Phylogeography and gene diversity of the gall-forming aphid *Aploneura lentisci* in the Mediterranean basin

Sagi Nahum[1]*, Moshe Inbar[1], Gidi Ne'eman[2] and Rachel Ben-Shlomo[2]

[1] Department of Evolutionary and Environmental Biology, University of Haifa, Haifa, 31905, Israel

[2] Department of Science Education-Biology, University of Haifa-Oranim, Tivon, 36006, Israel

Corresponding author:

Rachel Ben-Shlomo, Science Education-Biology, University of Haifa-Oranim,

Tivon, 36006, Israel

E-mail: ekly@research.haifa.ac.il

Tel: +972-4-9838921

Fax: +972-4-9832167

* Present address: Center for Translational Genetics, Bruce Rappaport Faculty of Medicine, Technion, Israel Institute of Technology, Haifa, Israel

ABSTRACT

Pistacia lentiscus L. (Anacardiaceae) is a common shrub in the Mediterranean vegetation. Throughout its distribution area this species is the obligate host of the kidney-shaped gall-forming aphid *Aploneura lentisci* (Homoptera: Fordinae). Despite the wide distribution of *Pistacia lentiscus* in diverse habitats along a climatic gradient in Israel, our previous study did not reveal any pattern of ecologically related genetic differentiation. Here we examined changes in gall density and genetic diversity of the gall forming aphid *A. lentisci* along its distribution gradient in Israel and in the neighboring island of Cyprus. Gall density and mass differed significantly among the Israeli populations and were positively correlated with annual precipitation and plants annual growth. Gene diversity of *A. lentisci* was very low in all populations and, as with its host plant, the genetic similarity was high within and among the Israeli populations and between the Israeli and Cyprian populations. To conclude, we found no evidence for any gene flow barrier and genetic differentiation among the aphids' populations.

Key words: Genetic diversity, gene flow, gall-forming aphid, *Pistacia*, plant–insect interactions

INTRODUCTION

Pistacia lentiscus L. (Anacardiaceae) is a typical thermophylic, evergreen and dioecious shrub in the Mediterranean vegetation, reaching an altitude of up to 500 meters. This lowland shrub is widely distributed throughout the Mediterranean region in southern Europe, the Levant and North Africa (Zohary, 1952). Such a wide distribution may well promote local adaptations, isolation and speciation. Indeed, genetic analysis of *P. lentiscus* samples from various locations throughout the Mediterranean basin showed some geographical differentiation (Barazani *et al.*, 2003). In Israel, *P. lentiscus* grows in diverse habitats along the North-South and West-East climatic and aridity gradients of increasing temperatures and decreasing precipitation. Differences in morphological and physiological (but not genetic) traits suggested ecotypic divergence, which enables *P. lentiscus* to cope with highly contrasting habitats in Israel (Shaviv, 1978). Shaviv (1978) described three distinct ecotypes: a) Mt. Gilboa ecotype, which is adapted to high temperatures and low humidity; b) Mt. Carmel ecotype, which is intolerant to high temperatures and is capable of growing slowly throughout the year; c) Coastal ecotype, which is tolerant to sea salt mist and has an extensive root system that improves water absorption in sandy soils. Using molecular tools (RAPD, AFLP; Nahum et al., 2008) we recently examined the occurrences of ecotypes in *P. lentiscus* in Israel. We found that gene diversity does not differ significantly between locations and there is no differentiation among Israeli populations. Gene flow estimates among all tested populations were high with no indication for isolation by distance. We found no pattern of ecologically related genetic differentiation; hence, the morphological and physiological differences probably reflect phenotypic plasticity (Nahum~~,~~ et al., 2008).

