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Research article

Genetic structure of *Zeugodacus tau* (Walker) in relation to cucurbit host plants

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Abstract

Zeugadacus tau (Walker) (Diptera: Tephritidae) is a polyphagous fruit pest that causes damage to agricultural and wild crops, especially in the family Cucurbitaceae. *Z. tau* is a complex species with a high level of genetic variation. The genetic structure was investigated of *Z. tau* populations collected from 10 different plant species using single strand conformation polymorphism (SSCP) analysis of the mitochondrial cytochrome c oxidase I (COI) sequences. In total, 489 *Z. tau* individuals showed 18 SSCP haplotype patterns, with a high level of genetic diversity. The clustering of COI sequences on the maximum likelihood tree and a haplotype network was consistent and showed that the *Z. tau* population collected from the wild crop of *Trichosanthes tricuspidata* had fixed haplotypes that were not found in *Z. tau* from agricultural crops. In addition, these fixed haplotypes were genetically different from the other haplotypes and are likely the cause of the genetic structure among *Z. tau* populations. The analysis of molecular variance revealed a significant genetic structure of *Z. tau* from cucurbit host plants (F-statistics [F_{ST}] = 0.6625, $p < 0.01$). Therefore, it can be concluded based on the sample populations used in this study that host plants play an important role in genetic differentiation among *Z. tau* populations and may be informative for genetic variation of other polyphagous insect pests to facilitate more efficient control and management.

Introduction

The tephritid fruit fly, *Zeugadacus tau* (Walker) is a pest of economically important crops in South Asia, East Asia, Southeast Asia and the South Pacific region (Jaleel et al., 2018). In 1849, *Z. tau* was first reported in China and then spread rapidly through the surrounding area (Jaleel et al., 2018). The rapid range expansions and strong adaptive ability of *Z. tau* are huge threats to agricultural resources (Tan et al., 2015). In the areas where outbreaks of this pest species have occurred, the quality and yield of commercial vegetables and fruits were reduced; therefore, *Z. tau* is listed as a quarantine species in several Asian countries and the USA (Ohno et al., 2008).

In Thailand, *Z. tau* was confirmed based on conventional and molecular techniques as a complex species (Baimai et al., 2000; Jamnongluk et al., 2003; Thanaphum and Thaenkham, 2003; Saelee et al., 2006; Kitthawee and Rungsri, 2011; Sumrandee et al., 2011; Zaelor and Kitthawee, 2018) with a high level of genetic diversity (Kitthawee and Julsirikul, 2019). Knowledge on the genetic diversity of insect pests is an important tool that can be used to optimize biological control strategies through understanding the population structure in different areas, including gene flow and migration. Understanding this makes it easier to determine the most efficient way to manage the pest fly.

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Several ecological factors affect the genetic differentiation of populations and one of these factors in insects is host species (Schluter, 2001), particularly regarding phytophagous insects (Mopper, 1996; Simon et al., 2015). *Z. tau* is a phytophagous and polyphagous fruit pest, infesting more than 80 species of agricultural and wild crops, especially in the family Cucurbitaceae (White and Elson-Harris, 1992; Singh et al., 2010; Jaleel et al., 2018). It was reported in several studies that *Z. tau* in Thailand showed a different phenetic structure (Kitthawee and Dujardin, 2010; Kitthawee and Rungsri, 2011; Dujardin and Kitthawee, 2013), as well as a different genetic structure (Baimai et al., 2000; Jamnongluk et al., 2003; Thanaphum and Thaenkham, 2003), depending on the type of host plant. Additionally, Baimai et al. (2000) suggested that host plant species may play an important role in genetic differentiation in *Z. tau* species. Furthermore, Shi et al. (2017) applied microsatellite markers and suggested that host plants affected the genetic variation of *Z. tau* under laboratory conditions. Different host plant species can generate selection pressure that prevents gene flow between insect populations in many polyphagous insects (Funk et al., 2002; Matsubayashi et al., 2010; Antwi et al., 2015).

