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Comparing the genetic structure of codling moth *Cydia pomonella* (L.) from Greece and France: long distance gene-flow in a sedentary pest species

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Abstract

Codling moth *Cydia pomonella* L. (Lepidoptera: Tortricidae) is the most important insect pest of apple production in Europe. Despite the economic importance of this pest, there is not information about the genetic structure of its population in Greece and the patterns of gene-flow which might affect the success of control programs. In this study, we analysed nine samples from apple, pear and walnut from various regions of mainland Greece using 11 microsatellite loci. Six samples from the aforementioned hosts from southern France were also examined for comparison. Bayesian clustering and genetic distance analyses separated the codling moth samples in two genetic clusters. The first cluster consisted mainly of the individuals from Greece, and the second of those from France, although admixture and miss-classified individuals were also observed. The low genetic differentiation among samples within each country was also revealed by F_{ST} statistics (0.009 among Greek samples and 0.0150 among French samples compared to 0.050 global value among all samples and 0.032 the mean of the pair-wise values between the two countries). These F_{ST} values suggest little structuring at large geographical scales in agreement with previous published studies. The host species and local factors (climatic conditions, topography, pest control programs) did not affect the genetic structure of codling moth populations within each country. The results are discussed in relation to human-made activities that promote gene-flow even at large geographic distances. Possible factors for the genetic differentiation between the two genetic clusters are also discussed.

Keywords: codling moth, population structure, gene-flow, apple, Torticidae, Lepidoptera

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Introduction

Codling moth *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) is a major insect pest of deciduous fruits in temperate areas worldwide. It infests the fruits of several cultivated tree species, mainly apple *Malus domestica* Borkh (Rosaceae), pear *Pyrus* spp. (Rosaceae), quince *Cydonia oblonga* Mill (Rosaceae) and walnut *Juglans* spp. (Juglandaceae) (Shel'Deshova, 1967; Barnes, 1991). The phenology of the species is quite variable with one to five generations per year. Temperature and photoperiod seem to be the major driving forces of this variation (Riedl & Croft, 1978), but pleiotropic effects of costly insecticide resistance mechanisms (Boivin *et al*., 2004) and adaptation to host-plants (Phillips & Barnes, 1975) are also involved. Codling moth has two traits which are interesting from both a theoretical and a practical point of view. First, it has developed resistance to various classes of chemical insecticides in several parts of the world (e.g. Europe: Sauphanor *et al*., 1998; USA: Knight *et al*., 1994, South America: Fuentes-Contreras *et al*., 2007). This has been attributed to the intensive chemical control against the pest. In some cases, insecticide treatments are the main force affecting the genetic structure and regulating the dynamics of codling moth populations (Franck *et al*., 2007). Second, the insect has achieved an almost globally distribution, presumably due to its potential to adapt in different environments. Reasons for this success have been reported by Thaler *et al*. (2008). Briefly, these are the re-emergence of two refugial haplotype clades with a long history of independent evolution leading to a diverse genetic heritage, the plasticity of the species to survive and reproduce under different climatic conditions and host-trees, the development of insecticide resistance and, lastly, the human activities which facilitate dispersal and intermixing of different genotypes and spread of resistance to insecticides.

These characteristics make codling moth a good model for studying ecological questions related to migration and gene flow among neighbouring or geographical distant populations. To shed light on dispersal process, several studies have analysed the structure of codling moth populations using either allozyme (Buès & Toubon, 1992; Buès *et al*., 1995) or more recently AFLP (Timm *et al*., 2006; Thaler *et al*., 2008) and microsatellite markers (Franck *et al*., 2007; Fuentes-Contreras *et al*., 2008; Chen & Dorn, 2010). Studies on temporal and spatial variation at molecular level could provide information for the pest fitness and success (Armstorng & Wratten, 1996), and they aid also the development of Integrated Pest Management strategies (Denholm & Rowland, 1992).

Theoretical aspects of spatial and temporal genetic patterns in relation to gene flow have been reviewed by Baur & Schmid (1996) and Roderick (1996). The strength of gene-flow among insect populations may be affected by the dispersal ability (distance and tendency for migration/dispersal) and limited gene flow is often observed in populations of relatively sedentary species. However, other biological traits, such as population increase and host-plant specialisation, and ecological factors related to habitat (landscape characteristics, geographical barriers, elevation, climatic constraints and host-plant isolation) can also often modulate gene-flow (Peterson & Denno, 1998a,b; Bohonak, 1999; Loxdale & Lushai, 2001). Knowledge about dispersal in insects and gene flow among their populations has been obtained mainly from two classes of methods. Directs methods, such as mark-release-recapture (MRR), can provide information on the contemporary pattern of migration, but they have some practical difficulties and limitations (e.g. dilution at large spatial distances). Also, they cannot estimate the contribution of emigrants to the local populations, as migration is not always translated into successful colonisation. Indirect methods use various genetic markers, and the required data (allele and genotype frequencies) for the estimation of gene-flow and migration rates are relatively easy to gather. These methods can provide long-term indirect estimates of gene flow, averaged over numerous generations and integrated geographically over many populations; but patterns of contemporary movements of individuals or propagules can be also addressed. Such information can assist in understanding the dynamics of pest expansion (Roderick, 1996; Peterson & Denno, 1998a,b; Bohonak, 1999; Loxdale & Lushai, 2001; Manel *et al*., 2005).

Cydia pomonella is traditionally considered as a sedentary species, and MRR experiments with males have shown that the majority disperse within 60–80 m, although a small proportion of males is able to fly up to several kilometres (Mani & Wildbolz, 1977; Keil *et al*., 2001). Laboratory experiments confirmed the results of MRR and showed also similar flight capacity for males and females (Schumacher *et al*., 1997). This variation in dispersal is considered adaptive, as it provides opportunities for survival in cases of habitat deterioration (Schumacher *et al*., 1997; Keil *et al*., 2001) and, along with transportation of infested material (fruits, harvest bins) (Hibgee *et al*., 2001), might have important implications on gene flow between distant populations. Studies with allozyme (Buès *et al*., 1995) and DNA (Timm *et al*., 2006; Franck *et al*., 2007; Fuentes-Contreras *et al*., 2008; Thaler *et al*., 2008; Chen & Dorn, 2010) markers on populations from various regions of the world have revealed variable results. Despite some evidence of genetic differentiation among geographically close populations with different kinds of markers, the general pattern is that genetic differentiation among distant population remains globally low (reviewed by Franck & Timm, 2010).