Throughout its distribution, *P. lentiscus* is the obligate host of the kidney-shaped gall-forming aphid *Aploneura lentisci* (Homoptera: Fordinae). Galls are formed on young leaflets in the spring by a single colonizing aphid (fundatrix), which later reproduces within the galls by parthenogenesis of wingless aphids. In the fall, the winged aphid generation emerges and disperses away from the galls (Wool and Manheim, 1986). The process of gall formation requires an intimate relationship between the aphid and its host plant (Weis et al., 198[illegible]. The fitness of the insect in the gall is highly influenced by the growing conditions of the host plant (Price, 1991). Indeed, Wool and Manheim (1988) reported significant differences in the reproductive success of *A. lentisci* growing in different habitats in Israel. Therefore, it is possible that the genetic structure of *A. lentisci* populations is influenced directly by habitat--specific abiotic factors and/or the typical traits of the host plant whether they are of genetic or environmental origin. In this study, we examined the genetic diversity of the gall forming aphid *A. lentisci* along its distribution gradient in Israel and in the neighboring island of Cyprus.

MATERIAL AND METHODS

Sampled populations

We sampled six natural populations of the gall forming aphid *A. lentisci*, representing the distribution range of *P. lentiscus* in Israel, and three additional populations in the island of Cyprus (Table 2). In each population, we collected galls and their aphid contents from six to nine shrubs. Additional four galls were collected in Kourbus, Cap Bon Peninsula, Tunisia, North Africa. The maximal distance between the Israeli populations was 165 km and between the Israeli and the Cyprian populations the maximal distance was about 500 km. The distance between Tunisia and Israel is about 2300 km and between Tunisia and Cyprus about 2000 km.

Gall density and reproductive success

As a measure of gall density, we counted the number of galls on a given shrub identified within two mi[illegible]. Gall density was measured for 10 shrubs in each population (total 90 shrubs). To obtain counting uniformity the same individual made all counts of all shrubs in all populations. In *A. lentisci* galls, gall mass is significantly correlated with the number of the enclosed aphids (Wool and Manheim, 1988), and thus gall mass can be used as a reliable measure of aphid fitness. We randomly collected 10 galls per shrub and 20 shrubs in all six Israeli population (total of 1200 ga[illegible] and weighed their dry mass. The aphids were extracted from the dissected galls that then were oven- dried (70^{o}C, 7 days) and weighed.

Correlative *Pistacia lentiscus* traits

The morphological and physiological measurements of *Pistacia lentiscus* traits are described in detail in Nahum et al. (2008). We measured the annual growth of 10 randomly selected young branches and pre-dawn water potential (using Pressure Chamber Instrument Model 600) in three to six randomly selected shoots of all sampled shrubs of all Israeli populations.

Genetic analysis

We extracted *A. lentisci* DNA using DNeasy Tissue Kit (Qiagen, Hilden, Germany) with minimal modifications. The genetic studies included amplified fragment length polymorphism (AFLP) fingerprint fragment analysis, and sequence analysis of 590 bp of the nuclear elongation factor 1-alfa (EF-1) and 983 bp of the mitochondrial genes cytochrome oxidase I and II (COI and COII).

***AFLP analysis*:** The AFLP method was carried out essentially as described by Vos et al. (1995). High quality genomic DNA (~200 ng) was digested with a pair of restriction enzymes (*Eco*RI/*Mse*I) at 37^{o}C for 4 hours, then ligated to double stranded *Eco*RI (E-) and *Mse*I (M-) adaptors. The resulting fragments were preamplified with nonselective primers, where the ligated adaptors served as target sites for primer annealing. Four selective primer combinations were used for AFLP amplification (Table 1). The selective *Eco*RI (E-) primers were labeled with florescent dye (6-Fam, Vic, Ned and Pet). PCR reactions were carried out in a total volume of 13 μl. PCR amplification cycles started at annealing temperature of 65^{o}C, after which the annealing temperature was lowered by 0.7^{o}C per cycle for 12 cycles (a touch- down phase of 13 cycles),

followed by 23 cycles at annealing temperature of 56^{o}C. Amplification products were visualized under a Florescence-Reader (Applied Biosystem). Allele identification and genotyping were determined directly from the chromatographs using Genotyper software (Applied Biosystem).