The partial sequence of the mitochondrial cytochrome c oxidase I (COI) has been widely used as a molecular marker in phylogenetic and population genetic studies of several species of tephritid fruit flies, especially for the Folmer region of the COI gene (Meeyen et al., 2013; Barr et al., 2014; Kunprom et al., 2015; Kunprom and Pramual, 2018; Kitthawee and Julsirikul, 2019). In addition, it has been proven to be useful for identification at the species level (Hebert et al., 2003). Kunprom and Pramual (2019) demonstrated the potential of this gene region to reveal cryptic diversity in some taxa of tephritid fruit flies. For *Z. tau*, the partial sequences of the Folmer region have been successfully used to discriminate at least three cryptic species within the *Z. tau* complex (Kitthawee and Julsirikul, 2019). A high level of sequence divergence of this gene region makes it appropriate to use as a genetic marker at the population scale (Moritz et al., 1987). However, the study of population genetic structure requires a large number of samples. A molecular technique, single strand conformation polymorphism (SSCP), that enables simultaneous detection of genetic variation in a large number of samples (Gasser et al., 2006), was used to quantify haplotype diversity for an informative segment of the mitochondrial COI gene to identify population structure. Therefore, the aim of the current study was to use SSCP analysis to investigate genetic variation in *Z. tau* populations collected from different cucurbit host fruits. The results of this study should indicate adaptation of this fruit fly species to the host plant at a local scale, which may be useful for pest management programs.

Materials and Methods

Z. tau collections

The *Z. tau* samples were collected from 10 different cucurbit fruits infested with fruit fly larvae from wild and agricultural areas in Loei province, Thailand. The scientific name of these cucurbit plants including the common name and abbreviation are: *Benincasa hispida* (winter melon, Bhi), *Coccinia grandis* (ivy gourd, Cgr), *Cucurbita maxima* (pumpkin, Cma), *Cucumis sativus* (cucumber, Csa), *Luffa acutangula* (angled luffa, Lac), *Luffa aegyptiaca* (luffa, Lae), *Lagenaria siceraria* (bottle gourd, Lsi), *Momordica charantia* (bitter gourd, Mch), *Momordica cochinchinensis* (gac fruit, Mco) and *Trichosanthes tricuspidata* (redball snake gourd, Ttr). Infested fruits of each plant species were kept in ventilated containers with sawdust at the bottom as a pupation medium. Each container was labeled with a collection code and kept at the Department of Biology, Mahidol University, Bangkok, Thailand until adult emergence. Adult flies were identified using the taxonomic keys from White and Elson-Harris (1992) and Sumrandee et al. (2011) and maintained at -20°C until DNA was extracted.

DNA extraction and single strand conformation polymorphism analysis

The head and thorax of individual *Z. tau* samples were subjected to total genomic DNA extraction using the method from Cockburn and Fritz (1996). The polymerase chain reaction (PCR) amplification of the partial cytochrome c oxidase subunit I gene (COI) was carried out with the forward primer: 5' TGG AGA CGA CCA AAT CTA 3' and reverse primer: 5' GAG GAA ATA CCA GCT AAA TG 3' (Somsill et al., 2015). The PCR program involved: 95°C for 3 min followed by 25 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min with a final extension of 72°C for 10 min. The PCR products were verified on 1% agarose gel electrophoresis that stained with ethidium bromide to assess the quality of the amplification. To perform the SSCP analysis, 5 μL of the PCR product was mixed with 15 μL of denaturing solution (98% formamide, 0.5M ethylenediaminetetraacetic acid, 0.05% bromphenol blue and 0.05% xylene cyanol) and heated at 96°C for 10 min before immediately cooling on ice. The denatured PCR products were loaded into 7% non-denaturing acrylamide gel and electrophoresed at 160 V for 3 hr 45 min at 4°C. After electrophoresis, the gels were stained with silver nitrate (Bassam et al., 1991). A 100-bp ladder was used as a reference for SSCP haplotype pattern evaluation. The unique SSCP haplotypes were bi-directionally sequenced at the Research Center of Ramathibodi Hospital, Bangkok, Thailand. Sequences were analyzed and trimmed to a length of 327 bp using the BioEdit version 7.2.5 software (Hall, 1999) for further analysis and submitted to the NCBI database (https://www.ncbi.nlm.nih.gov) under accession numbers: MT881615–MT881632.