In contrast to codling moth from western and Central Europe, there is no study on the genetic structure of populations from Greece. Codling moth in Greece is a major pest in apple orchards, and its control is based almost exclusively on chemical insecticides. Apple cultivation is distributed throughout mainland Greece at locations with different climatic conditions and landscape characteristics; and often apple and other fruits, hosts of codling moth, are cultivated in the same areas. These diversified habitats of codling moth may provide opportunities for population subdivisions and locally adapted populations. In addition, there is no information about the genetic similarity of codling moth populations from Greece with those from other European countries, such as France, where diversified habitats also exist and similar chemical control programs are applied. Comparative studies among populations from different countries can aid in the assessment of gene-flow over large spatial scales and shed light on the possible role of human activities, such as commerce, in pest dispersal.

Therefore, we used 11 microsatellites, which are highly polymoprhic codominant markers suitable for population genetic analysis (Behura, 2006), to assess the genetic structure of codling moth populations from different hosts and locations in Greece and southern France. An additional aim was to infer about potential gene flow over various geographical scales and adaptation to different hosts or environmental conditions. Such data might also be useful for the improvement of codling

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Table 1. Insect material. Orchard location and number of codling moth larvae (*N*) collected.

Code	Host	Collection year	Region*	Longitude	Latitude	*N*
AlexA	Apple	2007	Alexandria, NGR	22°28′ 64.2″	40°34′ 64.8″	24
AgAa	Apple	2007	Agia, CGR	22°39′ 54.4″	39°40′ 71.9″	54
AgAb	Apple	2007	Agia, CGR	22°39′ 38.2″	39°40′ 77.0″	44
PiA	Apple	2007	Zagora, CGR	23°3′ 57.4″	39°27′ 10.7″	19
TrA	Apple	2007	Tripoli, SGR	22°22′ 74.5″	37°31′ 81.4″	19
PiW	Walnut	2007	Drakia, CGR	23°2′ 51.4″	39°22′ 87.4″	29
AgW	Walnut	2007	Agia, CGR	22°40′10.2″	39°42′ 11.8″	25
PiP	Pear	2007	Lechonia, CGR	23°3′ 32.8″	39°19′ 04.7″	21
ArgP	Pear	2007	Argos, SGR	22°37′ 31.1″	37°36′ 12.1″	23
ValA	Apple	2005	Valence, SFR	4°55′ 42.5″	44°58′ 42.7″	36
AviA	Apple	2006	Avignon, SFR	4°53′ 36.3″	43°50′ 48.2″	20
MarW	Walnut	2003	St–Marcelin, SFR	5°16′ 26.2″	45°08′ 06.8″	29
ValW	Walnut	2005	Valence, SFR	4°56′ 03.2″	44°58′ 33.4″	20
ValP	Pear	2003	Valence, SFR	4°55′ 54.8″	44°58′ 32.4″	25
AviP	Pear	2006	Avignon, SFR	4°55′ 12.8″	43°49′ 32.4″	25

* NGR, north Greece; CGR, central Greece; SGR, south Greece; SFR, south France.

moth control programs, as they are often affected by the dispersal of the pest genotypes among orchards and regions.

Material and methods

Insect material

Fifteen samples of full grown diapausing codling moth larvae were collected from apple, walnut and pear orchards from various localities in Greece, with different altitudes (7–1122 m), covering a south-north cline. In addition, samples from the above hosts were collected in localities of southern France (altitude range 20–36 m). Larvae were collected using corrugated cardboard traps, and microsatellite genotyping analysis was performed on the emerging adults (table 1, fig. 1).

Microsatellite genotyping analysis

Total DNA was extracted from one leg of each moth using 200 µl of 10% Chelex 100 (Biorad, San Francisco, CA, USA) resin solution (Walsh *et al*., 1991). Eleven microsatellite loci (Cp3.180, Cp3.169, Cp2.129, Cp1.62, Cp5.24, Cp5.M, Cp4.S, Cp6.46, Cp2.131, Cp4.129, Cp6.32) (Franck *et al*., 2005, 2011) were examined in a total of 413 moths. Details on microsatellite loci amplification, analysis and visualization are presented in previous papers (Franck *et al*., 2007, 2011).

Basic statistics, Hardy-Weinberg equilibrium and linkage disequilibrium

We examined the data for presence of null alleles using Microchecker version 2.2.3 (van Oosterhout *et al*., 2004). In addition, we used the method of Brookfield (1996) as implemented in GENEPOP version 4.0 (Rousset, 2008) to calculate the frequency of null alleles (A_n). Due to the low frequencies of null alleles found (see results), data from all 11 loci were used in the subsequent analyses.

Allele frequencies, number of alleles per locus, observed (H_O) and expected (H_E) heterozygosity, as well as inbreeding coefficient (F_{IS}), were calculated using GENEPOP version 4.0. Allelic richness (*R*s, number of alleles independent of sample size) was calculated using FSTAT version 2.9.3.2 (Goudet, 1995). Deviation from Hardy-Weinberg equilibrium (HWE) at each locus and across all loci (*U* test and multisample score test, respectively; Raymond & Rousset, 1995) and linkage disequilibrium between pairs of microsatellite loci (*G* log-likelihood based exact test; Goudet *et al*., 1996) were tested using GENEPOP version 4.0.

Changes in the population effective sizes were examined using the software BOTTLENECK version 1.2.02 (Cornuet & Luikart, 1996). The observed gene diversities were compared to their expected values from the number of alleles in the samples under the assumption of mutation-migration-drift equilibrium. Gene diversities at the mutation-migration-drift equilibrium were computed for the two-phase mutation model (TPM; with 95% single-step mutations and 5% multiple-step mutations), which is considered appropriate for microsatellites (Piry *et al*., 1999). A significant excess of observed gene diversity relative to the expected gene diversity at the equilibrium may indicate a population size reduction, while a deficit may indicate that the population is growing. Significances were tested with two-tailed Wilcoxon's signed-rank test (Piry *et al*., 1999).

AMOVA and F_{ST} *analysis*

Population structure was assessed by calculating multilocus F_{ST} values (Weir & Cockerham, 1984) for pairwise comparisons of samples using ARLEQUIN version 3.11 (Schneider *et al*., 2000). Significance (*P*-value) was assessed with 10,000 permutations of diploid multilocus genotypes between samples. The structure of the data was also investigated by analysis of molecular variance (AMOVA) with ARLEQUIN version 3.11 using a permutation nonparametric approach (1000 permutations) to test for the significance of fixation indices (Excoffier *et al*., 1992). The partitioning of variance between groups, i.e. Greek and French samples, or three groups according to host-trees (apple, pear, walnut), among samples within groups, and within samples was examined.