***Nuclear EF-1 and Mitochondrial COI and COII sequencing*:** Fragments of each of the nuclear gene EF-1 and the mitochondrial genes COI and COII were amplified by PCR in a total volume of 13 μl and annealing temperature of 57^{o}C. The primers used for each gene are presented in Table 1. PCR products were purified (QIAquick columns – Qiagen) and sent for direct sequencing.

Genetic data analysis

AFLP analysis: Amplification products were scored as discrete character states (present/absent) and transformed into band frequencies. Diversity values were based on phenotype frequency (phenotypes defined by the band patterns produced by individual primer pairs). Data were analyzed by Tools for Population Genetic Analyses (TFPGA) software version 1.3 (Miller, 1997) and by GenAlEx (Peakall and Smouse, 2006). These programs consider AFLP bands as diploid-dominant markers in which the estimated allele frequencies are based on the square root of the frequency of the null (recessive) genotype. Population differentiation was tested by exact tests (1000 dememorization steps, 10 batches, 2000 permutations per batch: Raymond and Rousset 1995). Molecular analysis of variance (Nested AMOVA) was conducted using GenAlEx (Peakall and Smouse, 2006).

***Sequence analysis*:** All chromatographs were checked manually for their integrity. Heterozygotes of the nuclear gene EF-1 were clearly visualized and identified by the chromatographs. Sequences of every locus separately were aligned using the program ClustalX 1.81 (Thompson *et al.*, 1997). Following verification of each locus, we analyzed the combined sequences of CO genes. Neighbor Joining Trees were calculated by NJPLOT (part of ClusatalX 1.81; 1000 bootstrap trails). As outgroup we used the published sequences of *Slavum wertheimae* and *Baizongia pistaciae* (related aphid species that form galls on *P. atlantica* and *P. palestina,* respectively). Molecular analysis of variance (Nested AMOVA) was conducted using GenAlEx (Peakall and Smouse, 2006).

RESULTS

Gall density and weight

Gall density and mass differed significantly among the Israeli populations (One-way ANOVA, $F_{5,94}=24.175$, $P<0.01$ and $F_{5,90}=75.64$, $P<<0.01$, respectively; Fig. 1). Both galls density and mass were lowest in the relatively xeric sites (Bet Guvrin and Mt. Gilboa) and highest in the humid site (Mt. Carmel). Gall mass and density were positively correlated with plants' annual growth ($r^2=0.836$, $F=24.98$, $p<0.05$ and $r^2=0.702$, $F=9.464$, $p<0.05$, respectively). Gall mass was also significantly correlated with annual precipitation ($r^2=0.748$, $F=11.859$, $P<0.05$) and with *P. lentiscus* water potential (Mpa; $r^2=0.815$, $F=17.58$, $p<0.05$).

Genetic analysis

Parthenogenetically reproducing aphids from 49 galls representing six populations from Israel, 23 galls from three populations in Cyprus, and 4 galls from one population in Tunisia were analyzed by AFLP. The four selective primers pair combination yielded 80 loci. The levels of polymorphism and gene diversity for each population are summarized in Table 2. The frequency of polymorphic loci and the level of gene diversity (He) were relatively low for all tested populations, however, the Israeli populations exhibited the lowest level (P=13.75-21.25%; He=0.06-0.09), the Cypriots populations expressed an intermediate level (P=16.25-23.75%; He=0.08-0.11), and the Tunisian aphid population showed the highest level (P=33.75%; He=0.16), although only four galls were analyzed. The genetic differentiation among populations is presented in Table 3. Pairwise analysis (exact tests: Raymond and Rousset, 1995) showed no differentiation between populations. The Israeli and the Cypriot populations were similar (p = 1.00; Table 3). The genetic identity values between populations were high (Table 3, below diagonal), and the identity levels within the Israeli population, within the Cypriot populations as well as between the Israeli and the Cypriot populations were similar (I=0.92-0.98). The identity values between both the Israeli and the Cypriot populations and the Tunisian populations were slightly lower (I=0.80-0.85). The resultant dendogram is presented in Fig. 2A. Analysis of molecular variance (AMOVA, Fig. 2A) revealed that most of the calculated variance (70%) was within populations.