Data analysis

The SSCP haplotype sequences of *Z. tau* were aligned using ClustalW implemented in the BioEdit version 7.2.5 software (Hall, 1999). The COI sequences of *Z. tau* complex from GenBank (MH891801–MH891833; Kitthawee and Julsirikul 2019) were downloaded and used as reference data for complex species verification of *Z. tau* in the current study through phylogenetic relationship. In addition, the sequence variation of the partial COI gene was used to confirm the complex species verification with phylogenetic classification. Intraspecific and interspecific sequence differences and the maximum likelihood (ML) tree of COI sequences were conducted using the MEGA version 6.06 software (Tamura et al., 2013). The nucleotide substitution model with the lowest Bayesian information

criterion score (Tamura three-parameter model) was used for ML tree construction and all sequence positions containing gaps or missing data were eliminated from the dataset. Statistical support for each node was evaluated based on bootstrap analysis using 1,000 replications. The haplotype diversity (h), nucleotide diversity (π), number of polymorphic sites (S) and analysis of molecular variance (AMOVA) were performed using the Arlequin version 3.5 software (Excoffier and Lischer, 2010) to evaluate the genetic diversity and genetic structure of each *Z. tau* population, respectively. A haplotype network was constructed based on the median-joining method using the Network version 10.2.0.0 software (Bandelt et al., 1999) to analyze the genealogical relationship and distribution pattern among the COI haplotype sequences of *Z. tau*.

Results and Discussion

Mitochondrial cytochrome c oxidase I single strand conformation polymorphism haplotype and sequence analysis

The SSCP analysis of the partial COI sequences of the 489 individuals of *Z. tau* collected from the 10 different host types showed 18 unique haplotype patterns (haplotype a to haplotype r), as presented in Fig. 1. The distribution of these COI SSCP haplotypes and the genetic diversity values (Table 1) showed that haplotypes b, c and k were the most common and were shared among almost all populations of host plants, except for the Ttr population, whereas haplotypes o, p, q and r were restricted to some host plant populations. The noticeable restricted haplotypes were the haplotypes q and r, which were fixed at the Ttr population. The overall genetic diversity values of *Z. tau* collected from all host plant species combined were: haplotype diversity (h) = 0.9079, nucleotide diversity (π) = 0.0225 and number of polymorphic sites (S) = 47. The highest genetic diversity was in the Mco population (h = 0.9092, π = 0.0104, S = 16) and the lowest genetic diversity was in the Ttr population (h = 0.3068, π = 0.0077, S = 8). Although the average COI h value in the current study (0.9079) was in the same range as for other tephritid fruit flies such as *Ceratitis* spp. (h = 0.964, π = 0.014; Virgilio et al., 2008), *Bactrocera latifrons* (h = 0.8308, π = 0.0048; Meeyen et al., 2013), *Bactrocera dorsalis* (h = 0.984, π = 0.013; Choudhary et al., 2016) and *Zeugodacus cucurbitae* (h = 0.8802, π = 0.0044: Kunprom and Pramual, 2018), the average π value was relatively high compared to that of the above species (0.0225), as shown in Table 1. The high value of π was due to the nucleotide sequence of the fixed haplotypes, q and r, that were quite different from the other haplotypes (Fig. 2). Templeton (2006) suggested that new haplotypes are created by mutation and they are initially rare and restricted in the population; hence, they are valuable for gene flow studies. Therefore, the haplotypes q and r should benefit gene flow tracking among *Z. tau* from different host plant populations.

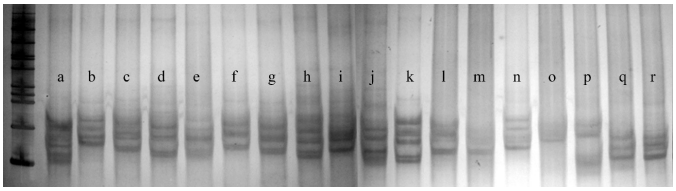

Fig. 1 Single strand conformation polymorphism analysis (SSCP) of 327 bp of mitochondrial cytochrome c oxidase I sequences of *Zeugodacus tau* collected from 10 cucurbit fruits showing 18 (a–r) different SSCP haplotype patterns

Table 1 Distribution of mitochondrial cytochrome c oxidase I single strand conformation polymorphism haplotypes, haplotype diversity (h), nucleotide diversity (π) and polymorphic site (S) of *Zeugodacus tau* from different cucurbit host plants

