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**Mitochondrial and nuclear diversity in the green oak leaf roller *Tortrix viridana* L. (Lepidoptera, Tortricidae) from Sardinia**

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The Green oak leaf roller *Tortrix viridana* is one of the most harmful pest of the Palearctic oaks. The young larvae of *T. viridana* develop inside the flushing buds and their egg hatch is synchronized with the bud burst of the oaks. The aim of the study was to investigate the genetic structure and variation of mitochondrial and nuclear loci in Sardinian populations associated with deciduous (*Quercus pubescens*) and evergreen (*Q. suber* and *Q. ilex*) oaks. We performed multilocus analyses (COI, COII mitochondrial genes and eight microsatellites) in about two hundreds individuals collected on all the three oak species coming from the most important forest districts in Sardinia Island, Italy. The populations were also characterized by egg hatching, after comparison in semi-field tests for egg development. Eighty-five mitochondrial haplotypes were found, with three ones shared among all populations, except the most southern one. A high haplotype diversity ( $H = 0.90$ ) and a low nucleotide diversity ( $\pi = 0.003$ ) have been observed. Microsatellites were highly variable with alleles ranging from 4 to 56 for locus and polymorphic information content values varying from 0.510 to 0.945. AMOVA tests were performed by structuring samples in different groups (geographic districts/oak species/egg development). The highest values of Fixation indexes resulted grouping populations according to early or late egg hatching. Furthermore, we tested for sex-biased dispersal, estimating pairwise  $F_{ST}$ ,  $F_{IS}$ , and relatedness  $r$  at nuclear loci separately for the sexes. We produced evidence for non random patterns of gene flow probably mediated by male biased dispersal, with values significantly higher in females than in males and females the phylopatric sex. In support of a male biased dispersal, nuclear and mitochondrial markers provided different results in the matter of genetic structure, with mitochondrial loci, unlike microsatellites, highlighting significant differentiation levels among populations.

# Mitochondrial and nuclear diversity in the green oak leaf roller *Tortrix viridana* L. (Lepidoptera, Tortricidae) from Sardinia

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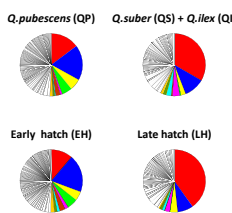
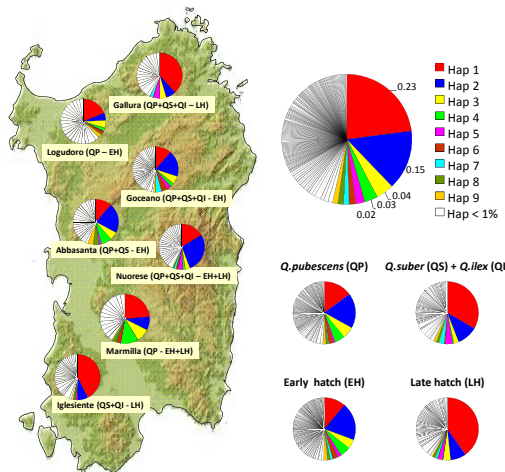
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The Green oak leaf roller *Tortrix viridana* is one of the most harmful pest of the Palearctic oaks. The young larvae of *T. viridana* develop inside the flushing buds and their egg hatch is synchronized with the bud burst of the oaks. The aim of the study was to investigate the genetic structure and variation of mitochondrial and nuclear loci in Sardinian populations associated with deciduous (*Quercus pubescens*) and evergreen (*Q. suber* and *Q. ilex*) oaks. The wide variability in budding within the different oak species determines the evolution of *T. viridana* populations which are adapted to a particular phenological type of oak and are characterized by different duration of summer diapause and hatching dates (Du Merle, 1999; Ivasov et al., 2002; Serra & Lentini, 2012).

We performed multilocus analyses in 362 individuals for COI, COII mitochondrial genes (total 2180 bp) and in 201 individuals for 8 microsatellites. Moths were collected in 24 localities with pubescent (QP), cork (QS) or holm oak (QI) of seven Sardinian forestry districts. Individuals were characterized for sex, geographic origin, host species, and early or late on the basis of the duration of the summer diapause and the hatching time of the eggs after comparison in semi-field tests for egg development (Serra & Lentini, 2012).