Sequence analysis of the nuclear gene EF-1 and the mitochondrial genes COI and COII also showed high similarity between populations. Among the 590 bp of the nuclear EF-1 gene only eight single nucleotide polymorphism (SNPs) were found. No

characteristic mutation for any of the populations or regions was found, and all sampled individuals from all regions and populations were clustered together. AMOVA revealed that 95% of the variance was within individuals and only 5% between populations (Fig. 2B). Similar results are evident for the 983 bp of the mitochondrial COI and COII genes (Fig. 2C). The variability in the mtDNA was very low and only five SNPs were found (four in the COI gene and one in COII), of which none is specific to a population or region. AMOVA revealed that 95% of the variance was within individuals and only 5% among regions.

DISCUSSION

The level of genetic variation

The Mediterranean region comprises a wide range environmental heterogeneity, with its eastern part (Israel and Cyprus) characterized by high temperature and low precipitation. *P. lentiscus* shrubs are widely distributed in these variable habitats. Plant morphology is significantly correlated with local environmental conditions, especially with annual precipitation and temperature; however, no genetic differentiations was found among Israeli populations nor between Israeli and Cyprian populations, and morphological differences appear to stem from phenotypic plasticity (Nahum et al., 2008).

The Fordini galling aphids are adapted to their particular *Pistacia* host plant (Wool, 2004). Our results indicated that gall traits (mass and density) are correlated with plants' morphology and are significantly associated with environmental conditions.

Gall density and mass were lowest in populations of xeric sites and highest under humid conditions (Fig. 1). These results are consistent with earlier findings by Wool and Manheim (1988) and generally support the plant vigor hypothesis (Price 1991) suggesting that vigorously growing plants (in better habitats) support larger populations of gall formers.

The north to south and west to east geographical gradients in Israel represent parallel wet to arid environmental gradients. Genetic diversity is expected to increase under stressful conditions and high environmental heterogeneity (Nevo *et al*., 1984; Linhart and Grant, 1996; Nevo, 2001). It is therefore expected that aphids' gene diversity would increase in association with increasing aridity along the environmental gradients. Our results showed that gene diversity in general was very low in all populations (Table 2) and the small differences in level of gene diversity among populations contradict the above-mentioned hypothesis. We found no evidence for any differences in either expected heterozygosity or polymorphism level (Table 2) that are associated with environmental conditions within Israel. The two populations growing under relatively stressful arid conditions of low annual rainfall and high temperatures (Bet Guvrin in the south and Mt. Gilboa in the north-east) showed the lowest values of gall density and mass (Fig. 1); however, the Bet Guvrin population exhibited the lowest and Mt. Gilboa the highest values of genetic diversity (He=0.06 and 0.09,respectively). Thus, in agreement with our previous results regarding the genetic diversity of the host plant *P. lentiscus* in Israel (Nahum et al., 2008), the genetic variability of the aphid *A. lentisci* is not influenced by abiotic habitat factors nor by the phenotypic characteristics of their host plant.

Genetic differentiation between populations

Environmental heterogeneity may underlie spatial differentiation between populations by creating barriers for gene flow (Linhart and Grant, 1996). The dispersal abilities of *A. lentisci* are mostly of the weak--flying insect type and thus are supposed to intensify differentiation. However, we did not find any pattern of ecologically related genetic differentiation; most of the molecular variance was evident within populations, and the samples from Israel, Cyprus and Tunisia were clustered together (Fig. 2). The clusters, originating from sequencing of 1573 bp of nuclear and mitochondrial genes (Fig. 2B and C), do not express separate groups, and analysis of variance (AMOVA Peakall and Smouse, 2006) revealed that 95% of the variance was located within individuals (nuclear EF-1 gene) or within populations (mitochondrial COI and II genes).

