

Research Article

Host adaptation in *Cydia pomonella* (Lepidoptera: Tortricidae) using microsatellite DNA markers

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Abstract: The codling moth (CM), *Cydia pomonella* (L.), is among the economically important pests of pome fruits. This moth causes tremendous crop losses worldwide annually. In the current study, 210 larvae from apple, pear, quince, and walnut orchards were collected from seven locations in Zanjan province, Iran. Four CM-specific microsatellite DNA loci, including Cyd10, Cyd11, Cyd12, and Cyd13, were analyzed by polymerase chain reaction (PCR). According to the results, the CM population sampled from quince showed the highest number of alleles per locus with the mean observed and effective allele numbers 1.75 and 1.51, respectively. The latter shows the number of alleles with equal frequencies that contributed the most to the observed heterozygosity. Also, the mean observed and expected heterozygosity for this population was 0.508 and 0.258, respectively. The increased observed heterozygosity confirms that the selection acts in favor of heterozygote genotypes. Large genetic distances were detected between the CM population from quince and the populations sampled from the other host plants, the largest between quince and walnut populations. Further, among-population diversity contributed the most to the insect's genetic diversity, which was 89%. Moreover, some of the populations had a deviation from the Hardy-Weinberg equilibrium ($p < 0.001$). While Cyd13 locus was more polymorphic than the other tested loci, Cyd11 locus was monomorphic. These findings reveal genetic variation in *C. pomonella*, collected from various fruit trees, indicating differences in some phenotypes noteworthy in integrated pest management.

Keywords: codling moth, host plant, SSR markers

Introduction

The codling moth (CM) *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) is a common fruit pest that causes extensive worldwide crop losses every year. The damage is caused by the larvae, which burrow into the fruit (Gratwick, 1992) and feed on the seeds. Substantial crop

loss occurs due to fruit abscission and premature ripening of infested fruits. CM is a polyphagous insect pest, and apple, pear, quince, and walnut trees are among its common hosts (Pajač *et al.*, 2011b).

Host plant preference is a known feature in local populations of polyphagous insects (Subramanian and Mohankumar, 2006). It is believed that host preferences originate in behavioral adaptations of the insect populations (Cunningham and Zalucki, 2014). An insect species' ability to adopt new adaptations profoundly affects the insect's

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success from the evolutionary perspective (Subramanian and Mohankumar, 2006). CM was characterized as an insect with a high capacity for adaptation to new environmental conditions (Pajač *et al.*, 2011b; Kadoić Balaško *et al.*, 2020). Elucidating polyphagous pests' population genetic structure can help understand the structure and population dynamics, behavior, and response to various selection pressures (Subramanian and Mohankumar, 2006). Once the host-adapted populations were characterized by *e.g.* genetic differentiation and limited gene flow, the next step would be considering or testing their response to various compounds and control agents at a population level manner in IPM programs.

Microsatellite DNA markers (Litt and Luty, 1989) (also known as simple sequence repeats, SSRs) are valuable tools in DNA-based insect studies, including their population genetic structure. These markers have many desirable features that make them attractive to researchers. They are co-dominant PCR-based markers (Abdurakhmonov, 2016). High polymorphisms, a high number of alleles/locus, and a high rate of mutation of SSRs have made them suitable DNA markers for studying the rapid evolutionary events in insect populations, including those associated with host plant adaptations. This research aimed to evaluate host-associated variability in CM populations using microsatellite markers, with possible application in pest management programs.

Experimental procedures

Insect sampling

A sampling of codling moth was carried out from apple *Malus domestica* Borkh., pear *Pyrus sp.* L., quince *Cydonia oblonga* Mill., and walnut *Juglans regia* L. orchards at seven locations in Zanjan province, Iran, including Abhar (36.1525°N, 49.2385°E), Zanjan (36.6830°N, 48.5087°E), Khorramdareh (36.2109°N, 49.1930°E), Sainqaleh (36.3035°N, 49.0767°E), Tarom (36.9668°N, 48.9143°E), Mahneshan (36.7421°N,

47.6721°E), and Nikpey (36.8481°N, 48.1764°E), during spring and summer, 2012. In each region, three orchards were sampled. Infested fruits were collected and transferred to the laboratory, and 52 larvae/host was collected except for apple, in which 54 larvae were sampled (30 larvae per region). Samples were kept at -20 °C until DNA extraction.