To test for isolation by distance (IBD) among codling moth samples a regression analysis of the linearised F_{ST} transformation ($F_{ST}/(1–F_{ST})$) onto the logarithm transformation of the geographical distance was performed (Rousset, 1997) using GENEPOP version 4.0. The significance of this regression was

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Fig. 1. Sampling sites and host-trees in Greece and France. 1, Alexandria (apple); 2, Agia (apple, walnut); 3, Zagora (apple), Lehonia (pear), Drakia (walnut); 4, Argos (pear); 5, Tripoli (apple); 6, St-Marcelin (walnut); 7, Valence (apple, pear, walnut); 8, Avignon (apple, pear).

based on Mantel's test (Mantel, 1967) using 1000 permutations of pair of samples.

Bayesian clustering and genetic distance analyses

Bayesian analysis implemented in STRUCTURE version 2.2 (Pritchard *et al*., 2000) was used to infer the number of *K* unknown genetic clusters in which the sampled multilocus genotypes can be split. The method also assigns a probability that the individuals belong to a certain cluster or to more than one cluster if they are admixed. We used both of the two ancestry models that STRUCTURE provides (admixture, each individual draws some fraction of its genome from each of the *K* populations; and no admixture, individuals are discretely from one population or another) without any prior information about the samples (i.e. sampling region, host-plant). In addition, two models for the allele frequencies (independent allele frequency, IAF; correlated allele frequency, CAF) were used for each of the two ancestry models. The IAF model assumes that the allele frequencies in each population are independent draws from a distribution, while the CAF model assumes that allele frequencies in the different populations are likely to be similar (probably due to migration or shared ancestry). In each case, we ran ten simulations (to check the consistency of our data) of 100,000 iterations following a burn-in period of 50,000 iterations for each *K*-value, and with values of *K* from 1 to 10. The pointers provided by Pritchard *et al*. (2000), Garnier *et al*. (2004) and Evanno *et al*. (2005) was used to select the *K*-value showing the best subdivision of our data. We examined the increase pattern of the estimated posterior probability of the data (mean LnPPD over ten runs divided by standard deviation) with *K* and the distribution of ΔLnPPD values (mean of the absolute values of the rate of change of LnPPD averaged over ten runs divided by standard deviation). The modal value of this distribution is located at the real *K* and its height is used as an indicator of the strength of the signal detected by STRUCTURE (Evanno *et al*., 2005).

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Table 2. Number of alleles, allelic richness and frquency of null alleles per loci over all samples from Greece ($n=258$) and France ($n=155$).

Locus	Number of alleles		Allelic Richness		Frequency of null alleles	
	Greece	France	Greece	France	Greece	France
Cp3.180	4	3	2.7	2.2	0.003	0.005
Cp3.169	24	23	8.0	8.6	0.000	0.000
Cp2.129	29	12	9.5	7.8	0.041	0.000
Cp1.62	22	12	8.8	6.1	0.020	0.000
Cp5.24	4	3	3.1	2.4	0.179	0.000
Cp5.M	24	25	13.0	15.5	0.012	0.021
Cp4.S	3	3	2.9	3.0	0.000	0.083
Cp6.46	26	22	12.4	10.4	0.099	0.028
Cp2.131	21	16	9.8	9.5	0.030	0.017
Cp4.129	8	6	5.0	3.5	0.052	0.000
Cp6.32	13	12	6.7	6.5	0.000	0.000

To verify the *K*-value found using STRUCTURE, we also analyse the multilocus genotypes with BAPS version 5.3 (Corander *et al*., 2008). In particular, we run a mixture analysis with the multilocus genotypes pre-assigned to the 15 samples of codling moth examined. Ten runs for each $K=2$–10 were performed.

In addition, the GeneClass2 software (Piry *et al*., 2004) was used for population assignment and exclusion tests, including the calculation of probability of origin for each individual moth. We used the Bayesian method of Rannala & Mountain (1997) as computation criterion. The probability for the assignment of the individuals to a given population was computed using the simulation algorithm of Paetkau *et al*. (2004) for Monte-Carlo re-sampling, and 1000 simulations were applied with an error rate of 0.01 for type I errors (assignment threshold probability). Assignment tests were also run without probability computation (without Monte-Carlo re-sampling). The score for each individual to the relative populations was calculated and the assignment threshold of scores was 0.05.

To further investigate the genetic relationship between populations, a neighbour joining (NJ) tree based on the allele shared distance (DAS) (Chakraborty & Jin, 1993) was constructed using the software POPULATIONS version 1.1.28 (http://bioinformatics.org/~tryphon/populations/). DAS distance counts the number of different alleles between multilocus genotypes. Bootstrap values were calculated by resampling loci, and are presented as percentages over 1000 replications. The tree was constructed using the software MEGA version 4.01 (Tamura *et al*., 2007). In addition, we used GENALEX 6.1 software (Peakall & Smouse, 2006) to calculated pairwise PhiPT distance values (an analogue of F_{ST}) in the examined populations. A principal components analysis (PCA) was performed using the matrix of the PhiPT values.

Results

Basic statistics, Hardy-Weinberg equilibrium and linkage disequilibrium

In total, 258 individuals from nine Greek localities and 155 individuals from six French localities were analysed. No evidence for null allele was found in the 11 loci using MICROCHECKER software. In addition, low frequencies of null alleles were calculated using GENEPOP, which ranged from 0.000 to 0.179 for each locus with mean values over all loci for the 15 populations 0.003–0.036. The number of alleles ranged from 3 to 29 and allelic richness from 2.2 to 15.5 (table 2). The mean (over all loci) number of alleles and allelic richness ranged among the Greek samples from 7.3 to 10.4 and from 6.8 to 7.8, respectively. The corresponding values for the samples from France ranged from 6.7 to 7.8 and from 6.1 to 6.6. Significant differences were found between Greek and French samples in the mean number of alleles (over all loci) (8.3 vs 7.1, $U=49.5$, $P=0.009$) and mean allelic richness (7.2 vs 6.3, $U=54.0$, $P=0.002$). By contrast, no differences were found between these two groups of samples in mean frequency of null alleles (0.024 vs 0.018, $U=33.5$, $P=0.477$), mean H_{EXP} (0.653 vs 0.638, $U=39.0$, $P=0.175$), mean H_O (0.626 vs 0.641, $U=21.0$, $P=0.517$) and mean F_{IS} (0.04 vs –0.01, $U=40.0$, $P=0.139$) (table 3, table S1 in the supplementary material).

Significant deviation from HWE was found in 26 (17 in Greek and nine in French samples) out of 165 locus/population combinations. Most of these deviations were associated with heterozygote deficiency. Significant multilocus deviations associated with heterozygote deficiency was observed in five populations, four from Greece (AgAb, AlexA, PiP, PiW) and one from France (AviP), and with heterozygote excess was indicated in one population from France (ValW) (table S1). BOTTLENECK software did not detect recent bottleneck effect in any of the populations examined. However, significant heterozygosity deficiency in the average observed gene diversity compared to the expected gene diversity under the TPM model was observed in two populations from Greece (PiP, $P=0.027$; TrA, $P=0.001$), which may suggest population expansion. Linkage disequilibrium analysis revealed 63 cases of significant associations between pairs of loci out of 825 pairwise comparisons. This value is above the expected number by chance in case of equilibrium ($0.05\times825=41.25$). Most pairs of loci with linkage disequilibrium were observed in one population from Greece (PiW, 15 pairs) and one from France (ValA, 16 pairs).