The AFLP fingerprint ~~technique~~ is considered a more sensitive technique for assessing population differentiation (Bensch and Akesson, 2005). Indeed, AFLP fingerprint clustered the samples from Tunisia (at a distance >2000 km) to a separate branch, and AMOVA showed that 19% of the variance appeared between regions. Nevertheless, the populations of Israel and Cyprus are clustered together (Fig. 2A). Similarly, the genetic identity among the Israeli populations, among the Cypriot populations and between Israel and Cyprus was high (I>0.9; Table 3) and the similarity between these populations and *A. lentisci* aphids from Tunisia, situated >2000 km apart, was slightly lower (I>0.8; Table 3). Exact test for pairwise comparison for population differentiation indicated lack of isolation.

The lack of differentiation among *A. lentisci* populations, despite being separated by thousands of kilometres, contradict results of genetic analyses of other

species of the Fordini galling aphids. Gene diversity of *Baizongia pistaciae*, form galls on *P. palestina* trees, showed aphids' differentiation among sites and even among trees that are separated by 150 m (Martinez et al., 2005). Cluster analysis of the galling aphid *Slavum wertheimae* form galls on *P. atlantica* showed differentiation between the south and the north of Israel (~200 km; Avrani, Ben-Shlomo and Inbar, unpublished data). The lack of differentiation over long distances characterizing *A. lentisci* is sustained by extensive gene flow among distant populations by yet unknown mechanism.

Gene diversity in Cyprus island populations

Island populations are expected to harbor lower genetic variation in comparison with their mainland counterparts (reviewed in: Frankham, 1997). Such loss of diversity is explained by small population size, recurrent demographic bottleneck events, and restricted external gene flow. Among insects this phenomenon was found most prevalent between different species of *Drosophila*, the meadow spittlebug *Philaenus spumarius*, the burying beetles *Nicrophorus americanus* and *N. orbicollis* (summarized in: Frankham, 1997), and the bumble bee *Bombus terrestris* (Estoup et al., 1996). However, our results are in disagreement with these theoretical assumptions and findings. We previously showed that the genetic variability of the host shrub *P. lentiscus* is similar in Cyprus and in the Israeli mainland (Nahum et al., 2008). In the current study we found that AFLP fingerprint genetic diversity of the galling aphids in Cyprus was slightly higher (He = 0.097±0.015) than the level in Israel (He = 0.077±0.014; Table 2; p=0.197, Mann-Whitney). At present, we do not have any good explanation for the different genetic patterns of the host plant and its gall-forming aphid.

Conclusions

As with their host plant, we found no evidence for genetic differentiation among the aphid populations in Israel. Such lack of distinct genetic structure may have several non-exclusive explanations: 1. Host plants are genetically similar. 2. The climatic differences are not sharp enough to favor local genetic adaptations. 3. High dispersal ability of the winged aphids may bridge large geographical distance. 4. Existing aphid populations on non-*Pistacia* hosts (secondary hosts) may promote gene flow among distinct aphid populations.

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Table 1:

Primes used in the genetic analyses

A. AFLP analysis

E-ACA	5'- GACTGCGTACCAATTCACA – 3'	- 6-Fam - Blue
M-CAC	5'- GATGAGTCCTGAGTAACA C – 3'	
E-ACG	5'- GACTGCGTACCAATTCACG – 3'	- Ned - Yellow
M-CTT	5'- GATGAGTCCTGAGTAACT T – 3'	
E-ACT	5'- GACTGCGTACCAATTCACT – 3'	- Vic - Green
M-CAA	5'- GATGAGTCCTGAGTAACAA – 3'	
E-ACC	5'- GACTGCGTACCAATTCACC – 3'	- Pet - Red
M-CTG	5'- GATGAGTCCTGAGTAACTG – 3'	

