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Original Research

Association analysis of tolerance to dieback phenomena and trunk form using ISSR markers in *Quercus brantii*

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Abstract: Oak decline is a complex syndrome in which several damaging agents interact and bring about a serious dieback in tree condition. Genetic diversity is a key factor for better adoption of natural populations to environmental stresses. The objective of this research was to identify the association of polymorphism patterns of different reproducible genomic Inter simple sequence repeats (ISSR markers) to level of dieback phenomena and also growth type in 18 different stands of Persian oak in central Zagros region. Totally, 180 trees were sampled and evaluated for growth type, tree diameter at breast height (DBH) and level of tree dieback. Genomic DNAs extracted of fresh leaves amplified using 15 multi-locus ISSR primers. The population structure determined using the Bayesian model-based clustering method implemented in STRUCTURE software by Monte Carlo Markov Chain (MCMC) method. Five distinct sub-populations (K=5) determined by the log likelihood of the data. Genome wide association study (GWAS) performed using the generalized linear model (GLM) and the mixed linear model (MLM) with Kinship matrix. Informative alleles recognized for level of tolerance to dieback and tree growth type traits. It was observed a significant co-segregation for phenotypic data and some of amplified fragments. Identification of these informative DNA markers can be utilized for pre-screening of high quality oak seedlings in early growth stages and better management in restoration of damaged stands.

Key words: Diversity; ISSR; Oak decline; Polymorphism; Tree growth type.

Introduction

Quercus brantii Lindl. is known as Persian oak, Brant's oak and Zagros oak. This forest tree species covers more than 50 percent of the Zagros forest area (1). Persian oaks have been affected by a condition known as chronic oak dieback or decline from 2000 (2, 3). This disorder is widespread, prolonged and complex. The causes of the condition often involve abiotic factors for example poor soils, recurrent drought, high winds, disturbed environments and air pollutants. Biotic agents that cause this disease include insects and fungi that are destructive to weakened trees (4).

The breeding programs of forest trees is greatly limited because of their long lifespan and the fact that most quantitative traits cannot be assessed until a seedling has matured physiologically. Marker-assisted selection (MAS) is a technology which may help to overcome the barriers to genetic improvement of forest species by accelerating the selection process (5).

Molecular markers are valuable tools in the characterization and assessment of genetic variation within and between plant species and populations (6, 7). It has been demonstrated that different DNA markers might reveal various classes of variation in genome (8, 9). Inter simple sequence repeat (ISSR)-PCR is a technique uses the microsatellite DNA sequences as primers to generate highly polymorphic multi-locus markers. ISSR markers are highly polymorphic and are useful in studies on genetics of populations, tagging of genes, genome mapping and evolutionary biology (10, 11).

Most important economic traits of horticultural and forest trees, such as wood properties, resistance to biotic and abiotic stresses, fruit quality and biomass traits, are controlled by polygenes. Thus, quantitative genetic strategies have been used to identify genes controlling these traits (12). Association mapping, also known as linkage disequilibrium (LD) mapping, is a valuable approach to overcome limitations of linkage mapping or pedigree-based quantitative trait loci (QTL) mapping. In AM, genotypic and phenotypic correlations are investigated in unrelated individuals. This methodology takes advantage of both LD and historical recombination present within the gene pool of an organism, thus utilizing a broader reference population. AM has been used in model species with available genomic resources. Recently, this approach has been applied to dissecting quantitative traits in many forest and fruit trees (13).

The objectives of this study was to assess the genetic structure in Persian oak population using ISSR marker and also the potential of Genetic association mapping to investigate the genetic control of tolerance to dieback phenomena in *Q. brantii* and also the growth type of stem in this species in natural stands of this forest tree.