AMOVA and F_{ST} analysis

The global estimate of F_{ST} over all 15 samples and the 11 microsatellite loci was 0.050 and significant different from zero ($P<0.001$), suggesting some genetic differentiation among samples. The hierarchical AMOVA over all loci and samples revealed small but significant variance (3.9% of the total variance, $P=0.006$) between groups (France vs Greece) with a global $F_{CT}=0.039$ ($P<0.001$), although most of the

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Table 3. Genetic diversity indices. Mean number of alleles (n), proportion of null alleles (A_n), allelic richness (R_S, number of alleles independent of sample size), heterozygosity expected (H_{EXP}) and heterozygosity observed (H_O) over all loci.

Samples	Country	Host	n	A_n	R_S	H_{EXP}	H_O
AlexA	N. Greece	Apple	7.5	0.024	6.8	0.666	0.650
AgAa	C. Greece	Apple	10.4	0.012	7.3	0.659	0.643
AgAb	C. Greece	Apple	9.5	0.030	7.2	0.671	0.610
PiA	C. Greece	Apple	7.3	0.020	7.1	0.699	0.694
TrA	S. Greece	Apple	7.6	0.014	7.3	0.626	0.636
PiW	C. Greece	Walnut	8.0	0.036	6.9	0.644	0.584
AgW	C. Greece	Walnut	8.5	0.013	7.5	0.641	0.646
PiP	C. Greece	Pear	7.4	0.021	7.0	0.598	0.561
ArgP	S. Greece	Pear	8.8	0.036	7.8	0.668	0.613
mean			*8.3*	*0.024*	*7.2*	*0.653*	*0.626*
ValA	S. France	Apple	7.3	0.004	6.1	0.647	0.656
AviA	S. France	Apple	6.7	0.014	6.4	0.639	0.662
MarW	S. France	Walnut	7.8	0.022	6.6	0.639	0.611
ValW	S. France	Walnut	6.8	0.010	6.4	0.654	0.705
ValP	S. France	Pear	7.1	0.030	6.3	0.635	0.629
AviP	S. France	Pear	7.0	0.030	6.2	0.605	0.582
mean			*7.1*	*0.018*	*6.3*	*0.638*	*0.641*

Table 4. AMOVA results for 11 microsatellite loci. The genetic variance was partitioned between two groups (Greece and France) and among samples within these two groups.

Locus	Within samples			Among Samples within groups			Among groups (Greece vs France)			
	df	Variance components	% variation	df	Variance components	% variation	df	Variance components	% variation	P
Cp3.180	801	0.162	98.9	13	0.001	0.3	1	0.001	0.8	0.081
Cp3.169	809	0.303	99.1	13	0.002	0.7	1	0.000	0.2	0.253
Cp2.129	807	0.398	98.1	13	0.005	1.2	1	0.003	0.7	0.126
Cp1.62	807	0.381	97.1	13	0.008	2.1	1	0.003	0.8	0.132
Cp5.24	805	0.219	84.9	13	0.003	1.4	1	0.035	**13.7**	**0.000**
Cp5M	811	0.452	97.3	13	0.005	1.1	1	0.007	1.6	**0.044**
Cp4S	799	0.220	97.3	13	0.003	1.4	1	0.003	1.3	0.072
Cp6.46	809	0.402	97.9	13	0.003	0.6	1	0.006	1.5	**0.036**
Cp2.131	811	0.385	94.1	13	0.006	1.4	1	0.018	4.5	**0.005**
Cp4.129	809	0.295	93.6	13	0.005	1.5	1	0.015	4.9	**0.004**
Cp6.32	809	0.347	86.4	13	0.001	0.3	1	0.053	**13.3**	**0.000**
Total	811	3.537	95.0	13	0.041	1.1	1	0.145	3.9	**0.006**

variance (95.0%) was within samples. The within group variance was low (1.1%) and non-significant ($P=0.074$). Global F_{ST} estimate among the Greek samples was 0.009 ($P<0.001$) and 0.015 ($P<0.001$) for the French samples. The mean value for the pair-wise comparisons between the samples from Greece and France was 0.032. The loci Cp5.24 and Cp6.32 may contribute more to the differentiation of the two groups, as they explain a high percentage of the between-group variance (13.7 and 13.3%, respectively) with F_{ST} values of 0.151 and 0.136, respectively (table 4). The pair-wise comparisons among samples resulted in 37 significant cases out of 51 with the F_{ST} values ranged in Greek and French samples 0.002–0.081 and 0.002–0.068, respectively (table 5). AMOVA on samples grouped by their host of origin (three groups) did not reveal significant variance in the genetic structure between the three hosts (–0.47% of the total variance, $P=0.569$; $F_{CT}=0.039$, $P=0.772$).

Mantel's test did not show significant correlation between the linearised F_{ST} values and the geographical distance among all samples ($P=0.562$, df = 105, $r=-0.023$), the Greek samples ($P=0.709$, df = 36, $r=0.105$) or the French samples ($P=0.699$, df = 15, $r=-0.046$).

Bayesian clustering and genetic distance analyses

Both admixture and non-admixture models showed the best solution of K to be 2 (results from the non-admixture model are presented; see fig. S1 in the supplementary material). There was a sharp increase of the LnPPD values, with K moving from 1 to 2, and then a plateau appeared to be reached. In addition, the highest (modal) value of the distribution of ΔLnPPD was observed at $K=2$ for both admixture and non-admixture models. It seems that splitting samples in two genetic clusters represents the optimal subdivision of the data and avoids unjustified and less informative over-splitting.

The first genetic cluster is mainly characterised by the individuals from Greece (Greek cluster) and the second by the individuals from France (French cluster) (fig. 2; the plot derived from no-admixture ancestry model and IAF model is

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Table 5. Bellow diagonal pairwise F_{ST} values among populations of *Cydia pomonella*.