## Mitochondrial analysis



## Mitochondrial genetic differentiation and diversity

Tab 1: Number of samples (N), number of haplotypes (NH), number of polymorphic sites (S), nucleotide diversity ( $\pi$ ) and haplotypic diversity (H).

	TOT	Gallura	Goceano	Logudoro	Abbasanta	Iglesiente	Nuorese	Marmilla
N	362	42	70	37	52	66	61	34
NH	149	22	41	29	30	31	33	20
S	119	32	52	43	39	39	47	30
$\pi$	0.00398	0.0028	0.0055	0.0052	0.0038	0.0026	0.0038	0.003
H	0.9215	0.854	0.953	0.96	0.94	0.82	0.89	0.92

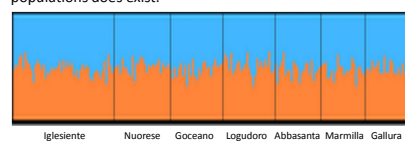


We found 149 haplotypes out of 362 collected specimens. Among them, two haplotypes (1, 2) showed a high frequency (23%, 15%, in red and blue respectively) and are present in all seven districts but with different frequency in each population. We found 140 haplotype with frequency <1%. The haplotype 1 (in red) was prevailing on evergreen (QI+QS) oaks populations in respect to deciduous (QP) population and in latter egg-hatching population in respect to early egg-hatching. The haplotype 2 (in blue) was prevailing in deciduous oak populations in respect to evergreen oaks populations and in early egg-hatching in respect to latter egg-hatching populations.

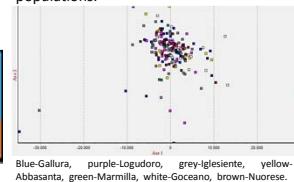
A high haplotype diversity ( $H = 0.92$ ) and a low nucleotide diversity ( $\pi = 0.004$ ) have been observed, with little differences across the observed populations (Table 1).

## Nuclear analysis

A Bayesian clustering method was used to examine population differentiation Applying STRUCTURE and the Evanno method to the entire data set, the best number of clusters given the microsatellite data was two. Although  $\Delta K$  procedure suggested that there were  $K = 2$  clusters, individual assignment probabilities to individual clusters were low and nearly uniform across clusters. This outcome indicates that the analysis procedure overestimated the true number of clusters and that a large admixture among populations does exist.



We used Factorial Correspondence Analysis (FCA) to examine genetic differentiation of multilocus genotypes. The analysis did not reveal any differentiation among populations that appeared clustered in an unique group. Outliers concern individuals from different populations.



## Genetic diversity at microsatellite loci

Tab 2: Number of samples (N), mean number of alleles (A), observed ( $H_o$ ) and expected heterozygosity ( $H_e$ ), inbreeding coefficient ( $F_{is}$ ) and its significance after 1000 permutations. Allelic richness (KAR) and private allelic richness (KPAR). In red significant values obtained after Bonferroni correction.

	TOT	Gallura	Goceano	Logudoro	Abbasanta	Iglesiente	Nuorese	Marmilla
N	201	30	23	26	23	50	27	22
A	30	3,3	15,1	14,6	15,1	19,6	15,1	14,2
$H_e$	0.85	0.83	0.82	0.82	0.83	0.84	0.83	0.84
$H_o$	0.69	0.70	0.77	0.78	0.81	0.66	0.72	0.66
$F_{is}$	0.19	0.14	0.13	0.22	0.21	0.22	0.15	0.23
KAR	5.40	5.40	5.27	5.27	5.52	5.41	5.35	5.54
KPAR	1.46	1.28	1.1	1.37	1.2	1.08	1.23	1.23
$F_{is}$ without null alleles	0.08	0.04	0.07	0.05	0.03	0.11	0.03	0.03

Microsatellites were highly variable with alleles ranging from 4 to 56 for locus and PIC (Polymorphic Information Content) values varying from 0.510 to 0.945. Two loci showed a high percentage of null alleles. Removing the two alleles from the analysis, all populations appeared at Hardy Weinberg equilibrium, with exception of Iglesiasiente that still showed a significant excess of homozygotes.

