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An initial examination of the population genetic structure of *Cydia pomonella* (Lepidoptera: Tortricidae) in Croatian apple orchards

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Abstract

Microsatellites were used to investigate the genetic structure and gene flow of three codling moth (*Cydia pomonella* Linnaeus; CM) populations in Croatia. Two populations were subjected to chemical control treatments for the last 15 years, and one population was not subjected to any chemical control treatment. During the assessment of genetic differentiation, only the males caught in pheromone traps were used. Ten variable microsatellites revealed strong allelic variation. The number of alleles (Na) varied from 2 (at loci Cyd 15 and Cyd 14) to 13 (at locus Cp 2.39), with an average value of 7.20 alleles per locus. The observed (H_O) and expected heterozygosity (H_E) per locus ranged from 0.5 to 0.76 and 0.10 to 0.84, respectively. The measures of pair-wise population genetic structure (F_{ST} = 0.02 - 0.04) were low and not significant in all of the comparisons (after correction for multiple comparisons). Additionally, an analysis of molecular variance (AMOVA) performed on the three populations, which were grouped geographically according to the type of management or according to the variation across generations (second or third generation individuals), revealed no significant variance in the genetic structure. However, the genetic variation was significantly partitioned within individuals (70-96%). Additionally, the tests for isolation by distance were not significant, suggesting a need to analyse the specifics of human-mediated CM transport over short and long distances within the Croatian apple-growing regions as a possible cause of gene flow. This study supports the hypothesis of genetic exchange in codling moths between orchards at moderate to potentially high levels.

Key words: Codling moth, microsatellite, genetic variation, F_{ST}, AMOVA, Tortricidae.

Introduction

Globally, the codling moth (*Cydia pomonella* Linnaeus; CM) is a key pest in pome fruit production, and this moth has a preference for apples. Recently, Croatian apple orchards have sustained large amounts of damage due to CMs. In Croatia, two generations of CMs appear per year; however, due to global warming in Croatia, a third generation is suspected to have appeared [1]. Despite a large number of chemical control treatments that have intensified several times in the past decade, damage to apple fruit occurs at levels greater than 1%. According to apple production guidelines, this is an unacceptable level of damage [2].

In the past decade, monitoring of CMs using pheromone traps has revealed earlier moth flight, which correlates with the greater ambient daily temperatures recorded during this period [1]. In 1999, CM males in Northwest Croatia were caught until the end of July [3]. However, more recently, male flight has been observed to last until the end of September. It is suspected that global warming and chemical-resistant CM biotypes are responsible for the longer flight period and observed increase in abundance of CMs.

The increase in the distribution and abundance of CMs in Croatia is congruent with data from other parts of Europe and the world [4-6]. Integrated measures of CM control that involve mating disruption and that have been used in Western Europe [6] have since been applied to small-area orchards in Croatia on a trial basis. This treatment has resulted in satisfactory control of CMs. However, products for wide-scale application of these integrated control measures, which involve mating disruption, are currently unavailable on the Croatian market. Furthermore, the number of available insecticides that are used to control CMs in Croatia is small due to strict sales and usage restrictions. Because of the increase in number of insecticide applications per year in the last few years, there are strict regulations to curtail the possibility of CMs developing resistance to insecticides. Cross-resistance among chitin synthesis inhibitors, moult-activating compounds such as tebufenozide and juvenile hormone analogues have been detected in CM populations in the Southeast of France [7, 8]. Additionally, resistance to various classes of insecticides has been documented in CM populations in Italy [9, 10].

Despite its economic importance, little is known regarding the genetic differentiation and gene flow of CM populations, both in Croatia and across Europe [11]. Due to climate change and frequent insecticide treatments, CM populations are assumed to have differentiated into many ecotypes that have different biological and physiological requirements for their development [12].

