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Research Article

Patterns of genetic variation among host-plant associated populations of the green oak leaf roller moth, *Tortrix viridana* (Lepidoptera: Tortricidae) in oak forests of northwestern Iran

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Abstract
The green oak leafroller moth, *Tortrix viridana* Linnaeus, 1758 (Lep. Tortricidae) is one of the key pests on *Quercus* spp. The larval instars of the moth feed on the developing buds and the leaves and cause oak trees to be leafless completely, significantly damaging the oak forest of northwestern Iran each year. The genetic differences in the host plant associated populations of oak leafroller moth have been assessed in the oak forests of northwestern Iran using inter-simple sequence repeat markers (ISSR). In this study 171 specimens representing nineteen populations feeding on various oak tree species including *Quercus brantii* Lindl., 1840, *Quercus infectoria* Oliv., 1801 and *Quercus libani* Oliv., 1801 were investigated. Results indicated that populations related to *Q. libani* had the highest genetic diversity, while populations related to *Q. brantii* and *Q. infectoria* had lower genetic diversity. Assessment of genetic structure of the populations showed that higher diversity was demonstrated in intrapopulations (92%) than interpopulations (8%) geographically, while strongly significant host-associated differentiation was also indicated by Approximate Bayesian Computation analyses among populations, with major clusters corresponded to the three species of host plants. These findings revealed that the host plant association can have a stronger effect on the genetic variations among the populations of *T. viridana* than the geographical barriers.
Key words: Defoliator moth, Interpopulation, Intrapopulation, ISSR, *Quercus*

الگوی تنوع‌ژنتیکی جمعیت‌های شب‌پره جوانه‌خوار بلوط *Tortrix viridana* (Lepidoptera: Tortricidae) در جنگل‌های بلوط شمال‌غرب ایران

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چکیده

شب‌پره جوانه‌خوار بلوط *Tortrix viridana*, Linnaeus, 1758 (Lep. Tortricidae) یکی از آفات مهم گونه‌های مختلف جنس بلوط .*Quercus* spp می‌باشد. لاروهای سنین مختلف این آفت از جوانه‌های در حال رشد و برگ‌های درختان بلوط تغذیه می‌نمایند و باعث بی‌برگی کامل درختان مورد نظر می شوند. در این تحقیق، تفاوت ژنتیکی پروانه جوانه‌خوار بلوط با استفاده از مارکرهای ISSR براساس میزبان‌های مختلف آن در جنگل‌های بلوط شمال‌غرب ایران مورد مطالعه قرار گرفت.

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۱۹ جمعیت شامل ۱۷۱ فرد که از سه گونه بلوط شامل *Quercus brantii* Lindl., 1840, *Quercus infectoria* Oliv., 1801 و *Quercus libani* Oliv., 1801 جمع آوری شـده بودند، مورد بررسـی قرار گرفت. نتایج نشـان داد که جمعیت های جمع آوری شده از گونه بلوط *Q. libani* دارای بیشترین تنوع‌ژنتیکی بودند، در حالیکه جمعیت‌های جمع‌آوری شده از دو گونه دیگر بلوط *Q. brantii* و *Q. infectoria* تنوع‌ژنتیکی کمتری را دارا بودند. بررسی ساختار ژنتیکی جمعیت‌های جمع آوری شده نشان داد که از نظر جغرافیایی تنوع‌ژنتیکی در درون جمعیت ها (۹۲ درصد) بسیار بیشتر از بین جمعیت‌ها (۸ درصد) می‌باشد، درحالیکه بررسی اثر گونه‌های مختلف بلوط بر تنوع‌ژنتیکی آفت موردنظر براساس روش تخمین بیزین نشان داد که جمعیت‌های جمع آوری شده از سه گونه مختلف بلوط در سه کلاستر مشخص (براساس سه گونه میزبان بلوط مورد نظر) قرارگرفتند. این یافته‌ها نشان از تخصص میزبانی معنی‌دار جمعیت‌های جمع‌آوری شده دارد و اینکه میزبان های مختلف آفت مذکور، بیشـتر از فواصـل و موانع‌جغرافیایی، تنوع‌ژنتیکی جمعیت‌های موردمطالعه را تحت‌تاثیر قرارداده اند.