DNA extraction

DNA was extracted from CM larva individuals using a CTAB method (Doyle and Doyle, 1987) with a mere modification. Extracted DNA was resolved in 50 µl Tris EDTA (TE) buffer and stored at -20 °C until PCR assay and diluted 1:50 with double-distilled deionized water before the assay.

Microsatellite loci PCR test

Four SSR loci, including Cyd10 (AY688624), Cyd11 (AY688625), Cyd12 (AY688635), and Cyd13 (AY688626) (Zhou *et al.*, 2005) for CM were analyzed in these populations. Briefly, PCR assay for the SSR loci was performed using the CM-specific primers. Each 15 µl reaction solution contained 5 µl PCR master mix (200 µM dNTPs, 1.5 mM MgCl₂, 1 U *Taq* polymerase) (Takapoo Zist, Tehran), 1 µl of 10 µM forward and reverse primers, and 1 µl of template DNA (~50 ng/µl). The PCR cycling program consisted of initial denaturing at 94 °C for 10 minutes, followed by 35 cycles of denaturing at 94 °C for the 30 s, annealing at 59 °C for Cyd10, 55 °C for Cyd11 and Cyd12, 51 °C for Cyd13, for 35 s, extension at 72 °C for 30 s, followed by a final extension at 72 °C for 10 m. Electrophoresis of PCR products was performed on 8% non-denaturing polyacrylamide gel. Scanning of the gel after staining with ethidium bromide was done using a gel doc device.

DATA analysis

The analysis, including allelic diversity, heterozygosity, genetic distance (Nei's similarity coefficient (Nei and Li, 1979)

among the populations, analysis of molecular variance, and Hardy Weinberg Equilibrium carried out using GenAlex ver. 6.5 (Peakall and Smouse, 2012). Multidimensional scaling was plotted using Xlstat (XLSTAT, 2020). The phylogenetic tree was plotted based on Nei's similarity coefficient using the neighbor-joining (NJ) method and 1000 bootstrapping by SplitsTree 4 (Huson and Bryant, 2006).

Results

Allelic diversity

Overall, the CM population sampled from quince had the highest number of alleles per

locus with the mean numbers of observed and effective allele numbers 1.75 and 1.51, respectively. Allelic diversity of CM populations sampled from walnut, apple, and pear were in descending order, respectively (Table 1). Among the loci, locus Cyd13 showed the highest allele numbers. The mean of observed and effective allele numbers for this locus were 2 and 1.792, respectively. In the second informative locus, Cyd12, the mean per locus number of observed and effective allele numbers was 1.75 and 1.739, respectively. Also, these values for the locus Cyd10 were 1.5 and 1.017, respectively. Locus Cyd11 was monomorphic (Table 1).

Table 1 Allelic diversity of *Cydia pomonella* populations sampled from quince, walnut, apple, and pear using microsatellite DNA markers.

Locus	N	Apple	Pear	Quince	Walnut	Mean	Allele size range (bp)
Cyd10	N _o	1	2	2	1	1.5	138-147
	N _e	1	1.034	1.034	1	1.017	
Cyd12	N _o	2	1	2	2	1.750	100-183
	N _e	1.956	1	2	2	1.739	
Cyd13	N _o	2	2	2	2	2	193-210
	N _e	1.385	2	2	1.780	1.792	
Cyd11	N _o	1	1	1	1	1	96 (monomorph)
	N _e	1	1	1	1	1	
Mean	N _o	1.5	1.5	1.75	1.50	-	
	N _e	1.34	1.26	1.51	1.45	-	

No and Ne indicates observed allele number and effective allele number, respectively.

Heterozygosity

According to the results, the *C. pomonella* population from quince showed the highest observed and expected heterozygosities of 0.508 and 0.258, respectively (Table 2). In the walnut population, the observed and expected heterozygosities were 0.412 and 0.234, respectively. These values for the apple population were 0.295 and 0.191, respectively. Moreover, the relevant values in *C. pomonella* from pear were 0.258 and 0.133 (Table 2). Also, the observed heterozygosities for the loci Cyd12 and Cyd13 in the quince population were 1. The

same value was seen for Cyd13 in the pear population and Cyd12 in the walnut samples. In comparison, Cyd12 and Cyd13 loci contributed to gene diversity more than the other studied loci.