Materials and Methods

Plant materials and sampling area

180 trees were sampled from central Zagros oak forests include three western provinces in Iran (Ker-

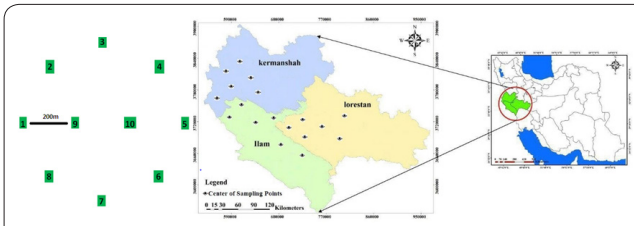

Figure 1. Geographical location of sampling sites in three central Zagros regions also the systematic sampling strategy used for collection of plant materials.

manshah, Ilam and Lorestan) during March till May, 2016. Six different populations per each province which previously reported as damaged areas selected. At least 50 km was the distance between stands. A systematic strategy carried out for selection 10 trees per stand so that the first tree selected randomly and the next ones have 200 m distance with each other (Figure 1). Sampling area per stand was approximately 30 hectares. Fresh leaves collected from each tree and wrapped in aluminum foils and transferred to nitrogen tank. All trees evaluated for some phenotypic traits like diameter at breast height, the percentage of died shoots and growth form. There was at least some fresh weight on tree and no one of them was totally dead. The criterion for oak decline is the proportion of dry dead shoots to all shoots of tree which measured as percent value. We prepared a scale from 0 to ten. Each number equals to ten percent.

DNA extraction

100mg fresh leaf tissue of each genotype used for DNA extraction based on Doyle & Doyle (1987) protocol (15). DNA quality and quantity examined using 0.8% agarose gel electrophoresis and spectrophotometry, respectively.

PCR Reactions

15 high reproducible polymorph ISSR primers (table 2) have been used to conduct PCR reactions in 20ul volume include: 1.5ul DNA, 20ul PCR buffer 10x, 1.8ul $MgCl_2$ (20mM), 0.4um dNTP (1mM), 1.2ul primer (10pM), 0.3ul *Taq* DNA Polymerase (5unit). PCR cycles carried out using a Bio-Rad c1000 thermal cycler. The PCR program was: 94 °C as the primary denaturation: 10cycles (touchdown) with 0.5 °C per cycle decrease: 25 Normal PCR cycles.: Denaturation (94 °C for 30sec): Annealing (Primer specific Tm °C for 45sec): Extension (72 °C for 2min). Final Extension time was 2 °C for 7min.

Electrophoresis

Agarose gel 2% electrophoresis conducted to separate amplified products in each reaction. Ethidium Bro-

Table 1. Geographical characteristics of 18 natural stands of *Q. brantii* located at central Zagros.

NO	Region	Stand	Abbreviation	Sample number	Elevation (m)	Geographic location			
1	Kermanshah- Gilangharb	Kalkosh	K1	10	1386	47" 33	21′ 06	34° 46	N E
2	Kermanshah - Sarpol Zahab	Imamieh	K2	10	1390	85 89	06 27	34 46	N E
3	Kermanshah- Gilangharb	Avalviar	K3	10	1310	33 95	06 03	34 46	N E
4	Ilam – Ivan	Sarab	I1	10	1586	66 90	13 40	34 46	N E
5	Ilam – Ilam	Tajarian	I2	10	1064	63 32	14 02	34 46	N E
6	Ilam – Shirvan	Kalilali	I3	10	1215	40 46	08 10	34 46	N E
7	Ilam – Chardavol	Mamd-Gholi	I4	10	948	54 42	16 07	33 47	N E
8	Lorestan-Kuhdasht	Bwineh	L1	10	1012	78 38	05 20	33 47	N E
9	Lorestan-Kuhdasht	Komeil-Malmir	L2	10	1191	64 09	48 30	33 46	N E
10	Lorestan dowreh Chegeni	Benarkooh	L3	10	1256	49 42	42 33	33 46	N E
11	Lorestan-Khorramabad	Shourab	L4	10	1155	91 97	42 19	33 46	N E
12	Lorestan-Poldokhtar	Chameshahran	L5	10	1318	82 92	44 21	33 46	N E
13	Lorestan-Kuhdasht	Dargonbad	L6	10	917	32 57	39 07	33 47	N E
14	Ilam – Abdanan	Dinarkooh	I5	10	1277	37 58	30 31	33 47	N E
15	Ilam – Badrah	Haranmar	I6	10	1291	82 49	31 52	33 47	N E
16	Kermanshah - Kermanshah	Charzebar	K4	10	1213	06 97	26 11	33 48	N E
17	Kermanshah - Dalahu	Sorkhalizheh	K5	10	903	64 60	20 55	33 47	N E
18	Kermanshah - Islamabadgarb	Aliabad	K6	10	944	57 48	40 05	33 47	N E

Table 2. The characteristics of the ISSR primers and amplified fragments in PCR reactions.