واژه‌های کلیدی: بلوط، برون جمعیت، درون جمعیت، شب پره برگخوار، ISSR

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Introduction

Tortrix viridana, Linnaeus, 1758 (Lep. Tortricidae), is a significant forest pest and its natural distribution in the west Palearctic region including Iran, Turkey, Europe, Russia, and the Near East (Fazeli & Abai, 1990; Hunter, 1990; Markov, 1993; Kalapanida & Glavendekic, 2002; Ghobari *et al*., 2007; Schroeder & Degen, 2008a). The pest is oligophagous, mainly feeding on the species of the genus *Quercus* (Hunter, 1990). The early larval instars feed on the developing buds, while the late larval instars feed on the leaves (Du Merle, 1999). In western provinces of Iran, where the oak forest dominates, the periodic outbreaks of *T. viridana* pose a severe risk (Fazeli & Abaei, 1990; Ghobari *et al*., 2007; Yazdanfar *et al*., 2015a; Yazdanfar *et al*., 2015b). Therefore, understanding the biology of the outbreak is of the utmost importance to the forestry sectors of this region.

Genetic variations in *T. viridana* populations have been well-shown to be associated with different species of *Quercus*, and their geographical distribution (Simchuk *et al*., 1999; Du Merle, 1999; Schroeder & Degan, 2008a; Serra *et al*., 2014; Serra *et al*., 2019). An initial study by Simchuk *et al*. (1999) showed the effects of genetic patterns on the population dynamics of *T. viridana* using isozyme loci Est-4 and Pts-4 loci. Their results revealed the heterozygosity of the loci on the outbreak or depression phases of *T. viridana*. Schroeder & Degan (2008b) assessed the genetic variation of *T. viridana* and one of its hosts, *Q. robur*, and they found that the host shows different patterns of gene variation regarding the type of molecular markers used. They also found that the mitochondrial and AFLP markers indicated a high genetic variation within populations of *T. viridana*. Schroeder *et al*. (2010) showed that genetic difference among the isolated populations of *T. viridana* was unexpectedly higher than within the non-isolated ones. They further showed that the bottleneck effect did not decrease the genetic variation of the pest. Their results suggest that balancing natural selection causes unexpectedly high genetic variation among the populations of *T. viridana*. In the latest research by Serra *et al.* (2019), the genetic structure of *T. viridana* was studied

in relation to geographical distances and host phylogeny using COI and COII mitochondrial genes and eight nuclear microsatellites. Their results indicated that oak budburst phonologies affected the mitochondrial variation of *T. viridana* and the long distances separated populations of the pest.

Inter Simple Sequence Repeats (ISSRs) among different markers, have some beneficial characteristics for use in analyzing a population's genetic diversity, including low cost, technical simplicity, reliability, repeatability and independence of any prior DNA sequence information (Nagaraju *et al*., 2001; Luque *et al*., 2009). ISSRs are widely used for studying genetic variations of different species of moths (Vijayan, 2005; Roux *et al*., 2007).

Since *T. viridana* shows ecological and genetic adaptations to different hosts within the genus *Quercus*, this can form a basis for outbreak events and the oak's decline (Du Merle, 1999; Schroeder & Degan, 2008b). Therefore, knowledge of genetic variation among populations of this pest inhabiting various host species can help manage its outbreak in the oak forests. Ghiasoddin *et al* (2020) assessed the population genetics of oak leaf roller moth, by ISSR and RAPD in Mariwan (Kurdistan, NE Iran). In this study, we investigated a much wider area by considering host tree (*Q. libani*, *Q. infectoria*, *Q. brantii*) using ISSR marker. This study aims to determine the genetic variations within and among populations of *T. viridana* affecting the oak forests of northwestern Iran and further identify the existence of potentially different sympatric races feeding on other host plants of the *Quercus* species.

Materials and methods

Sample collection

171 fifth instar larvae of *T. viridana* were gathered at ten sites in an area of approximately 17500 km^2, stretching from Kurdistan province to western parts of the West Azerbaijan province, northwestern Iran (Figure 1). At each site, nine individuals (fifth instar larvae) related to each oak host species, *Q. brantii*, *Q. infectoria* and *Q. libani* were collected (Table 1). The three mentioned oak species are native to the oak forests of northwestern of Iran, although the distribution of *Q. libani* is much more limited than the other two species, therefore the limited distribution of *Q. libani*, samples from the host could only be gathered from three sites.