	AlexA	AgAa	AgAb	PiA	TrA	AgW	PiW	PiP	ArgP	ValA	AviA	MarW	ValW	ValP	AviP
AlexA[1]	–														
AgAa	0.011 ***	–													
AgAb	0.012 **	−0.002 NS	–												
PiA	0.079 ***	0.056 ***	0.053 ***	–											
TrA	0.013 **	0.006 NS	0.005 NS	0.054 ***	–										
AgW	0.081 ***	0.057 ***	0.057 ***	0.023 ***	0.063 ***	–									
PiW	0.057 ***	0.043 ***	0.044 ***	0.003 NS	0.045 ***	0.002 NS	–								
PiP	0.021 ***	0.011 **	0.012 ***	0.066 ***	0.020 ***	0.050 ***	0.045 ***	–							
ArgP	0.011 *	0.009 *	0.005 NS	0.051 ***	0.005 NS	0.046 ***	0.043 ***	0.018 **	–						
ValA	0.058 ***	0.042 ***	0.039 ***	0.018 **	0.051 ***	0.027 ***	0.008 NS	0.030 ***	0.045 ***	–					
AviA	0.062 ***	0.047 ***	0.046 ***	0.015 ***	0.055 ***	0.015 **	0.003 NS	0.038 ***	0.049 ***	0.012 **	–				
MarW	0.021 ***	0.002 NS	−0.001 NS	0.079 **	0.010 ***	0.078 ***	0.069 **	0.014 ***	0.013 *	0.058 ***	0.068 ***	–			
ValW	0.015 *	0.007 *	0.011 *	0.083 ***	0.025 ***	0.077 ***	0.062 ***	0.007 NS	0.033 ***	0.045 ***	0.064 ***	0.014 *	–		
ValP	0.045 ***	0.026 ***	0.033 ***	0.025 ***	0.033 ***	0.020 **	0.012 *	0.026 ***	0.038 ***	0.015 *	0.021 ***	0.051 ***	0.038 ***	–	
AviP	0.012 *	0.008 *	0.005 NS	0.056 ***	0.015 *	0.055 ***	0.038 ***	0.002 NS	0.019 **	0.026 **	0.040 ***	0.009 NS	0.002 NS	0.025 ***	–

[1] Abbreviations are defined in table 1. [2] NS, non significant, $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

presented). However, both of them are not pure, as there are individuals from Greece with high membership coefficient to the French cluster and *vice versa*. This is demonstrated also by the mean membership coefficients of the samples from Greece and France to the two clusters (table 6). The assignment of the individuals to their country of origin were further examined, and they were assigned to a single cluster (Greek or French) when their proportion of ancestry in that cluster was greater than 80%, otherwise they were considered as admixed. This empirical threshold was determined after analysing the distribution of mean ancestry coefficients of the individuals. The no-admixture ancestry model provided much better assignment of the individuals to their country of origin (Greek individuals: 77.9 and 81.0% with CAF and IAF allele frequency models, respectively; French individuals: 89.0 and 87.7% with the respective allele models) compared to the admixture model (Greek individuals: 58.5 and 21.3% with CAF and IAF allele frequency models; French individuals: 59.4 and 42.6% with the respective allele models), and this is illustrated also in the mean membership coefficient of the samples examined (table 6).

In agreement to the results from STRUCTURE, BAPS revealed the highest posterior probability ($P=1.000$) for a structure with two genetic clusters ($K=2$). The first cluster contained the Greek samples, and the second those from France. We also examined the assignment of the individuals in these two genetic clusters (Greek and French) using GeneClass2. The percentage of corrected classified individuals was 85.5% (98.4 and 63.9% for Greek and French individuals, respectively) using the simulation algorithm of Paetkau *et al.* (2004) to compute the probability that an individual belongs to a reference population. The percentage of corrected classified individuals was increased when the assignment was based on a score test without probability computation, reaching 91.8% (92.2 and 91.0% for Greek and French individuals, respectively). In general, the assignment rates obtained by GeneClass2 were close to those obtained using STRUCTURE with the no-admixture ancestry model.

The NJ tree based on DAS (fig. 3) provided similar results with the Bayesian clustering analysis. The tree resulted in two major clusters, the first consists of the samples from France and the second those from Greece. Two major clusters were also revealed by PCA on PhiPT distance values. The separation was due to the scores of the first PCA axis, which explained 66.0% of the total variance. Variation within the two clusters was observed due to the scores of the second PCA axis, which explained a lower percentage (13.7%) of the total variance (fig. 4).

Discussion

In the present study, we investigated the genetic relationships among codling moth samples from three hosts from Greece and France, analysing 11 microsatellite loci, which is the highest number of loci compared to previous microsatellite studies on this insect species. Population structuring was found, with the most obvious case the separation of the Greek samples from those from France. However, important gene-flow was recorded among populations within countries and at a lower extent between countries.

Genetic diversity, Hardy-Weinberg equilibrium

The samples from Greece showed adequate genetic diversity, as indicated by the mean allelic richness and mean

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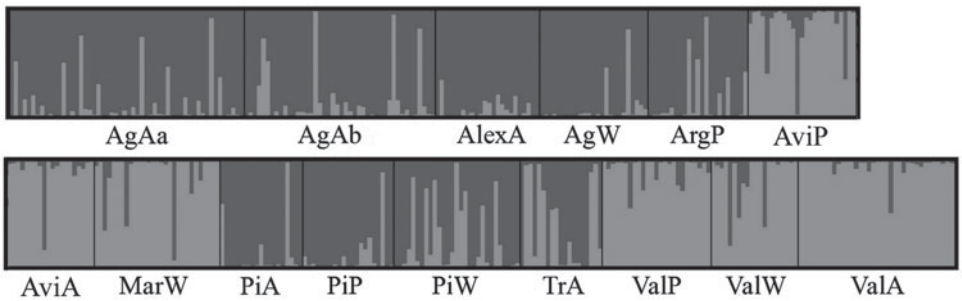

Fig. 2. Partition of genetic variation. Clustering plot of the 15 *Cydia pomonella* samples examined using no-admixture ancestry model and independent frequency allele model. Number of genetic clusters, $K=2$; Cluster 1 (Greek cluster), dark grey colour; Cluster 2 (French cluster), light grey colour. Each individual moth is represented as a vertical bar partitioned into two segments. The lengths of each segment are proportional to the estimated membership coefficients of the individual in each of the two clusters. Individuals of different samples are separated by black vertical lines. AgAa , AgAb, AlexA, AgW, ArgP, PiA, PiP, PiW and TrA are samples from Greece, AviP, AviA, MarW, ValP, ValW and ValA are samples from France; for details see table 1.

number of alleles over all loci which ranged from 6.8 to 7.8 and from 7.3 to 10.4 and the high number of alleles observed especially in seven (13–29 alleles) out of the 11 loci examined. The values are in the same order or even higher than those recorded in previous studies for codling moth populations from Europe (France, Armenia, Italy: Franck *et al*. (2007); Switzerland: Chen & Dorn (2010)) and Chile (Fuentes-Contreras *et al*., 2008). In addition, the Greek samples showed significantly higher diversity, in terms of mean number of alleles and allelic richness, than those from France examined here. Nevertheless, the samples from both countries showed adequate molecular variance, and no recent bottlenecks were detected, suggesting a lively demographic history which is characterised by recent demographic expansions in some Greek populations.