Haplotype	Host plant type										Total
	Agricultural									Wild	
	Bhi	Cgr	Cma	Csa	Lac	Lae	Lsi	Mch	Mco	Ttr	
(N)	(53)	(53)	(44)	(59)	(51)	(47)	(38)	(57)	(54)	(33)	(489)
a	8	23		2	8			23	5		69
b	6	3	6	1	2	10	12	4	6		50
c	18	3	3	4	7	16	7	3	2		63
d	9	2					1	2	2		16
e	4	6	20	8		7	8		6		59
f		8	1	11	3	8	3		10		44
g				1	1	2		1	3		8
h	2	1	2		3		4	9	3		24
i		1		3	1		1	1			7
j	2			4	5	1		1	4		17
k	4	6	5	5	16	2	1	10	7		56
l			6	14	2		1	1			24
m				6		1		1	1		9
n			1		1			1			3
o									5		5
p					2						2
q										27	27
r										6	6
h	0.8215	0.7692	0.7526	0.8743	0.8525	0.8002	0.8236	0.7845	0.9092	0.3068	0.9079
π	0.0084	0.0097	0.0067	0.0065	0.0086	0.0057	0.0076	0.0094	0.0104	0.0077	0.0225
S	12	14	9	14	14	11	12	14	16	8	47

Bhi = *Benincasa hispida* (winter melon); Cgr = *Coccinia grandis* (ivy gourd); Cma = *Cucurbita maxima* (pumpkin); Csa = *Cucumis sativus* (cucumber); Lac = *Luffa acutangula* (angled luffa); Lae = *Luffa aegyptiaca* (luffa); Lsi = *Lagenaria siceraria* (bottle gourd); Mch = *Momordica charantia* (bitter gourd); Mco = *Momordica cochinchinensis* (gac fruit); Ttr = *Trichosanthes tricuspidata* (redball snake gourd)

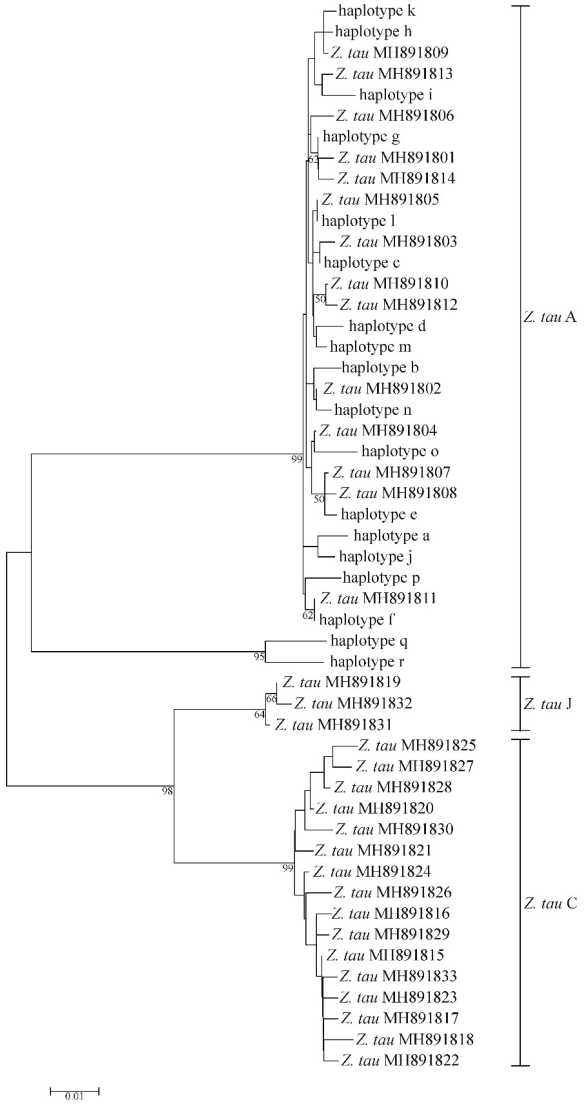

Fig. 2 Maximum likelihood tree of 327 bp of mitochondrial cytochrome c oxidase I sequences of *Zeugodacus tau* representing phylogenetic relationship of 18 single strand conformation polymorphism haplotype sequences in current study and reference sequences, showing bootstrap support (>50%) using the Tamura 3-parameter distance at each branch, scale bar indicates 0.01 substitution per site and *Z. tau* species complex clades are marked with vertical lines to the right of corresponding sequences and labelled as *Z. tau* A, C and J