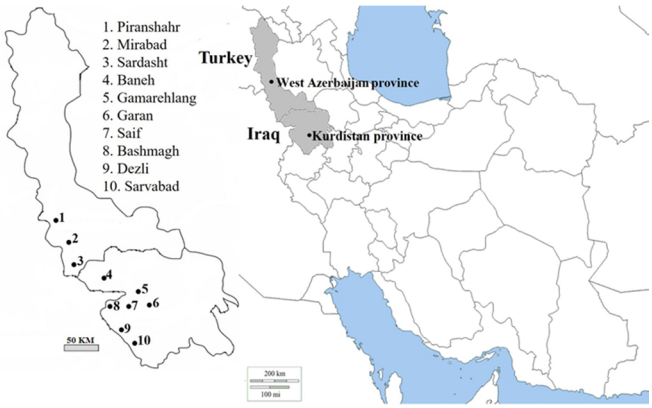

Fig. 1. Map of sampled sites in the forests of Kurdistan and West Azerbaijan provinces (northwestern Iran).

The selected oaks were chosen 10 m apart to decrease the probability of larvae migrating among the crowns of trees. To ensure that the collected individuals related to each of the mentioned three species of host and the ease of rearing, *T. viridana* fifth (final) instar larvae were sampled. The collected larvae were reared on the oak leaves to adults under room condition in laboratory of Department of Plant Protection, University of Kurdistan.

Table 1. Geographical coordinate of 10 sampled sites and the type of host species in each site in the forests of Kurdistan and west Azerbaijan provinces (northwestern Iran).

Collection site	Longitude Latitude	ID	Host species *Q. brantii* B	 *Q. infectoria* I	 *Q. libani* L
Piranshahr	36° 20′ 24″N 45° 25′ 24″E	1	B1	I1	L1
Mirabad	36° 29′ 09″N 45° 18′ 18″E	2	B2	I2	
Sardasht	36° 16′ 98″N 45° 50′ 57″E	3	B3	I3	
Baneh	36° 02′ 97″N 45° 93′ 70″E	4	B4		
Gomarehlang	36° 63′ 25″N 46° 29′ 24″E	5	B5	I5	L5
Garan	35° 32′ 01″N 46° 08′ 07″E	6	B6	I6	
Seif	35° 36′ 18″N 46° 08′ 07″E	7	B7	I7	
Bashmagh	35° 34′ 51″N 46° 07′ 15″E	8		I8	L8
Dezli	35° 38′ 07″N 46° 18′ 08″E	9	B9		
Sarvabad	35° 31′ 52″N 46° 36′ 58″E	10		I10	
Number of individuals per host			72	72	27
Total number of individuals				171	

DNA extraction

We ground the head and thorax of larva in liquid nitrogen to extract their total genomic DNA via a modified CTAB method (Dumolin *et al*., 1995; Schroeder & Degen, 2008a). The quality of extracted DNA was assessed using agarose gel electrophoresis and it's quantity determined by Eppendorf BioPhotometer Plus.

PCR

The ISSR markers amplify inter-microsatellite regions of mtDNA or nDNA without any prior sequence knowledge. Three primers out of 20 primers were used for PCR amplification based on band resolution, repeatability and polymorphism: ISSR1 (AG(8)C), ISSR2 (AG(8)G), ISSR3 (AG(8)T), and combined primers included ISSR4 (ISSR1+ISSR2), ISSR5 (ISSR1+ISSR3), ISSR6 (ISSR2+ISSR3). We carried out all PCR amplification with a 20 µL volume containing 10 ng of template DNA, 2 µL 10× PCR buffer, 1 µL (10 pmol) of primer, 1.5 mM of MgCl2, 0.75 U *Taq* DNA polymerase, dNTP (10 mM) 0.5 µLand ddH2O to the final volume in a PCR thermal cycler (Bio-Rad C1000-TM, USA). The amplification conditions were one cycle 94ºC for 5 min, 35 cycles included 94ºC for 45s, annealing at 50ºC for 45 s for all six used primers, and 72ºC for the 60s, and one cycle final extension at 72ºC for 10 min. We assessed the results' precision by considering each primer's negative control and band pattern replication. To analyze the PCR product, 5 µl of the product with 1 µl of 6X loading dye was loaded on 2% agarose gel in 1X TAE buffer (Tris/Acetic acid/EDTA) electrophoresis buffer. For visualization of DNA, we added SYBR safe stain to agarose gels, utilizing a gel documentation system (UVItec of Cambridge, UK).