## AMOVA tests for mitochondrial data

Source of variation	Geographic districts		Evergreen and deciduous oaks		Early-late hatching populations	
	Percentage of variation	Fixation index	Percentage of variation	Fixation index	Percentage of variation	Fixation index
Among groups			14.32	$\Phi_{CT}=0.143$	21.32	$\Phi_{CT}=0.213$
Among populations within groups	4.13		9.86	$\Phi_{SC}=0.115$	6.22	$\Phi_{SC}=0.079$
Within populations	95.87	$\Phi_{ST}=0.041$	75.82	$\Phi_{ST}=0.242$	72.45	$\Phi_{ST}=0.275$

AMOVA tests were carried out grouping: seven geographic districts, evergreen vs deciduous oaks, egg development (early, late egg hatching insect population). The highest values of Fixation indexes resulted grouping populations according to early or late egg hatching. The global estimate of  $\Phi_{ST}$  from the mitochondrial data indicated that significant genetic structure existed. However, in comparison with the mitochondrial analysis, the microsatellite differentiation was generally small and reflected subtle differences in allele frequencies among populations.

## AMOVA tests for microsatellites data

Source of variation	Geographic districts		Evergreen and deciduous oaks		Early-late hatching populations	
	Percentage of variation	Fixation index	Percentage of variation	Fixation index	Percentage of variation	Fixation index
Among groups			0.16	$F_{CT}=0.002$	-0.01	$F_{CT}=-0.00008$
Among populations within groups	0.38		1.1	$F_{SC}=0.011$	1.19	$F_{SC}=0.01194$
Within populations	99.62	$F_{ST}=0.0038$	98.74	$F_{ST}=0.013$	98.81	$F_{ST}=0.01186$

	Gallura	Marmilla	Nuorese	Abbasanta	Goceano	Logudoro	Iglesiente
Gallura	0	0.00993	0.0391	0.04331	0.03819	0.0125	-0.00339
Marmilla	0.10243	0	0.01801	0.00418	0.00624	-0.00717	0.02362
Nuorese	0.1576	-0.00383	0	-0.00154	-0.00021	0.03846	0.05519
Abbasanta	0.11257	-0.02079	-0.006	0	-0.00474	0.00716	0.06041
Goceano	0.16273	-0.00618	-0.00968	-0.00437	0	0.00441	0.05711
Logudoro	0.1506	0.00112	-0.01169	0.00121	-0.00984	0	0.02794
Iglesiente	0.04142	0.27823	0.3272	0.28022	0.33232	0.32675	0

$F_{ST}$  values above diagonal;  $\Phi_{ST}$  values below diagonal

## Population Pairwise $F_{ST}$ , $R_{ST}$ and $\Phi_{ST}$

Significant  $\Phi_{ST}$  values (in red) were mainly obtained between Gallura/Iglesiente versus all other localities for mitochondrial data, although no significant divergence was detected among them. They appeared as the most differentiated populations, probably because they are characterized by a) late egg hatching *T. viridana* populations, and b) dominance of evergreen oaks.

Significant differences in allele haplotype frequencies were obtained among some other localities. Conversely, no significant values were obtained in the comparison of allele frequencies of microsatellite data both for  $F_{ST}$  and  $R_{ST}$ . This is probably due to the gene flow associated with male dispersal, which masks structure at nuclear traits (see sex dispersal rate).