The genetic structure of CM populations have been studied

using allozyme markers, but low genetic differentiation was observed among the sampled populations [13]. Using Amplified Fragment Length Polymorphism molecular markers (AFLP), differences among sampled CM populations (even those separated by small geographic distances) were successfully established [14]. AFLP markers were also used to study the molecular phylogenies and genetic structures of CMs. The application of AFLP markers has identified the recent evolutionary history of CMs, including the Pleistocenic splitting of CMs into two refugial clades, the interbreeding of mitochondrial haplotypes in the Holocene and finally, the complete human-aided intermixing and splitting of the populations into many locally adapted populations [12]. Recently, co-dominant markers (microsatellites) have been isolated from CMs [15, 16]. These microsatellites were used to estimate genetic structure across French CM populations [11]. The results revealed low genetic differentiation across the populations. However, the authors observed a marginal impact of insecticide treatment on the allelic richness of the CMs. Similarly, low genetic variation (F_{ST} = 0.002) was found in populations sampled from abandoned orchards (without insecticide treatment) and production orchards (organic and conventional production orchards) in Chile [17]. Conversely, studies using the same markers have reported significant genetic differentiation across CM populations sampled from various host plants, and they were able to distinguish local populations sampled from the same host within a distance of 10 km [18]. In a recent study, CM males that were sampled (using pheromone traps) from two French apple orchards (one organic orchard and another orchard that was treated with chemical insecticides) situated 30 km apart were used for genetic structure analyses [19].

Bearing in mind diversity of the data obtained by different authors, the aim of this study was to use pheromone-trapped CM males to estimate the genetic structure of CM populations across Croatian apple orchards with various pest management control practices.

Materials and Methods

Sample collection and processing: Codling moth individuals were collected from apple orchards in three locations in Croatia during the 2008 growing season (Fig. 1). The largest apple production region is in the Northwest of Croatia, near the borders of Hungary and Slovenia.

The orchards in this region range from 70 to 250 ha, and the apples are produced according to the principles of the Integrated Pest Management (IPM). The three sampled orchards were differentiated based on their insecticide treatments. Orchard 1 (Beloslavec) was characterised by a low cultivation level, without insecticide treatment, and the apple production in this orchard was classified as organic (untreated orchard). This orchard is surrounded by natural pasture without any organised agricultural production. Orchards 2 (Kloštar Ivanić) and 3 (Nedelišće) were characterised by intensive cultivation in accordance with integrated control procedures and sprayed with organophosphates, insect growth regulator (IGR) insecticides and neonicotinoid insecticides seven times per year or during the growing season (treated orchards).

Kloštar Ivanić was established 15 years ago, and the Nedelišće orchard had the longest integrated control programme. The distances between the orchards are as follows: Beloslavec to Kloštar Ivanić, 55 km; Beloslavec to Nedelišće, 60 km; Kloštar Ivanić to Nedelišće, 110 km. Therefore, the CM populations are assumed to be isolated from one another. All three orchards grew Idared, Golden Delicious and Jonagold apples.

CM samples were collected using pheromone traps (Csalomon®), and only the males of this species were collected. The pheromone traps were placed in the middle of the orchards because their attractant range was approximately 500 m. It was assumed that the sampled and, subsequently, genotyped moths were from the population that existed in the orchards. Each sampled male moth was marked and placed in 100% ethanol, pending genetic analysis. At the end of the season, a diagram of the male moths flight activity was created. Three adult CM flights were noted (data not shown). The second-generation peak (flight) appeared from June 11 to July 7, and the third-generation peak appeared from August 10 to August 20. At each location (Beloslavec, Kloštar Ivanić and Nedelišće), approximately thirty CMs were selected for genetic analysis (15 second-generation moths and 15 from the third generation). A total of 86 moths were selected as the best candidates for further analysis.