Statistical Analyses

Using 259 multiple bands from 171 individuals, a presence-or-absence (1/0) matrix was prepared. Genetic variability within and among populations was evaluated using GenAlEx 6.502 software (Peakall & Smouse, 2006) in order to find the percentage of polymorphic bands (PP), band frequency [total band number (TBN)], number of different alleles (Na), number of effective alleles (Ne), expected heterozygosity (He) and Shannon's information index. Using Nei's genetic distance (Nei, 1978), we calculated the genetic relationships among populations with GenAlEx 6.502 software. We performed Principal Coordinates Analysis (PCoA) to visualize genetic relationships among all populations associated with the hosts. Based on a pairwise population matrix of Nei's genetic distance, we used analysis of molecular variance (AMOVA) to apportion genetic variance within and among populations that calculated by GenAlEx 6.502 software. Gene flow was calculated using the formula Nm (Number of immigrants) = (1- FST)/4FST (McDermott & McDonald, 1993) for discrete populations based on host plants. We further used the phylogenetic analysis to show genetic variation among the populations of *T. viridana* associated with different oak host species. We produced the phylogenetic tree based on the Bayesian method using MrBayes v3.2.7a (Huelsenbeck & Ronquist, 2001). Four simultaneous Monte Carlo Markov chains of 10,000,000 cycles were implemented and sampled at intervals of 100 generations. Each analysis did not consider the first 10,000 trees (as "burn-in"). Log-likelihood stability was achieved after 100,000 generations. Support for BI tree nodes was determined based on the values of Bayesian posterior probabilities. The final tree was drawn using FigTree v.1.4.2 (Rambaut, 2009).

Results

The six ISSR primers produced 259 different bands in 171 individuals across the ten geographic populations associated with the three host oak species. The ISSR4 and ISSR1 primers had the highest (127) and lowest (96) number of bands, respectively. The number of polymorphic bands per primer ranged from 30 for ISSR1 on the *Q. infectoria* host to 45 for ISSR4 on *Q. infectoria* host. The highest average number of polymorphic bands of 37.8 was obtained in the collected samples from *Q. infectoria*. The average number of polymorphic bands in the collected samples associated with *Q. brantii* and *Q. libani* was 36.5 (Table 2). Among the investigated populations, the highest (*i.e*. 158) and lowest (*i.e.* 88) number of bands were amplified in L8 and B7, respectively (Figure 2).

Table 2. Performance of each tested primer on *T. viridana* with the number of total band pruduced by each primer (TNB), number of diffrient allels (TPB), number of private band (PB), polymorphism percentage (PP).

Q. libani			*Q. infectoria*			*Q. brantii*				Host
PP	PB	NA	PP	PB	NA	PP	PB	NA	TNB	Primer
92.11	2	35	78.95	0	30	81.58	2	31	38	ISSR1
75.00	1	36	85.42	2	41	75.00	3	36	48	ISSR2
94.87	2	37	89.74	1	35	89.74	1	35	39	ISSR3
88.00	1	44	90.00	2	45	76.00	0	38	50	ISSR4
75.56	1	34	91.11	0	41	97.78	3	44	45	ISSR5
84.62	3	33	89.74	1	35	89.74	0	35	39	ISSR6
84.56		36.5	87.64		37.83	84.56		36.5	43.16	Mean
-	10	219	-	6	227	-	9	219	259	Total