We also observed cases of both single and multi locus significant deviations from HWE, mostly associated with heterozygote deficiency. HWE deviations have been recorded in other microsatellite studies on codling moth populations from Switzerland (Chen & Dorn, 2010) and Armenia (Franck *et al*., 2007) but not in populations from France, Italy (Franck *et al*., 2007) and Chile (Fuentes-Contreras *et al*., 2008). In various microsatellite studies on lepidopteran species, including codling moth (Chen & Dorn, 2010), the significant departures from HWE (heterozygote deficiency) have been attributed to the presence of null alleles, whose frequency seems to be greater in Lepidoptera than in other insect orders (Meglécz & Solignac, 1998; Keyghobadi *et al*., 1999; Meglécz *et al*., 2004; Endersby, *et al*., 2006; Orsini *et al*., 2008). In our study, MICRO-CHECKER did not find evidence for null allele at the 11 loci examined, and frequencies of null allele according to the method of Brookfield (1996) were generally lower than that reported in previous microsatellite studies on codling moth (Franck *et al*., 2007; Fuentes-Contreras *et al*., 2008; Chen & Dorn, 2010). Therefore, other factors should be responsible for the deviations from HWE observed here. In other insect orders, such as aphids, significant heterozygote deficiency in sexual populations have been attributed to selection, Wahlund effect, inbreeding and other population effects (e.g. Delmotte *et al*., 2002; Fenton *et al*., 2003). Selection of insecticide resistant genotypes due to intensive chemical control and their inbreeding could account for the HWE deviations (heterozygote deficiency) observed in the present study. The cases of linkage disequilibrium found support the possibility of inbreeding and non-random mating. Codling moth is a sedentary species, and this behaviour might enhance the inbred of selected resistant genotypes. However, Wahlund effect of sampling from distinct gene pools in the same population cannot be excluded. In support of this, Bayesian clustering analysis showed that some populations contain members of both genetic clusters into which the multilocus genotypes were split (see fig. 2 and below).

Genetic structure, gene-flow

Both Bayesian clustering and genetic distance analyses revealed a genetic differentiation between the samples from Greece and those from southern France. This was also supported by AMOVA and F_{ST} analysis and somehow by the significant differences in some of the genetic diversity indices examined. Part of the genetic differentiation between the French and the Greek samples could be attributed to historical events. An example might be a different level of admixture in these countries of the two geographically isolated mitochondrial genotype clades which were split during the Pleistocene but come into contact after disappearance of the geographic barriers due to climatic changes (Thaler *et al*., 2008). Geographic distance might then have enhanced genetic differentiation through genetic drift or local adaptation phenomena. Selection forces related to environment and to pest management practices can shape the genetic structure of local populations (Thaler *et al*., 2008). Recently, Franck *et al*. (2007) have shown that codling moth populations from France and other countries were mainly structured according to the history of insecticide applications. In support of this, the *kdr* allele, which confers resistance to pyrethroids, has been recorded often in France, but not yet in numerous samples from Greece, including those examined in the present study (Voudouris *et al*., 2011).

Although we sampled codling moth from diverse habitats in Greece, in terms of geography, environmental conditions and host-trees, the majority of the individuals were grouped in one genetic cluster. The same was observed in the samples from southern France examined here. Supporting evidence for the lack of substantial population structuring within the two countries compared to the genetic variation between countries were provided also by the AMOVA results and the F_{ST} values (see below). These findings are in accordance with previous

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Table 6. Average membership coefficients for the two genetic clusters obtained from STRUCTURE for the 15 samples of *Cydia pomonella* examined.

Population	Host	Region	Admixture ancestry model				No-admixture ancestry model			
			CAF		IAF		CAF		IAF	
			G	F	G	F	G	F	G	F
AlexA	Apple	N. Greece	0.773	0.227	0.659	0.341	0.920	0.080	0.941	0.059
AgAa	Apple	C. Greece	0.745	0.255	0.661	0.339	0.861	0.139	0.890	0.110
AgAb	Apple	C. Greece	0.740	0.260	0.659	0.341	0.854	0.146	0.867	0.133
PiA	Apple	C. Greece	0.793	0.207	0.680	0.320	0.902	0.098	0.900	0.100
TrA	Apple	S. Greece	0.598	0.402	0.566	0.434	0.619	0.381	0.622	0.378
AgW	Walnut	C. Greece	0.837	0.163	0.737	0.263	0.927	0.073	0.932	0.068
PiW	Walnut	C. Greece	0.659	0.341	0.595	0.405	0.727	0.273	0.750	0.250
PiP	Pear	C. Greece	0.791	0.209	0.696	0.304	0.898	0.102	0.915	0.085
ArgP	Pear	S. Greece	0.749	0.251	0.676	0.324	0.848	0.152	0.854	0.146
ValA	Apple	S. France	0.127	0.873	0.222	0.778	0.033	0.967	0.030	0.970
AviA	Apple	S. France	0.162	0.838	0.235	0.765	0.069	0.931	0.069	0.931
MarW	Walnut	S. France	0.184	0.816	0.268	0.732	0.093	0.907	0.103	0.897
ValW	Walnut	S. France	0.183	0.817	0.264	0.736	0.121	0.879	0.129	0.871
ValP	Pear	S. France	0.170	0.830	0.261	0.739	0.067	0.933	0.070	0.930
AviP	Pear	S. France	0.231	0.769	0.309	0.691	0.138	0.862	0.134	0.866

CAF, correlated frequency allele model; IAF, independent frequency allele model.
F, French cluster; G, Greek cluster.

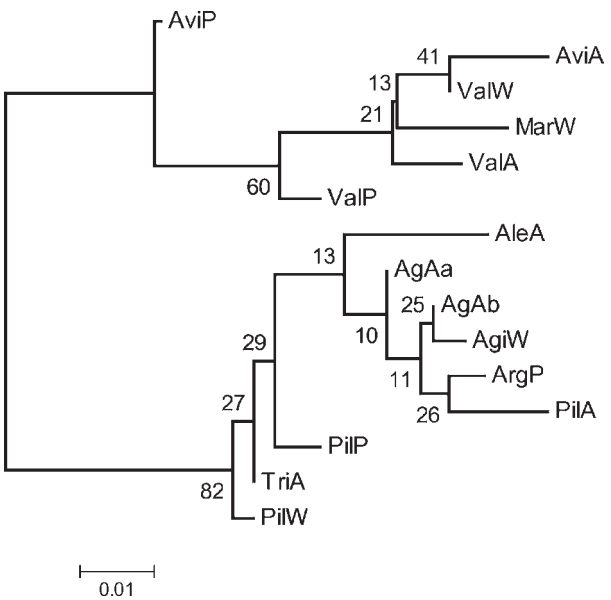

Fig. 3. Neighbour joining tree based on shared allele distance (DAS) among 15 samples of *Cydia pomonella*. Numbers denotes bootstrap percentages (1000 resamplings). AgAa, AgAb, AlexA, AgW, ArgP, PiA, PiP, PiW and TrA are samples from Greece; AviP, AviA, MarW, ValP, ValW and ValA are samples from France; for details see table 1.

