentiated using genetic differentiation (F_{ST}) values. The pair-wise estimates of F_{ST} among subspecies of beach mice was found to be 0.23-0.63 (Mullen et al. 2009). Putative subspecies of *M. vitrata*, which cannot be differentiated based on morphological characters in Asia and sub-Saharan Africa was indicated by the high pairwise F_{ST} values of 0.44–0.85 (Malini et al. 2015). Hence, a pairwise F_{ST} value of 0.44–0.45 among the Taiwan, Cambodia and Malaysia populations of *P. xylostella* in the current study should be carefully considered for additional sampling and further analysis to determine if there are genetically distinct *P. xylostella* populations are existing in these countries, since the pest management strategies should be precisely developed and adopted according to the genetic differences of the pest. It should be noted that pairwise analysis of the mitochondrial genes indicated that *P. xylostella* populations from the southern region of China were more differentiated than those from the northern region (Wei et al. 2013). Consequently, it is possible to expect genetically distinct *P. xylostella* populations within Southeast Asia.

In conclusion, this study confirmed the presence of genetically distinct *P. xylostella* in Southeast Asia, but it requires additional studies with more populations, especially from Cambodia, Lao PDR, Vietnam, Thailand and Taiwan. Therefore, the genetic differences in *P. xylostella* population should be carefully considered while designing the pest management strategies in different geographical regions.

Acknowledgements

This work was supported by the Federal Ministry for Economic Cooperation and Development (GIZ Project number 13.1432.7-001.00), Germany.

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