B. Nuclear gene sequencing

EF-1-F	5'- CCCGGACACAGAGATTTCAT – 3'
EF-1-FR	5'- TTTCCACTCGTCCTATGGG – 3'

C. Mitochondrial genes sequencing

COI-F	5'- TGGTACAGGAACAGGATGAACA -3'
COI-R	5'- TGCTCAAATAATTGATGGGGA -3'
COII-F	5'- AAGATTTCAAAATAGAAATTCACCTC -3'
COII-R	5'- CGTCCTGGGATTGCATCTAT -3'

Table 2:

Gene diversity (Unbiased heterozygosity, He) and percent polymorphic loci (P; 95% criterion) of the sampled *A. lentisci* populations in Israel, Cyprus and Tunisia

Population	**N**	**He**	**P (%)**
Kziv River	8	0.06	13.75
Turan	8	0.08	20.00
Mt. Carmel	9	0.08	18.75
Mt. Gilboa	7	0.09	21.25
Hadera	8	0.09	20.00
Bet Guvrin	9	0.06	16.25
Polis (9)	6	0.11	23.75
Kivisili	9	0.10	23.75
Aphrodite Rock	8	0.08	16.25
Tunisia	4	0.16	33.75

Table 3:

Genetic differentiation among populations: Combined probabilities for each pairwise comparison (Exact tests for population differentiation; above diagonal) and genetic identity (below diagonal)

	Kziv River	Turan	Carmel	Mt. Gilboa	Hadera	Bet Guvrin	Kivisili	Aphrodite	Polis	Tunisia
Kziv River	*****	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.1180
Turan	0.9600	*****	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.2070
Carmel	0.9577	0.9552	*****	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.2820
Mt. Gilboa	0.9497	0.9658	0.9502	*****	1.0000	1.0000	1.0000	1.0000	1.0000	0.8860
Hadera	0.9663	0.9560	0.9687	0.9753	*****	1.0000	1.0000	1.0000	1.0000	0.7010
Bet Guvrin	0.9667	0.9618	0.9786	0.9554	0.9711	*****	1.0000	1.0000	1.0000	0.1090
Kivisili	0.9702	0.9573	0.9554	0.9532	0.9585	0.9677	*****	1.0000	1.0000	0.2780
Aphrodite	0.9750	0.9698	0.9701	0.9692	0.9756	0.9777	0.9769	*****	1.0000	0.4420
Polis	0.9311	0.9136	0.9235	0.9135	0.9255	0.9387	0.9364	0.9325	*****	0.9990
Tunisia	0.7945	0.7891	0.7997	0.8245	0.8073	0.7906	0.8240	0.8055	0.8390	*****

Figure legend:

Fig. 1

Average (± SE) gall mass and density on *P. lentiscus* shrubs in Israel. Columns with common letters are not significantly different (Duncan Post-hoc test, $p<0.05$).