Genetic distance and Multidimensional scaling

In this research, the greatest genetic distance was observed between the populations sampled from walnut and quince. On the other hand, the populations from walnut and apples showed high genetic similarity. The high genetic similarity was evident in apple-pear and pear-

walnut CM population pairs (Table 3). Also, multidimensional scaling and cluster analyses showed two main groups in which apple, walnut, and pear populations were grouped, and the population from quince constituted a separate group (Figs. 1 and 2). According to the phylogenetic tree, CM individuals from pear, walnut, and apple trees were grouped in distinct groups, the latter two with more remarkable similarity, as mentioned above.

Analysis of molecular variance and Hardy-Weinberg equilibrium

According to the results, within-population variation was only 11%, but most of the variance was contributed by between populations variation, which was estimated at 89% (Table 4). Also, the chi-square test showed that the loci Cyd12 and Cyd13 in CM population from walnut, the locus Cyd13 in the pear population, the locus Cyd12 in the apple population, and the loci Cyd12 and Cyd13 in the population from quince had a deviation from the Hardy-Weinberg equilibrium ($p < 0.001$). However, the locus Cyd10 in the pear and quince populations and the locus Cyd13 in the apple population were in equilibrium (Table 5).

Table 2 Heterozygosity of *Cydia pomonella* populations sampled from quince, walnut, apple, and pear using microsatellite DNA markers.

Locus	H	Apple	Pear	Quince	Walnut	Mean
Cyd10	H _o	0	0.033	0.033	0	0.0165
	H _e	0	0.033	0.033	0	0.0165
Cyd12	H _o	0.850	0	1	1	0.712
	H _e	0.489	0	0.5	0.499	0.372
Cyd13	H _o	0.333	1	1	0.650	0.745
	H _e	0.278	0.5	0.5	0.439	0.429
Cyd11	H _o	0	0	0	0	0
	H _e	0	0	0	0	0
Mean	H _o	0.296	0.258	0.508	0.412	-
	H _e	0.192	0.133	0.258	0.234	-

H_o and H_e stand for observed and expected heterozygosity, respectively.

Table 3 The population genetic distance of *Cydia pomonella* using microsatellite DNA markers based on Nei's similarity coefficient.

Population 1	Population 2	Genetic distance
Quince	Apple	0.460417
Walnut	Apple	0.017217
Pear	Apple	0.029866
Walnut	Quince	0.551616
Pear	Quince	0.389799
Pear	Walnut	0.032284

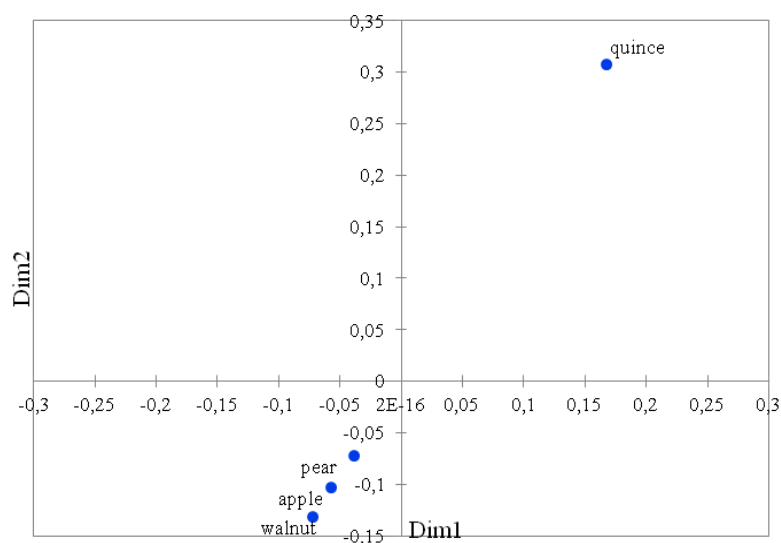


Figure 1 Multidimensional scaling of *Cydia pomonella* populations sampled from quince, walnut, apple, and pear using microsatellite DNA markers based on Nei's Similarity coefficient.