Table 2 Intraspecific (on diagonal) and interspecific (below diagonal) average percentage nucleotide difference (mean±SE) based on 327 bp of the mitochondrial cytochrome c oxidase I sequence using Tamura 3-parameter model

	1	2	3
1. *Zeugodacus tau* A (GenBank)	0.79±0.24		
2. *Zeugodacus tau* C (GenBank)	12.88±2.05	1.19±0.30	
3. *Zeugodacus tau* (Current study)	2.21±0.35	13.07±1.95	3.41±0.52

The sequences of the 18 COI SSCP haplotypes of *Z. tau* and the COI sequences retrieved from GenBank (MH891801–MH891833) were used to identify the phylogenetic relationship. The *Z. tau* sequences from GenBank were reported as complex species of *Z. tau* A, C and J by Kitthawee and Julsirikul (2019) and Z. *tau* A is a *Z. tau sensu stricto*. Individuals of *Z. tau* collected in the current study were initially identified (based on external morphology) as *Z. tau* A, and their COI sequences on the ML tree group within the *Z. tau* A clade (Fig. 2), were consistent with morphological classification. The values of intraspecific and interspecific sequence divergences between *Z. tau* individuals also supported the external morphology and phylogenetic classifications (Table 2). The average Tamura 3-parameter interspecific nucleotide difference between *Z. tau* in the current study and *Z. tau* A from GenBank (2.21±0.35%) was close to the intraspecific nucleotide difference (3.41±0.52%) and did not exceed a cut-off value for DNA barcode identification (Hebert et al., 2003). In addition, the interspecific nucleotide difference between *Z. tau* in the current study and *Z. tau* C from GenBank was rather high (13.07±1.95%) and similar to the interspecific COI sequence divergence between different tephritid species (5.1–16.7%; Hu et al., 2008; Jiang et al., 2014; Bartolini et al., 2020; Dong et al., 2020). Furthermore, the intraspecific COI sequence difference among the investigated *Z. tau* populations (3.41±0.52%) revealed a high level of genetic variation, likely caused by the haplotypes q and r, as was noticed in the ML tree and the haplotype network that yielded congruent topologies. The haplotypes q and r were separated as a subclade of *Z. tau* A in the ML tree (Fig.2) and separated by many mutational steps from other haplotypes in the haplotype network (Fig. 3), implying genetic differentiation between *Z. tau* populations. Over a long period of time, these differences may prevent gene flow between populations and promote speciation (Slatkin, 1987).

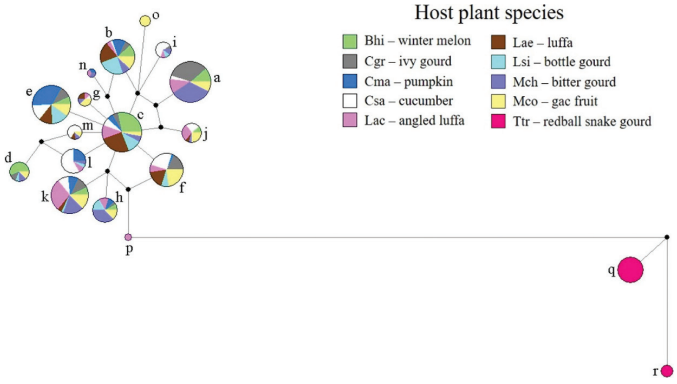

Fig. 3 Median-joining network among mitochondrial cytochrome c oxidase I haplotype sequences of *Zeugodacus tau*, where each circle represents a different haplotype, the size of circles in the network corresponds to the number of individuals presenting that particular haplotype, pie charts within each circle show the frequency of haplotypes present in different host plant species and median vectors are indicated by black dots and connecting lines are proportional to mutation steps between haplotypes

Table 3 Analysis of molecular variance among *Zeugodacus tau* populations

Source of variation	Degrees of freedom	Variance components	Percentage of variation	F-statistic
Among populations	9	2.6161	66.25	0.6625 ($p < 0.01$)
Within populations	479	1.3326	33.75	
Total	488	3.9487		

Genetic structure of Z. tau populations

The AMOVA analysis of *Z. tau* populations indicated significant variation between populations from different host plant species (F_{ST} = 0.6625, $p < 0.01$) (Table 3). Most of genetic differentiation was found between populations (66.25% of the total variation). The genetic structure of *Z. tau* that was related to the host plant species was primarily caused by the genetic variation found in the haplotypes q and r that were fixed in the Ttr population.