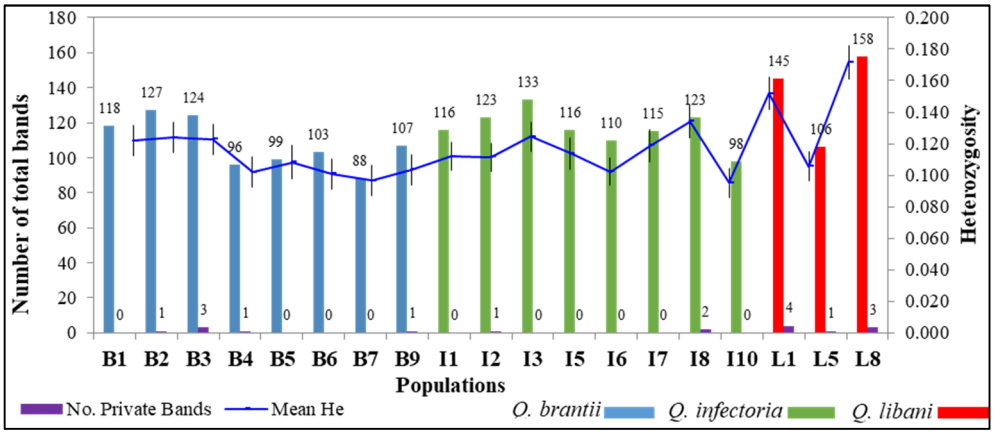

Fig. 2. Comparison of genetic diversity of *T. viridana* in northern Zagros. The number of total bands, the number of private bands, and the expected heterozygosity

Results showed that the highest genetic diversity was in the populations associated with *Q. libani* (I = 0.28, He = 0.16.), while populations associated with *Q. brantii* (I = 0.23, He = 0.13) and *Q, infectoria* (I = 0.23, He = 0.13) had lower genetic diversity (Table 3). In the populations associated with *Q. infectoria,* the highest percentage of polymorphic loci was observed (%PP = 87.6). In contrast, the populations associated with *Q. brantii* and *Q. libani* had similar and lower polymorphic loci (%PP = 84.6) (Table 3).

Table 3. Comparison of genetic diversity of *T. viridana* on different collection site and three *Qurecus* host species (PP%, percentage of polymorphic loci; I, mean Shannon's information index; He, mean expected heterozygosity).

Host	Collection site	Ne	PP	I	He	ID
Q. brantii	-	1.18	84.6	0.23	0.13	B
	Piranshahr	1.19	47.9	0.19	0.12	B1
	Mirabad	1.19	44.8	0.20	0.12	B2
	Sardasht	1.18	47.1	0.20	0.12	B3
	Baneh	1.16	35.9	0.16	0.10	B4
	Gamarelang	1.18	36.3	0.17	0.11	B5
	Garan	1.16	39.8	0.16	0.10	B6
	Seif	1.15	32.8	0.15	0.10	B7
	Dezli	1.16	41.3	0.17	0.10	B9
Q. infectoria	-	1.17	87.6	0.23	0.13	I
	Piranshahr	1.17	44.4	0.18	0.11	I1
	Mirabad	1.16	47.1	0.18	0.11	I2
	Sardasht	1.18	51.4	0.20	0.13	I3
	Gamarelang	1.18	43.3	0.18	0.11	I5
	Garan	1.15	41.7	0.17	0.10	I6
	Seif	1.19	44.0	0.19	0.12	I7
	Bashmagh	1.22	46.7	0.21	0.13	I8
	Sarvabad	1.15	37.8	0.15	0.10	I10
Q. libani	-	1.22	84.6	0.28	0.16	L
	Piranshahr	1.24	55.2	0.24	0.15	L1
	Gamarelang	1.16	40.5	0.17	0.11	L5
	Bashmagh	1.27	59.9	0.27	0.17	L8
Total (geographic different)	-	1.81	44.0	0.19	0.12	-
Total (host)	-	1.19	85.6	0.25	0.14	-

Nei's genetic distance range was 0.03 to 0.06 among the populations associated with *Q. brantii* and 0.01 to 0.05 among those associated with *Q. infectoria* and *Q. libani*. The PCoA, based on Nei's genetic distance, separated the collected populations based on the three oak host species (Figure 3). From the sites associated with *Q. brantii,* B4, B6 and B5 clustered closer together and B7, B9 and B3 together. However, there was a clear separation of these sites from those associated with other oak host species. From those sites associated with *Q. libani* sites, L1, L5 and L8 clustered together with L1 and L5 being much closer. All 3 sites clearly separated from those associated with other oak host species. Sites I5 and I8 associated with *Q. infectoria* clearly separated from other sites associated with *Q. brantii* and *Q. libani*.