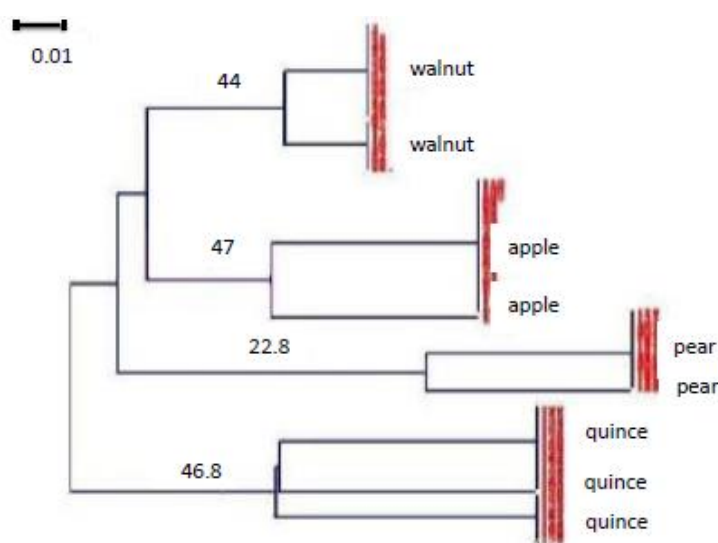


Figure 2 Phylogenetic tree showing *Cydia pomonella* populations similarity sampled from quince, walnut, apple, and pear using microsatellite DNA markers based on Nei's similarity coefficient.

Table 4 The AMOVA of *Cydia pomonella* populations sampled from quince, walnut, apple, and pear using microsatellite DNA markers.

Source of variation	df	SS	MS	Estimated variance	Molecular variance (%)
Between populations	3	285794.20	951640746	925.087	89
Within population	416	46966.07	112.899	112.899	11
Total	419	33276.31		1037.987	100

Table 5 Hardy-Weinberg equilibrium (HWE) of *Cydia pomonella* populations sampled from quince, walnut, apple, and pear using microsatellite DNA markers.

Population	Locus	df	Chi-square	Probability	Significance
Walnut	Cyd10 ^m				
	Cyd11 ^m				
	Cyd12	1	60	0	not in HWE
	Cyd13	1	13.91	0	not in HWE
Pear	Cyd10	1	0.017	0.896	in HWE
	Cyd11 ^m				
	Cyd12 ^m				
Apple	Cyd13	1	60	0	not in HWE
	Cyd10 ^m				
	Cyd11 ^m				
	Cyd12	1	32.78	0	not in HWE
Quince	Cyd13	1	2.40	0.121	in HWE
	Cyd10	1	0.009	0.926	in HWE
	Cyd11 ^m				
	Cyd12	1	30	0	not in HWE
	Cyd13	1	30	0	not in HWE

^m: Monomorphic.

Discussion

Allelic diversity and heterozygosity

The low allelic diversity seen in the current study might be connected with the Cyd set of primers used, as suggested by Pajač *et al.* (2011a). Further work using other sets of primers, *e.g.*, Cp series, seems necessary to achieve complementary data. CM population from quince showed the highest observed and expected heterozygosities of 0.508 and 0.258, respectively (Table 2). This heterozygosity evidently, reveals high genetic diversity in the mentioned population. The high genetic diversity observed in the quince population sampled in the current study might be explained by considering the population's relative isolation from the other host-associated populations of codling moth.

Moreover, the increased observed heterozygosity in the studied populations confirms that the selection favors heterozygote

genotypes. This finding is congruent with previous reports discussing the capabilities of CM in confronting the unprecedented environmental factors (Kadoić Balaško *et al.*, 2020; Pajač Živković *et al.*, 2019; Zhu *et al.*, 2017; Khani and Moharramipour, 2010; Boivin *et al.*, 2005), considering the heterozygote advantage in insects (Jingade *et al.*, 2011; Groot *et al.*, 2014).

Genetic distance and Multidimensional scaling

Population structuring of the codling moth, observed in the current study, seems to be in line with previous reports suggesting strong adaptation ability of codling moth to the new condition including host plants and forming host-associated races and populations, and reports on limited flight and gene flow in *C. pomonella* (Chen and Dorn, 2010; Thaler *et al.*, 2008; Meraner *et al.*, 2008; Pajač *et al.*, 2011b). In multidimensional scaling and cluster analyses, the quince and apple populations were grouped separately and showed evident genetic distance (Figs. 1 and 2, Table 3), although both host plants belong to pome fruits. Both apple and quince cultivations have a long history in Asia, including Iran (Cornille *et al.*, 2014; Cornille *et al.*, 2012; Postman, 2009), and host-related populations from the early times of establishment the orchard might be a reason for this observation. The highest genetic distance was observed between quince and walnut populations. This genetic distance might be illustrated considering that the walnut population originated from the apple population (Chen and Dorn, 2010). So, this probably has exerted further differentiation between the quince population and the resulting walnut population. Similarly, Phillips and Barnes (1975) have reported a plum fruit race in codling moths with an origin from the California walnut feeding population (reviewed in Wearing *et al.*, 2001).