The 10 investigated cucurbit host plant species were classified based on cultivation type as wild or agricultural host plants (Table 1). Nine host plant species were categorized as agricultural crops, while the redball snake gourd (Ttr) was categorized as a wild-growing plant, as it is not an economically cultivated crop in Thailand (Duyfjes and Pruesapan, 2004). The distribution of the haplotypes present in the different host plant species on the haplotype network demonstrated that there were two haplotype clusters (Fig. 3). The haplotypes a–p were distributed in agricultural crops while the haplotypes q and r were fixed for the wild-growing plant, the Ttr population. In addition, the network revealed a large genetic difference between the haplotypes q and r and the others. The restricted and genetically distinct haplotypes had an effect on the population structure. This observation was supported by the AMOVA analysis, when the Ttr population was excluded, as that AMOVA result showed a rather weak genetic structure with a low level of variation (9.33%), indicating that genetic variation within the Ttr population was important for the differentiation of genetic structure between *Z. tau* populations.

In Thailand, several studies have shown that *Z. tau* genetic variation is related to the host plant species (Baimai et al., 2000; Jamnongluk et al., 2003; Thanaphum and Thaenkham, 2003; Kitthawee and Dujardin, 2010; Kitthawee and Rungsri, 2011; Dujardin and Kitthawee, 2013). However, those studies focused on the genetic diversity within the species complex of *Z. tau* without separation based on host plant species. Therefore, the current study revealed a correlation between host plant types and genetic variation within a single species of *Z. tau* (A). A comprehensive review by Jaleel et al. (2018) showed that *Z. tau* had a wide range of host plant species. An earlier report on the *Z. tau* complex and associated host plants in Thailand showed that most of the host species of *Z. tau* A were agricultural plants, except *Trichosanthes cordata* and *T. tricuspidata* (Baimai et al., 2000). Host plants are an ecological factor that play an important role for herbivorous insects, such as site of feeding, mating, oviposition and development (Funk et al., 2002). The close relationship with the host plant during every part of the insects life cycle promotes adaptation and creates conditions favorable for reproductive isolation to develop (Funk et al., 2002; Simon et al., 2015). In addition, phytophagous insects have a high genetic diversification rate that is prone to occur by selective pressure from different host plants, also known as host-shift (Mopper, 1996; Simon et al., 2015). In the current study, there was only one species of wild-growing host plant (*T. tricuspidata*). However, the *Z. tau* population from this plant species exhibited distinct genetic differences from those of agricultural host plant populations, consistent with Saelee et al. (2006). A study on genetic variation of the *Z. tau* complex in Thailand (Saelee et al., 2006) demonstrated that *Z. tau* A collected from one population of *T. cordata* and five geographically different populations of *T. tricuspidata* had high genetic similarity, but were different from those collected from agricultural plants. This may imply that the geographical distance is unlikely to influence genetic differentiation in this fruit fly species, consistent with a previous suggestion by Kitthawee and Julsirikul (2019) that the genetic structure of the *Z. tau* complex in Thailand is more correlated with host plant species than the geographical distance. Shi et al. (2017) used a novel host (banana) to rear *Z. tau* in the laboratory and found that there was a significant genetic difference between the populations from novel and traditional host plants. Although the host plants in the current study were not novel, the genetic structure of *Z. tau*, taking the haplotype distribution and sequence data into consideration, indicated a substantial influence of host plant species on the gene flow and genetic differentiation of *Z. tau* populations in Thailand. However, the genetic structure of *Z. tau* in the current study was analyzed based on the sample populations, so the impact of wild-growing host plant on the genetic differentiation of *Z. tau* could not be strongly determined due to the limited *Z. tau* collection. It would be better if more populations of *Z. tau* from wild-growing plant species were added for a more robust conclusion.

In conclusion, the genetic diversity of *Z. tau* may be caused by several factors. However, the rather high value of genetic differentiation (F_{ST} = 0.6625) among *Z. tau* populations revealed that different host plant species may be a barrier to gene flow and dispersion, creating a genetic structure among *Z. tau* populations. The investigation on the genetic structure of *Z. tau* in relation to different host plant species provides information about one of the factors that affect genetic variation of insect pests and can be used to facilitate more efficient pest control and management.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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