studies on French populations with allozyme (Bués & Toubon, 1992; Buès *et al*., 1995) or microsatellite markers (Franck *et al*., 2007) and on Chilean populations with microsatellite markers (Fuentes-Contreras *et al*., 2008). However, recent studies on populations from Central Europe (Germany, Austria, northern Italy) (Thaler *et al*., 2008) and South Africa (Timm *et al*., 2006) with AFLP markers showed high degree of genetic differentiation among populations, even at local geographical scales (e.g. <1 km in South Africa). Apart from the limited moth dispersal, variation in microclimate, habitat-specific conditions, geographic isolation, host-adaptation and pest control practices in Central Europe and relative isolation of pome fruit production areas along with absence of wild hosts in South Africa are possible reasons for these structuring patterns. Franck & Timm (2010) have suggested that these discrepancies among studies could also be attributed to the different affinities of the primers used. However, a recent microsatellite study on populations from Switzerland reported also important genetic differentiation at local geographic scale (even less than 10 km), which was mostly attributed to the sedentary behaviour of codling moth (Chen & Dorn, 2010).

The global F_{ST} values (0.050 among all 15 samples; 0.009 among Greek samples; 0.015 among French samples; 0.032 mean value for the pair-wise comparisons between samples from Greece and France; $F_{CT}=0.039$ between the groups of French and Greek samples), as well as the pair-wise values found here, are in the range of those reported in previous microsattelite studies on codling moth populations from other countries [$F_{ST}=0.066$ among samples from France, Italy, Armenia and Chile, and $F_{ST}=0.006$ among samples from France (Franck *et al*., 2007); $F_{ST}=0.026$ among samples from central Chile (Fuentes-Contreras *et al*., 2008); $F_{ST}=0.016$–0.128, with a mean 0.064 in samples from Switzerland (Chen & Dorn, 2010)]. In addition, early studies using allozyme loci found similar F_{ST} values with the aforementioned ones [$F_{ST}=0.03$ for samples from France (Buès *et al*., 1995); $F_{ST}=0.066$ at an intercontinental level (Pashley & Bush, 1979)]. The F_{ST} values found here contribute to the view of Franck & Timm (2010) that, globally, the codling moth populations appear to be only slightly structured among regions or countries.

It is worth also noticing that the F_{ST} values found here for a sedentary insect are quite lower than those reported in microsatellite studies on other sedentary lepidopteran species, such as *Melitaea cinxia* L. (Nymphalidae) ($F_{ST}=0.200$; Palo *et al*., 1995) and *Polyommatus bellargus* Rottemburg

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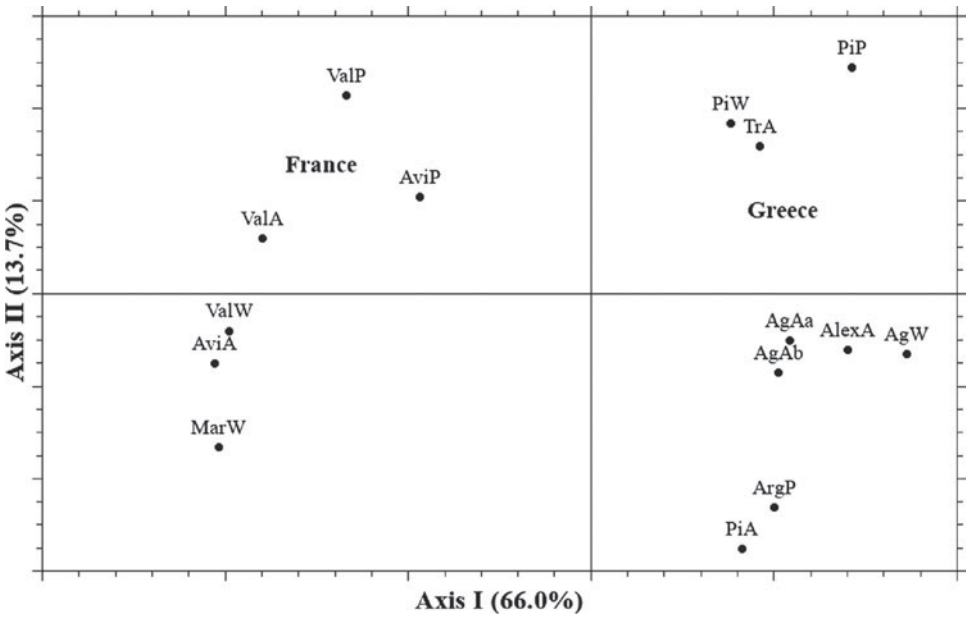

Fig. 4. Plot of the scores of the two Axes of principal component analysis of molecular variance using the PhiPT pairwise distance matrix. The two axes explain 79.7% of the total variance. AgAa, AgAb, AlexA, AgW, ArgP, PiA, PiP, PiW and TrA are samples from Greece; AviP, AviA, MarW, ValP, ValW and ValA are samples from France; for details see table 1.

(Lycaeninae) (F_{ST} = 0.127; Harper *et al*., 2003). By contrast, our values are closer to those reported for highly dispersive lepidopteran species in studies using microsatelitte [*Plutella xylostella* (L.) (Yponomeutidae), F_{ST} = 0.005 (Endersby *et al*., 2006); *Helicoverpa armigera* (Hübner) (Noctuidae), F_{ST} = 0.002 (Endersby *et al*., 2007)] or allozyme [*Heliothis virescens* (Fabricius) (Noctuidae), F_{ST} = 0.002 (Schneider, 1999); *Ostrinia nubilalis* (Hubner) (Crambidae) F_{ST} = 0.024 (Coates *et al*., 2004)] markers. In general, dispersive species show reduced F_{ST} values compared to those of reduced dispersal ability (reviewed by Bohonak, 1999). Nevertheless, the different analyses performed in the present study (including the assignment tests; see below for discussion) come to the same conclusion, i.e. there is gene-flow at various geographic scales (mostly within the two countries) in a sedentary species, even between samples which are hundreds or thousands kilometres apart, separated by many physical barriers. It should be mentioned that sedentary behaviour has been proved also for Greek strains and populations with mark-release-recapture experiments and kinship analysis based on microsatellite markers (maximum dispersal distance ~200 m; Voudouris *et al*., unpublished data). This gene-flow could not be attributed to active dispersion of codling moth individuals. The most possible explanation is passive dispersion due to human activities related to transportation over long distances of infested fruits and harvest bins, which are known to frequently carry diapausing larvae (Higbee *et al*., 2001; Fuentes-Contreras *et al*., 2008). However, some individuals of codling moth are able to undertake flights of several kilometres (Mani & Wildbolz, 1977; Schumacher *et al*., 1997) and, given that commercial pome and walnut production and domestic culture of host-trees of codling moth are widespread in Greece and southern France, a 'stepping-stone' model of expansion could not be totally excluded. The gene-flow described by the 'stepping-stone' hypothesis in other sedentary species had a significant influence on the genetic homogenisation over large geographical distances (Peterson, 1996).