Analysis of molecular variance and Hardy-Weinberg equilibrium

The 89% contribution of population variations in molecular variance seen in our research might be attributed to host-related differentiation. Thus,

the results can be considered as a consequence of the codling moth characteristics on its affinity to specific host adaptation, sedentary behavior, and limited gene flow, as mentioned above. Thaler *et al.* (2008), using AFLP markers, reported a high degree of genetic differentiation between the analyzed strains and populations, despite the low genetic variation within the individual strains and populations (Thaler *et al.*, 2008). On the other hand, Voudouris *et al.* (2012) suggested that agents like host species and local factors (climatic conditions, topography, and pest control programs) do not affect the genetic structure of codling moth populations within each country. However, most of the findings (Chen and Dorn, 2010; Thaler *et al.*, 2008; Meraner *et al.*, 2008; Duan *et al.*, 2016) disagree with this view. Moreover, six out of nine polymorphic alleles showed deviation from Hardy-Weinberg equilibrium. The population history of this insect might be a reason for these departures (Waples, 2015). For instance, it was reported that pesticide application affected the genetic structure of *C. pomonella* (Franck *et al.*, 2007; Wan *et al.*, 2019). Also, Wan *et al.* (2019) demonstrated genetic basis in CM new adaptations on its olfactory capability in which duplication in olfactory receptor gene (*OR3*) enhances the ability of CM to exploit kairomones and pheromones in locating both host plants and mates. Further, the pattern of differences in forewing shape related to control practice type was reported in CM (Pajač Živković *et al.*, 2019) and in the shape of hind-wing *Diabrotica virgifera* concerning resistance type (Mikac *et al.*, 2019). Therefore, the variability of *C. pomonella* seen in our study seems reasonable.

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Statement of Conflicting Interests

The authors state that there is no conflict of interest.

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شناسایی جمعیت‌های میزبانی کرم سیب (*Cydia pomonella* (Lepidoptera: Tortricidae) با نشانگر ریزماهوره

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چکیده: کرم سیب، (*Cydia pomonella* (L.) یکی از آفات مهم میوه‌های دانه‌دار است که سالانه در جهان، خسارت هنگفتی را باعث می‌شود. در این پژوهش، ۲۱۰ نمونه کرم سیب از باغات سیب، گلابی، به و گردو از ۷ شهرستان استان زنجان جمع‌آوری شد و دی‌ان‌آی آنها در ۴ جایگاه ریزماهوره‌ای اختصاصی کرم سیب به‌روش واکنش‌های زنجیره‌ای پلیمرز (PCR) مورد بررسی قرار گرفت. براساس نتایج به‌دست آمده، جمعیت کرم سیب جمع‌آوری شده از درخت به، به‌ترتیب با میانگین تعداد آلل مشاهده شده و مؤثر ۱/۷۵ و ۱/۵۱، دارای بیش‌ترین تعداد آلل در هر جایگاه بود. عدد اخیر، نشانگر تعداد آلل‌هایی با فراوانی یکسان است که بیش‌ترین سهم را در هتروزیگوسیتی مشاهده شده دارند. علاوه براین، میانگین هتروزیگوسیتی مشاهده شده و مورد انتظار در این جمعیت به‌ترتیب ۰/۵۰۸ و ۰/۲۵۸ ثبت شد. افزایش در میانگین هتروزیگوت‌های مشاهده شده مؤید این است که انتخاب طبیعی به‌نفع ژنوتیپ‌های هتروزیگوت عمل کرده است. فواصل ژنتیک فاحشی بین جمعیت کرم سیب جمع‌آوری شده از به و جمعیت سایر میزبان‌ها مشاهده شد که بیش‌ترین آن بین جمعیت‌های به و گردو بود. هم‌چنین، تنوع بین جمعیتی بیش‌ترین تأثیر را در تنوع ژنتیکی این حشره داشت. بعضی از جمعیت‌ها در تعادل هاردی-واینبرگ نبودند ($P < 0.001$). درحالی‌که بیش‌ترین چندشکلی مربوط به جایگاه Cyd13 بود، جایگاه Cyd11 تک‌شکل بود. در این بررسی، در جمعیت‌های کرم سیب جمع‌آوری شده از میزبان‌های مختلف، تنوع ژنتیکی مشاهده گردید و این تنوع ممکن است نشان‌دهنده وجود تنوع فنوتیپی دارای اهمیت در کنترل تلفیقی آفت باشد.

واژگان کلیدی: کرم سیب، گیاه میزبان، نشانگر ریزماهوره