Fig. 2

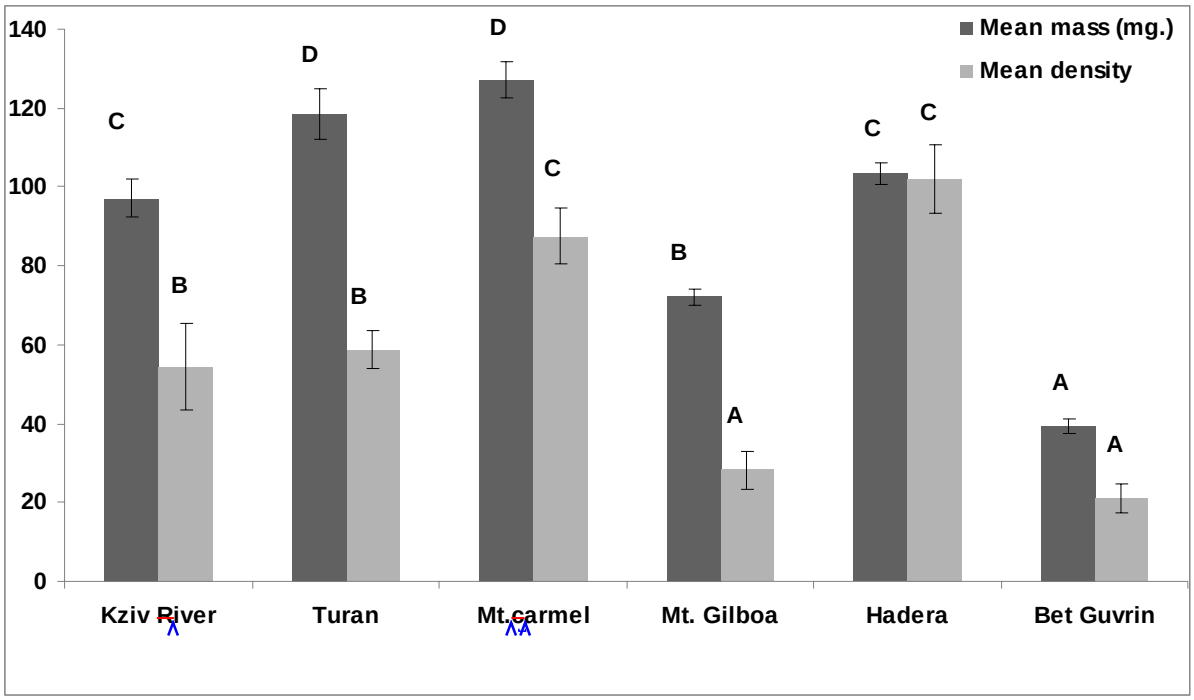
Genetic differentiation among *A. lentisci* populations. Dendogram based on Nei's (1978) unbiased genetic distance among populations, and molecular analysis of variance (Nested AMOVA) partitioned among regions, among populations, within populations and within individuals (I- stands for Israel, C- for Cyprus and T- for Tunisia).