Another point of discussion is that assignment tests revealed individuals that are not assigned to their country of origin (Greece or France) and might represent hybrids between populations from these two or other countries. We cannot, however, discriminate between these two alternatives or clarify the patterns of codling moth dispersal across southern Europe, as it was not possible to analyse samples from localities spanning the geographic distance between Greece and southern France. Nevertheless, this finding could be attributed to recent immigration and supports also the view that gene-flow occur also over large geographical distances (Franck *et al*., 2007; Franck & Timm, 2010). It highlights also the impact of globalisation of commerce on the rather recent range expansion of this insect pest. Franck *et al*. (2007) found also individuals from France and Italy that were hybrids between populations from different countries as a result of recent migration. In addition, Pashley & Bush (1979) suggested a European origin of Canadian and southern African populations, with New Zealand being an additional source for the latter populations. Recently, Timm *et al*. (2006) come to similar conclusions after the analysis of samples from Canada, England and South Africa.

Isolation by distance

The theory of an isolation-by-distance (IBD) effect from a source area (Wright, 1943; Slatkin, 1993) predict that gene flow is most common over short geographic distances and decreases as geographic distance increases. IBD effect has been detected in several insect species (reviewed by Peterson &

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Denno, 1998a). However, we observed no significant correlation between genetic and geographic distance either among all 15 samples or among the nine Greek and among the six French samples. Other studies using microsatellite (Chen & Dorn, 2010) or AFLP (Timm *et al*., 2006; Thaler *et al*. 2008) markers failed also to find a significant IBD effect at various geographic scales in populations from Central Europe or from South Africa. On the other hand, two studies using microsatellites reported significant IBD effect in populations from Chile (Fuentes-Contreras *et al*., 2008) or from a larger spatial scale (populations from France, Italy Armenia and Chile) (Franck *et al*., 2007). Chen & Dorn (2010) suggested that the discrepancy among studies about IBD effect could be related to the innate dispersal capacity of codling moth individuals and to anthropogenic influence on the orchard agro ecosystem. Human-mediated long distance dispersal of codling moth through transportation of infested material (reviewed by Franck & Timm, 2010) could be another factor for the lack of IBD observed here or in other studies. Similarly, Peterson & Denno (1998a) suggested that IBD was not pronounced in highly mobile insects as a result of low genetic differentiation (due to gene-flow) even between distant populations. In our case, however, the lack of samples covering the distance between France and Greece might have also contributed to the non-significant IBD effect.

Host-related genetic differences

Both Bayesian clustering and genetic distance analyses, including AMOVA and F_{ST} statistics, failed to reveal host-associated differences among the populations examined here, and thus we have not evidence of host-race formation in codling moth from Greece or southern France. Similarly, allozyme polymorphism analysis on French populations from apple, pear, quince, walnut and apricot *Prunus armeniaca* L. did not reveal genetic difference related to host-tree (Buès & Toubon, 1992; Buès *et al*., 1995). In addition, Timm *et al*. (2006) did not find host-specific differences in AFLP patterns among samples from apple, pear and stone fruit from South Africa. However, early behavioural and physiological studies on codling moth from California reported differences between populations from apple, walnut and a stone fruit species (plum), and it was suggested that there are three well-defined host-races (Phillips & Barnes, 1975). Toward this direction were the results of two recent studies using DNA markers. Thaler *et al*. (2008) found that individuals of a codling moth population from walnut trees in northern Italy had a different AFLP pattern than a neighbouring population from apple trees. Chen & Dorn (2010) reported also genetic differentiation among populations from three host-trees (apple, apricot and walnut) in Switzerland. It seems that the genetic studies, including ours, provide contradictory results about the existence of host-adapted population or races in codling moth, and only one-third of them report host-associated differences. Chen & Dorn (2010) suggested that the lack of host-associated difference in the aforementioned studies maybe be due to the use of allozyme loci (Buès & Toubon, 1992; Buès *et al*., 1995), which are less polymorphic than microsatellite markers, or to the small sample sizes examined per tree species and location (Timm *et al*., 2006). In our study, regardless the use of more microsatellite loci than Chen & Dorn (2010) (11 vs 9) and similar or higher sample sizes per host-tree, host-related genetic differences were not found. It seems, therefore, that host-adapted populations of codling moth are not quite spread; and, until now, they have been only recorded in Central Europe and California. The reason for this remains to be found, although factors related to climate and to agricultural landscape (e.g. distribution and size of the orchards of the various host-trees) might be involved. According to Barnes (1991), a host-associated race or population of codling moth could only evolve in an orchard of sufficient area of dominance.

Conclusions

The results of the present study suggested important gene-flow among samples from various regions of Greece from three hosts. The same pattern was observed in samples from southern France which were examined for comparison purposes. On the other hand, population structuring between the two countries was found. Historical events along with the relative geographic isolation, genetic drift and local adaptation phenomena might have contributed to this genetic differentiation. However, cases of gene-flow between samples from Greece and France were also detected. These results add to the growing body of evidence that codling moth populations are slightly structured at either country or global level (reviewed by Franck & Timm, 2010), which reflects the globalisation of this insect pest. Given the sedentary nature of the species, which has been proved also for Greek strains and populations (Voudouris *et al*., unpublished data), the patterns of gene-flow observed here highlight the importance of agricultural practices and commerce in the passive dispersal of individuals over large distances. Our findings may have important implications for the chemical control of the pest and insecticide resistance management, as they are often affected by the dispersal of codling moth. A recent study showed that insecticide resistance is widespread in codling moth populations in the main apple-growing regions of Greece (Voudouris *et al*., 2011). Apart from local factors related to insecticide selection pressure, this might have resulted from the gene-flow among populations recorded in the present study. Lastly, we didn't find evidence for host-adapted populations either in Greece or in France, suggesting regular gene-flow among populations from the three host-trees examined, although future research shall include samples from additional cultivated, domesticated and wild tree species.

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Supplementary material

The online table and figure can be viewed at http://journals.combridge.org/ber.

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