The CM-infested (damaged) fruits were evaluated before apple

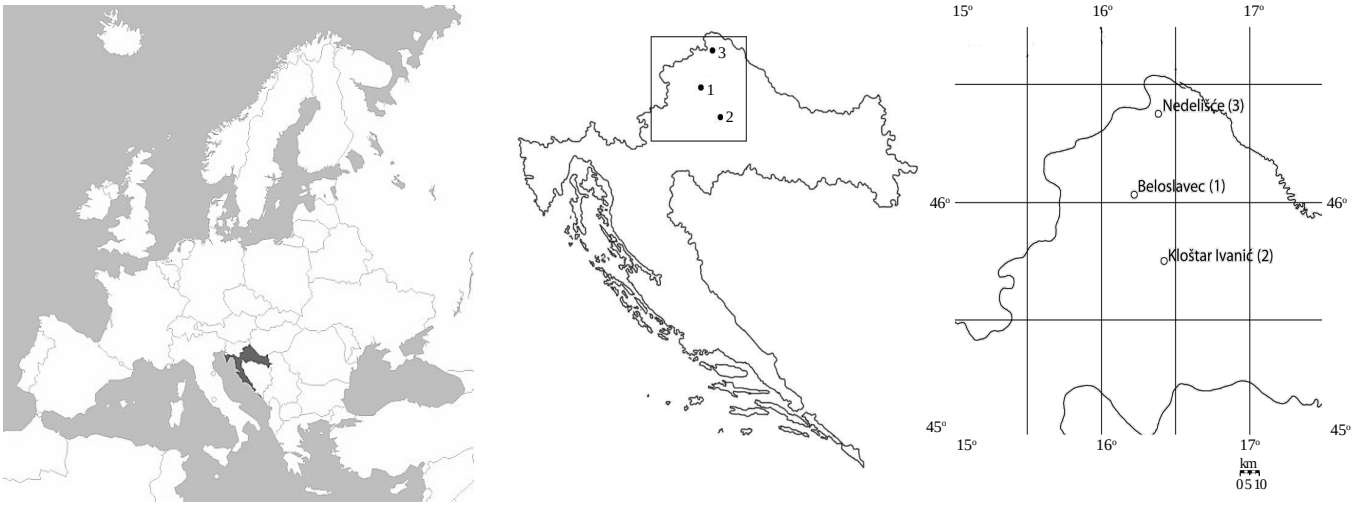

Figure 1. The location of Croatia in Europe and the three study sites (populations) from which *Cydia pomonella* individuals were collected for genetic analysis. 1 Beloslavec, an untreated orchard; 2 Kloštar Ivanić, a treated orchard; 3 Nedelišće, a treated orchard.

picking on the basis of 1000 fruit per orchard.

Total genomic DNA was extracted from the individual CMs using the GenElute™ Mammalian Genomic DNA Kit (Sigma-Aldrich). Individual CMs were genotyped at 10 microsatellite loci: Cp 5.24, Cp 2.39, Cp 3.180, Cp 2.129, Cp 3.169, Cp 1.62, Cp 1.60 [15], Cyd 15, Cyd 16, and Cyd 14 [16].

The PCR reactions for the first set of previously described primers [15] were carried out in 10-µl reaction volumes containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 50 µM of each dNTP, 0.4 µM of each primer, 0.5 units of *Taq* DNA polymerase (Sigma-Aldrich) and 20 ng of the DNA template. The amplifications were performed using a Veriti 96-Well Thermal Cycler (Applied Biosystems) under the following conditions: 2 min at 94°C, 33 cycles of 30 seconds at 94°C, 40 seconds at the appropriate annealing temperature for each primer set, and 40 seconds at 72°C, with a final extension of 10 min at 72°C for all primers. The annealing temperature was 60°C for primers Cp 5.24, Cp 2.39 and Cp 2.129, 58°C for primers Cp 3.180 and Cp 1.62 and 57°C for primers Cp 3.169 and Cp 1.60.

The PCR reactions for the second set of previously described primers [16] were carried out in 25-µl reaction volumes containing 1x PCR buffer (Sigma-Aldrich), 2.0 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM of each primer, 0.5 units of *Taq* DNA polymerase (Sigma-Aldrich) and 20 ng of the DNA template. The amplifications were performed using a Veriti 96-Well Thermal Cycler (Applied Biosystems) under the following conditions: 5 min at 94°C, 33 cycles of 30 seconds at 95°C, 30 seconds at an annealing temperature of 53°C for the Cyd 15 and Cyd 16 primers, 44°C for the Cyd 14 primer and 30 s at 72°C, with a final extension at 72°C for 10 min for all of the primers.