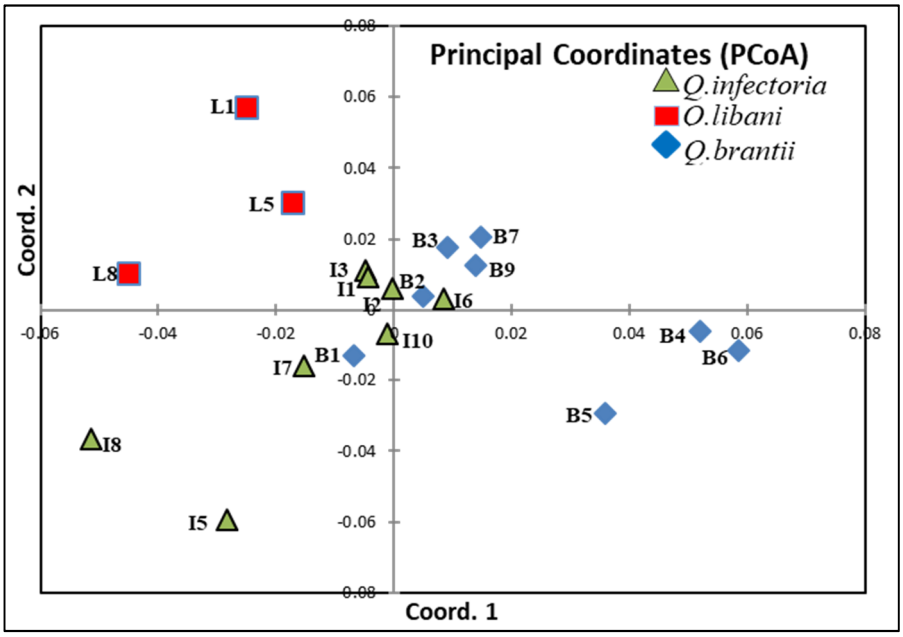

Fig. 3. Principal coordinate analysis (PCoA) of 19 *T. viridana* populations from 10 sampled sites and three host plants based on Nei genetic distance matrix.

There was some close clustering among the sites associated with *Q. infectoria* and *Q. brantii*, sites I1, I2, I3, I6, I7, I10, B1 and B2. Results of PCoA indicates that the host association is independent of sites geography and most likely related to host species, since sites that clustered (or separated for that matter) were from different geographical locations. The AMOVA data indicated that there was significantly higher genetic variability within the populations (*i.e.* associated with the host) than among the geographically separated populations (P = 0.01). 92% of the total genetic variability calculated within the collected populations, and only 8% was among them. The Fst (fixation index) as a gene flow index was 0.09, and Nm (the number of migrants) was 2.9.

The most parsimonious produced phylogenetic tree supported the results of PCoA and further showed that individuals from each host were placed near together. Except for site I8 associated with *Q. brantii* there was a clear clustering of the collected individuals related to the three host species, independent of their geographical locations (Figure 4).

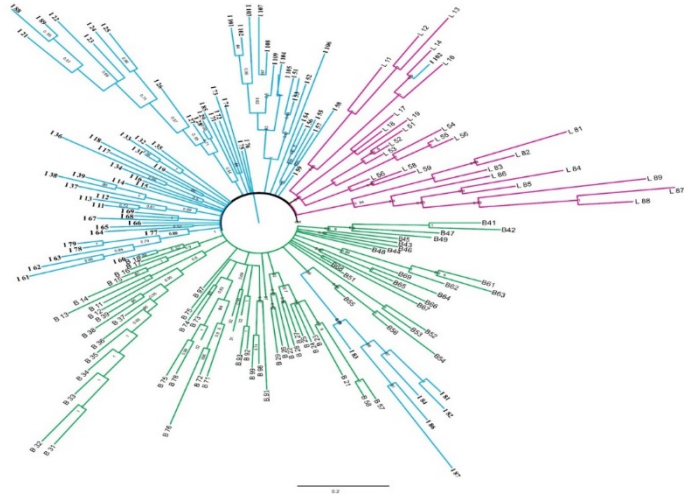

Fig. 4. Phylogenetic tree from Bayesian analysis of 1/0 matrix of the collected populations of *T. viridana* associated with three oak species (*Q. brantii, Q. infectoria,* and *Q. libani*) based on the six ISSR markers (The values among the branches indicate clades with posterior probability>0.5). The green branches indicate the samples collected from *Q. brantii*; the blue branches indicate the samples collected from *Q. infectoria,* and the red branches indicate the samples collected from *Q. libani.*

Discussion

The importance of the oak leaf roller moth as one of the key pests of oak forests has prompted the worldwide assessment of its genetic variation and ecological knowledge. The new findings obtained from these studies offer essential guidelines to manage and control the pest species. Through the same context, we studied the genetic variation of pest populations related to their three host oak species in the oak forests of northwestern Iran.