A. AFLP analysis

B. The nuclear EF-1 gene sequencing

C. The mitochondrial COI and COII gene sequencing

Figure 1


140
120
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Mean mass (mg.)
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C
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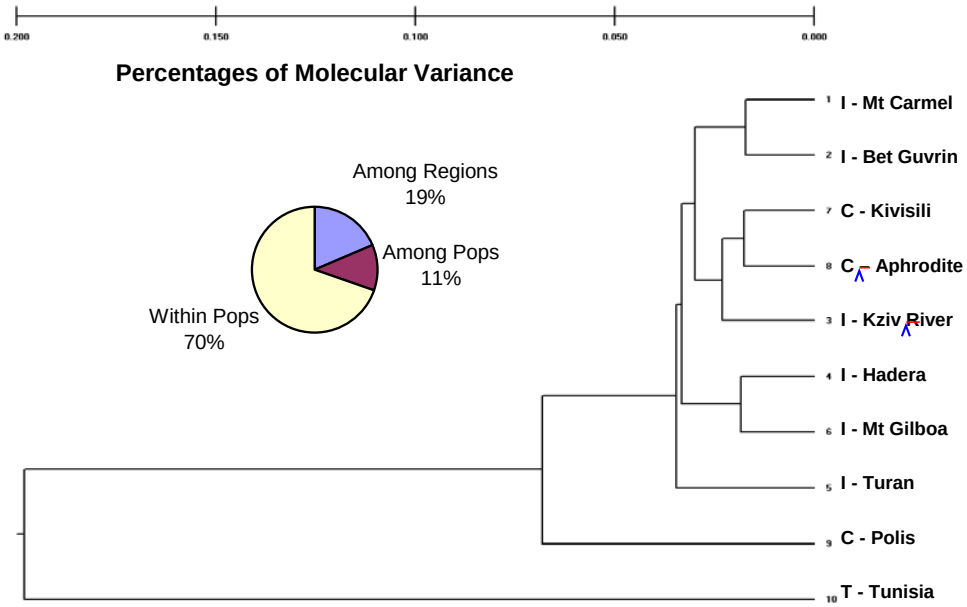
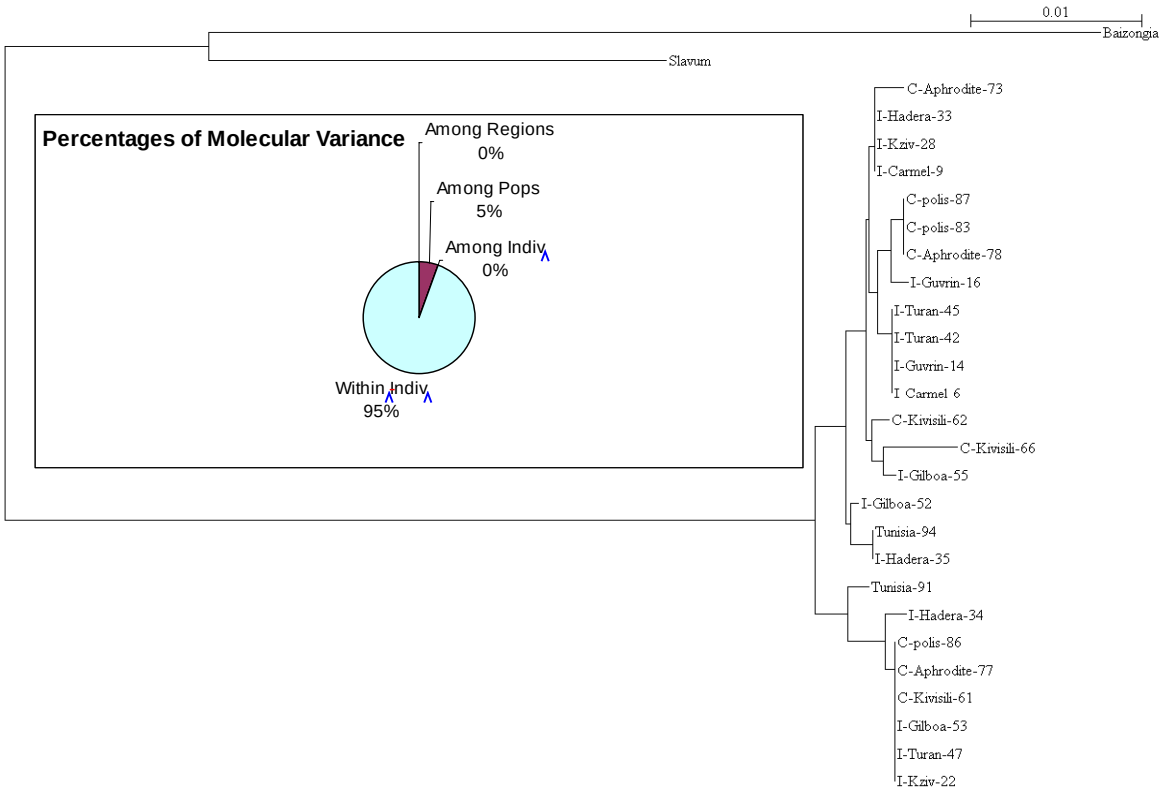
Kziv River
Turan
Mt.carmel
Mt. Gilboa
Hadera
Bet Guvrin

Figure 2

A. AFLP



B. EF-1


0.01
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Among Pops
5%
Among Indiv
0%
Within Indiv
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C-Aphrodite-73
I-Hadera-33
I-Kziv-28
I-Carmel-9
C-polis-87
C-polis-83
C-Aphrodite-78
I-Guvrin-16
I-Turan-45
I-Turan-42
I-Guvrin-14
I Carmel 6
C-Kivisili-62
C-Kivisili-66
I-Gilboa-55
I-Gilboa-52
Tunisia-94
I-Hadera-35
Tunisia-91
I-Hadera-34
C-polis-86
C-Aphrodite-77
C-Kivisili-61
I-Gilboa-53
I-Turan-47
I-Kziv-22

C. COI and COII