The PCR products were separated using 6% polyacrylamide gels (8 M urea) on an S2 vertical electrophoresis unit (Gibco-BRL Life Technologies, Paisley, UK) at a constant power of 45 W. After separation, the gels were silver-stained [20].

Biostatistical analysis of the CM populations: Allelic diversity (the number of alleles per locus), allelic richness (AR), and Nei's unbiased expected (H_E) and observed (H_O) heterozygosity and fixation indices (F_{IS}) were calculated for each locus using FSTAT version 2.9.3 [21].

The number of null alleles per locus and per population was calculated using the MICRO-CHECKER program [22].

Deviations from Hardy-Weinberg equilibrium (HWE) and genotypic linkage disequilibrium were performed using Markov chain methods (5000 dememorisations, 500 batches, and 1000 iterations) using GENEPOP 1.2 [23]. The same program was used to test for linkage disequilibrium between all of the pairs of loci.

Tests for significant differentiation (Fisher's exact tests) for all of the populations' loci and population pairs were also performed using GENEPOP 1.2 [23]. The significance tests for genetic differentiation were corrected for multiple comparisons using the Bonferroni procedure (10,000 permutations) [24]. F_{ST}, estimated as θ, was calculated among population pairs using FSTAT version 2.9.3 [25,26].

GenAlEx 6.2 [27] was used to conduct an analysis of molecular variance (AMOVA) [28] and to estimate ΦST (9999 permutations). For AMOVA genetic structure was partitioned with regard to the geographic region/management type (because each management type occurred in a different geographic region, this category was combined and simply referred to as the management type; see Figs 1 and 2 the differences between the second and third CM generations of the populations with specific management types (untreated population: Beloslavec; treated populations: Kloštar Ivanić and Nedelišće) [27]. The total genetic variation was partitioned into three levels: 1) Between populations, 2) among individuals, and 3) within individuals.

Isolation by distance was computed using FSTAT version 2.9.3 [21]. A Mantel test was calculated to determine whether there was a significant positive correlation between F_{ST} as estimated by F_{ST} ($1/F_{ST}$ -1) and the natural logarithm (Ln) of the geographic distance between populations (in km).

Evidence for recent bottlenecks in the populations was assessed using BOTTLENECK 1.2 [26]. This analysis incorporated a stepwise mutation model (SMM) [29] and a two-phase model (TPM) [30], in which 90% of the mutations follow the SMM and 10% represent multistep changes [31]. Wilcoxon signed-rank tests were used to determine whether the deviations of the observed heterozygosity relative to that which is expected at drift-mutation equilibrium were significant ($P < 0.05$). A modal shift in the allelic frequency distribution was used as an indicator of a population bottleneck [32].

Results and Discussion

The average percentages of fruit that were attacked before apple picking in 2008 were 0.8% and 1.09% in the treated orchards and 4.94% in the untreated orchard.

Genetic diversity: After correction for multiple comparisons (n=86), significant linkage disequilibrium was only detected for the paired loci Cp2.129/Cp1.60 ($P<0.01$). However, this only involved one population (Beloslavec). Therefore, these loci were retained in all of the subsequent analyses.

Significant deviation from HWE was observed at the Cp 5.24, Cp 3.169, Cp 1.62 and Cp 1.60 loci after corrections for multiple comparisons (n=3, $P<0.01$). MICRO-CHECKER indicated the presence of null alleles in the Cp 5.24 locus in the Kloštar Ivanić population, the Cp 1.60 locus in the Nedelišće population, the Cp 3.169 locus in the Beloslavec and Kloštar Ivanić populations and the Cp 1.62 locus in all of the populations.

The first set of previously described primers (Cp 5.24, Cp 2.39, Cp 3.180, Cp 2.129, Cp 3.169, Cp 1.62 and Cp 1.60) [15] had more alleles than the second set of primers (Cyd 15, Cyd 16 and Cyd 14) [16] (Table 1). Two to 13 alleles per locus were found in all of the loci, and locus Cp 2.39 had the highest number of alleles. The average number of alleles across all of the loci was 7.20.