Our results indicate that among the studied populations of *T. viridana*, those that were associated with *Q. libani* (L8) included the highest number of polymorphic bands (TNB = 158) and the B7 population associated with *Q. brantii* had the least genetic diversity (TNB = 88). The *Q. libani* prefers higher altitudes with rich and moist soil and is more sensitive to its local environmental conditions (especially drought and poor soils) than the other studied oak species (Fattahi, 2000). These preferences can produce more palatable leaf tissue with lower tannin content, the highest levels of nitrogen, total protein, total carbohydrate, and water content in comparison to *Q. brantii* and *Q. infectoria*, making it the favored host plant for *T. viridana* and more susceptible to *T. viridana* grazing at the same time (Yazdanfar *et al*., 2015a, 2015b).

The host-specificity of *T. viridana* has been observed in other geographical locations. When observing fundamental differences in the defoliation level of *Quercus robur* trees in Germany, Schroeder *et al*. (2011) found that larvae significantly prefer *Q. robur* over other oak species. Female of *T. viridana* have been observed to favor susceptible oaks because of their explicit profile of herbivore-induced volatiles (Kersten *et al*., 2013).

The phenological stage of a host plant may also cause change in a host preference of the pest (Stalhandske et al., 2016). The growth preference of *Q. libani* may change its phenological stage compared to the other two host trees, and making it a more palatable and preferred host than the other two hosts.

Therefore, individual moths observed in this study may prefer *Q. libani* over the other two hosts and prefer to migrate from them to *Q. libani*. This host preference may explain the highest genetic variation of the population related to *Q. libani*.

Based on the observed significant differences between and within the populations related to the host species obtained from AMOVA (Table 4), the differentiation among individuals could determine their host's preference and moderate gene flow among populations. The low values of Wright's F index (0.08) and a number of migrants (2.9) confirm these results. The results of PCoA based on Nei's genetic distance revealed that the genetic distances of individuals gathered from various hosts in one region were higher than those collected from similar hosts in different sites. Therefore, the kind of the host species has more influence on grouping the collected individuals than geographic conditions.

The fact that individuals are genetically more similar to trees standing close to each other may also be due to a kind of adaptation of green oak leaf roller groups to individual host trees (Schroeder & Degen, 2008b). Adapting to single (or few) host trees may influence the dispersal behavior of *T. viridana*. Local adaptation to a host plant is generally limited by the herbivore's genetic diversity, gene flow and/or phenotypic plasticity (Ruiz-Montoya & Nunez-Farfan, 2013).

Such an adaptation could be investigated using candidate genes correlated with oaks' susceptibility (or tolerance) to insect grub, which we will do in a future project. Adaptation to single host trees may therefore lead to identifying more or less susceptible phenotypes (and maybe genotypes) of oaks which could result in a sounder insight of this insect-plant interaction.

Based on Bayesian analysis, the resulted phylogenetic tree also confirmed that the populations associated with the three host species were separated, while separation of populations based on geography didn't occur. This may be caused by some sort of adaptation of the sampled moth populations to the host trees (Schroeder & Degan, 2008b). That is a tendency of adult females to lay eggs on the trees that have passed their immature stages (Du Merle *et al*., 1985) and the high ability of adult males to fly long distances, causing high gene flow among geographical conditions (Serra *et al*., 2019).

An important factor to consider is the phenology of different oak populations resulting in the close relation of the sampled pest populations with the host. Comparing the structure and variation of mitochondrial haplotypes in *T. viridana* populations related to the deciduous *Quercus pubescens* and evergreens *Quercus suber* and *Quercus ilex*, Serra *et al*. (2019)

showed that genetic differentiation among populations is possibly associated with the egg development and essentially in the bud-burst phenology of various oak populations.

Our results indicate that the host association can strongly affect the genetic variations in populations of *T. viridana* in the oak forest of Northwestern Iran. These variations are related to host association and are independent of geographical location. These findings may open a new perspective and change approaches toward managing this pest, particularly in the forests where different species of oak are adjacent.

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