	Gallura	Logudoro	Abbasanta	Iglesiente	Nuorese	Goceano	Marmilla
Gallura	0	0.03348	0.00355	0.00031	-0.00968	-0.01481	0.00758
Logudoro	0.00488	0	0.08852	0.03791	0.04614	0.02414	0.04555
Abbasanta	0.00511	0.00587	0	0.03702	0.00456	0.02014	0.01602
Iglesiente	0.00593	0.00296	0.00134	0	-0.00672	0.00216	-0.00295
Nuorese	0.00135	0.00203	-0.00154	-0.00028	0	-0.00189	-0.00554
Goceano	0.01405	0.00503	0.00074	0.00598	0.0031	0	0.01334
Marmilla	0.00306	0.00812	0.00769	0.00582	0.00303	0.00736	0

$F_{ST}$  values below diagonal,  $R_{ST}$  values above diagonal

## Sex dispersal rate

	Males	Females
$F_{ST}$	0.04	0.34
$F_{ST}$	0.008	0.120
R	0.090	1.100

We produced evidence for non random patterns of gene flow probably mediated by male biased dispersal, with values significantly higher in females than in males ( $p < 0.05$ ), showing females more phylopatric than males.

In support of a male biased dispersal, nuclear and mitochondrial markers provided different results: a genetic structure could be found in mitochondrial but not in microsatellites profiles.

## Dispersal

Many populations have not high proportion of not migrants (< 3.6% except Iglesiasiente). The population with a very high self recruitment is Iglesiasiente (0.966). The red high values corresponding to Iglesiasiente % of immigrant individuals from all populations but the high violet value means that it doesn't send individuals to the other populations. The dispersal rate is low and never is higher than 0.10 (with the exception of Iglesiasiente). It is not well understandable the reason of this predominant unidirectional migration to the southern forestry districts. However, Iglesiasiente is characterized by only evergreen oaks and late egg hatching moth populations. In addition, climatic conditions, like the predominant mistral wind that drives towards the Island, and an ecological barrier consisting of a strip of about 20km without oaks between Iglesiasiente and the others districts, may limit dispersal along the S-N direction.

	0	1	2	3	4	5	6
m[0][0]:	0.6879	m[0][1]: 0.0365	m[0][2]: 0.0367	m[0][3]: 0.1596	m[0][4]: 0.0366	m[0][5]: 0.0363	m[0][6]: 0.0364
m[1][0]:	0.0114	m[1][1]: 0.6787	m[1][2]: 0.0114	m[1][3]: 0.2649	m[1][4]: 0.0112	m[1][5]: 0.0112	m[1][6]: 0.0112
m[2][0]:	0.0124	m[2][1]: 0.0127	m[2][2]: 0.6794	m[2][3]: 0.2586	m[2][4]: 0.0124	m[2][5]: 0.0123	m[2][6]: 0.0122
m[3][0]:	0.0055	m[3][1]: 0.0059	m[3][2]: 0.0058	m[3][3]: 0.9666	m[3][4]: 0.0055	m[3][5]: 0.0054	m[3][6]: 0.0053
m[4][0]:	0.0109	m[4][1]: 0.0113	m[4][2]: 0.0108	m[4][3]: 0.2672	m[4][4]: 0.0109	m[4][5]: 0.0110	m[4][6]: 0.0109
m[5][0]:	0.0118	m[5][1]: 0.0120	m[5][2]: 0.0119	m[5][3]: 0.2616	m[5][4]: 0.0118	m[5][5]: 0.0119	m[5][6]: 0.0118
m[6][0]:	0.0125	m[6][1]: 0.0132	m[6][2]: 0.0125	m[6][3]: 0.2573	m[6][4]: 0.0123	m[6][5]: 0.0125	m[6][6]: 0.0125

0=Gallura, 1=Logudoro, 2=Abbasanta, 3=Iglesiente, 4=Nuorese, 5=Goceano, 6=Marmilla  
The numbers in violet are the % of individuals not migrant.

Results suggested that genetic differentiation among Sardinian populations of *Tortrix viridana* are associated with egg development and, consequently, with bud burst phenology of different oak populations. In addition, geographic origin, latitude and altitude contribute to differentiate late-early egg hatching populations on the same oak species, with a genetic structure more identifiable at mitochondrial loci than on nuclear traits.

Accordingly with ecological studies that described females as badly flyers and stationary and males as good flyers (Du Merle & Pinguet, 1982), differences in structure of nuclear traits associated to sex indicated that green oak leaf rollers are characterized with a sex male-biased dispersal.