The CMs from the Beloslavec orchard had the greatest average allelic diversity (6.2 alleles across all of the loci) compared to the other two locations. Both of these populations had an average of 5.3 alleles across all of the loci (Table 1).

The most allelic richness across all of the loci was observed in the CMs from the Beloslavec orchard. However, locus Cyd 15 from the Beloslavec and Kloštar Ivanić orchards and locus Cyd 14 from all of the locations had the least allelic richness (Table 1).

Mean expected heterozygosity (H_E) ranged from 0.10 to 0.84, while the mean observed heterozygosity (H_O) ranged from 0.5 to 0.76 in all of the loci across all of the populations (Table 1).

The inbreeding index (F_{IS}) ranged from 0.1 to 0.79 in all of the loci across all of the populations. The highest average F_{IS} was observed in the Kloštar Ivanić population (mean F_{IS}=0.22 across

Table 1. The number of alleles per locus (Na), allelic richness (AR), the expected (H_E) and observed (H_O) heterozygosity and Weir and Cockerham's [25] inbreeding coefficient (F_{IS}) at 10 microsatellite loci from three populations of *Cydia pomonella*.

		Microsatellite loci										
Population		Cyd 15	Cyd 16	Cyd 14	Cp 5.24	Cp 2.39	Cp 3.180	Cp 2.129	Cp 3.169	Cp 1.62	Cp 1.60	Mean
Beloslavec (n=29)	Na	2	3	2	3	13	4	12	10	9	4	6.20
	AR	2.00	3.00	2.00	3.00	12.47	3.90	11.76	9.57	8.85	3.99	6.06
	H_E	0.43	0.54	0.13	0.67*	0.84	0.49	0.81	0.79*	0.82*	0.43*	0.59
	H_O	0.39	0.38	0.14	0.76	0.72	0.35	0.72	0.55	0.57	0.10	0.47
	F_{IS}	0.09	0.31	-0.06	-0.14	0.14	0.29	0.10	0.31	0.31	0.76	0.21
Kloštar Ivanić (n=28)	Na	2	3	2	4	10	3	8	9	7	5	5.30
	AR	2.00	3.00	2.00	3.93	9.91	3.00	7.85	8.85	6.92	5.00	5.25
	H_E	0.44	0.47	0.10	0.66*	0.84	0.31	0.76	0.78	0.75*	0.55*	0.57
	H_O	0.33	0.48	0.11	0.54	0.64	0.29	0.68	0.54	0.5	0.12	0.42
	F_{IS}	0.25	-0.03	-0.04	0.19	0.23	0.09	0.11	0.32	0.34	0.79	0.22
Nedelišće (n=29)	Na	3	3	2	4	10	3	9	6	8	5	5.30
	AR	2.90	3.00	2.00	3.93	9.88	2.90	8.78	5.99	7.85	5.00	5.22
	H_E	0.53	0.61	0.10	0.68	0.81	0.22	0.76	0.77	0.75*	0.76*	0.60
	H_O	0.52	0.64	0.11	0.75	0.69	0.10	0.59	0.56	0.48	0.33	0.48
	F_{IS}	0.01	-0.07	-0.04	-0.11	0.15	0.53	0.23	0.28	0.36	0.56	0.19
All populations (n=86)	Na	3	3	2	4	13	4	14	12	12	5	7.20
	AR	2.31	3	1.98	3.52	10.61	3.30	10.28	8.89	8.38	4.97	5.72
	H_E	0.47	0.54	0.11	0.67	0.83	0.34	0.78	0.78	0.77*	0.58*	0.59
	H_O	0.42	0.50	0.12	0.68	0.68	0.24	0.66	0.55	0.52	0.19	0.46
	F_{IS}	0.12	0.07	-0.04	-0.02	0.17	0.31	0.15	0.30	0.34	0.71	0.21

*Significant deviation from Hardy-Weinberg equilibrium.

all of the loci). Population pair-wise genetic differentiation, estimated as F_{ST}, was low, ranged from 0.02 to 0.04 and was not significant after corrections for multiple comparisons (n=3, P<0.01) (Table 2).

The AMOVA indicated that the majority of the genotypic variation was significant and partitioned across individuals from the different management types (treated *vs.* untreated apple orchards: 86% variation, p<0.01), from the second and third generations in all of the populations (86% variation, p<0.01), from the untreated population from Beloslavec (92%, p<0.01) and from the treated populations from Kloštar Ivanić (98%, p<0.01) and Nedelišće (70%, p<0.01).

A Mantel test of isolation by distance revealed an insignificant negative relationship between Slatkin's linearised F_{ST} $[F_{ST}(1/F_{ST}-1)]$ [33] and the Ln of the geographic distance (in km) in all of the populations (r=-0.53; p=0.65). This suggests that the pattern of gene flow described by an isolation-by-distance model is unlikely to have occurred.

Table 2. Pair-wise estimates of Weir and Cockerham's[25] θ (F_{ST}; below the diagonal) and distance (km; above the diagonal) for the *Cydia pomonella* populations from the treated (Kloštar Ivanić and Nedelišće) and untreated (Beloslavec) orchards.

Population	Beloslavec	Kloštar Ivanić	Nedelišće
Beloslavec	-	55	60
Kloštar Ivanić	0.019	-	110
Nedelišće	0.037	0.035	-

There was no evidence suggesting that the examined populations had undergone a bottleneck event because each population exhibited a normal L-shaped allelic distribution.

The CM populations from the three Croatian locations revealed low estimates of genetic structure despite differences in the type of control/management (treated vs. untreated apple orchards), indicating high levels of gene flow and movement. The genetic differentiation across the studied geographical regions was low (F_{ST} values ranging from 0.02 to 0.04), which is consistent with previous studies on CMs in Europe and elsewhere [11, 13, 17, 19]. This confirms the general hypothesis that there is a lack of genetic differentiation across populations of Lepidoptera pests. Although the variability among the orchards was relatively low, the genetic variation was significantly partitioned within the individuals in all of the examined categories (management type and the second and third generations of each population), indicating high allelic diversity. Despite the fact that the differences in allelic richness were not statistically significant, the CM population from Beloslavec (the untreated orchard) had the greatest average allelic diversity compared to the other two locations (the treated orchards). This indicates that insecticide treatments potentially lower allelic richness.

A different situation has been reported for apple-growing areas in South Africa [14] and Switzerland [18], where significant genetic differentiation has occurred between populations sampled from different geographical regions. This difference could be due to differences in the molecular markers (such as AFLP) used for the study conducted in South Africa. However, in the Swiss study [18] the same microsatellite markers that had previously detected low levels of genetic structural variation in French and Chilean

populations were used [11, 17, 19].

CMs are considered to be "sedentary insects" [34], but studies on their flight capacity have demonstrated that some individuals are able to disperse across several kilometres [35]. In this study, there was no evidence for an isolation by distance effect (based on the very low F_{ST} estimates), suggesting that other mechanisms, such as human-mediated transport, may assist the movement of CMs across short and long distances within the Croatian apple-growing regions. Human-mediated dispersal could be an effective means of dispersal for CM populations that are pre-adapted to different climatic conditions. However, further investigations need to be conducted to specifically test the hypothesis that CMs use human-mediated transport in Croatia and elsewhere across their distribution. Nevertheless, wood bins used for apple transport are known to frequently carry diapausing CM larvae and possibly facilitate dispersal across large distances [17].

Certain studies have indicated that insecticide administration is a factor that has shaped temporal genetic variation in the investigated populations [11, 19]. However, its resulting impact on the genetic diversity found from the various geographical locations investigated in the aforementioned study was relatively low, probably because of high gene flow and migration rates [11]. Further research, that incorporates more populations across the geographic range of the Croatian pome fruit production areas, is needed to better understand the CM population genetic structure in Croatia and the natural and/or anthropogenic factors that shape it.

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