NO	Primer	Sequence	Tm (°C)	Number of alleles	Fragment Size	PIC- Value
1	UBC 840	5′- GAGAGAGAGAGAGAGAYT-3′	53	8	1700- 250	0.121
2	UBC 836	5′-AGAGAGAGAGAGAGACYA-3′	53	11	2000 - 250	0.259
3	ISSR 17	5′-CACACACACACACACAG-3′	52	10	2000 - 400	0.294
4	UBC 809	5′-AGAGAGAGAGAGAGAGG-3′	52	9	1000 - 200	0.326
5	ISSR 155	5′-TGT GTGTGT GTG TGT GGG-3′	56	11	1500 - 300	0.288
6	ISSR 165	5′-AGGAGAGAGAGAGAGCC-3′	56	14	1400 - 250	0.401
7	UBC 895	5′-AGAGTTGGTAGCTCTTGATC-3′	56	11	1600 - 390	0.052
8	UBC 841	5′-GAGAGAGAGAGAGAGAYC-3′	55	7	1500 - 250	0.274
9	UBC 842	5′-GAGAGAGAGAGAGAGAYG-3′	55	9	1800 - 150	0.305
10	UBC 835	5′-AGAGAGAGAGAGAGACYC-3′	55	10	1600 - 300	0.224
11	ISSR 08	5′-GAGAGAGAGAGAGAGAT-3′	50	14	1600 - 300	0.379
12	UBC 807	5′-AGAGAGAGAGAGAGAGT-3′	50	11	1600 - 300	0.425
13	UBC 810	5′-GAGAGAGAGAGAGAGAT-3′	50	13	1400 - 350	0.360
14	UBC 814	5′-CTC TCT CTCTCTCTCTA -3′	50	4	1500 - 350	0.246
15	ISSR 16	5′- GAGAGAGAGAGAGAG -3′	50	17	1400- 300	0.210

Table 3. Diversity measures calculated based on 157 detected alleles for 15 ISSR loci.

Population	Sample No	Shannon Index (I)± SE			Heterogeny (He)± SE			PolymorphicAlleles (%)
I1	10	0.308	±	0.022	0.211	±	0.016	65.61
I2	10	0.291	±	0.021	0.199	±	0.016	63.06
I3	10	0.311	±	0.021	0.212	±	0.016	65.15
I4	10	0.313	±	0.022	0.216	±	0.016	64.97
I5	10	0.301	±	0.022	0.208	±	0.017	63.69
I6	10	0.280	±	0.021	0.190	±	0.015	62.42
K1	10	0.286	±	0.022	0.200	±	0.017	57.96
K2	10	0.319	±	0.021	0.219	±	0.016	68.15
K3	10	0.291	±	0.022	0.200	±	0.016	62.42
K4	10	0.203	±	0.020	0.136	±	0.014	47.13
K5	10	0.221	±	0.021	0.152	±	0.016	45.86
K6	10	0.240	±	0.022	0.165	±	0.016	50.32
L1	10	0.309	±	0.021	0.212	±	0.016	66.24
L2	10	0.296	±	0.021	0.202	±	0.016	64.97
L3	10	0.300	±	0.021	0.206	±	0.016	63.06
L4	10	0.285	±	0.021	0.194	±	0.015	61.75
L5	10	0.271	±	0.022	0.188	±	0.016	55.41
L6	10	0.285	±	0.023	0.200	±	0.017	55.05
Total	**180**	**0.284**	**±**	**0.005**	**0.195**	**±**	**0.004**	**61.1**

I= Ilam, K= Kermanshah, L= Lorestan

mide staining used for detecting the fragments. Presence and absence of each fragment scored as 1/0 for all genotypes (supplementary data file).

Molecular and Statistical Analyses

polymorphism information content (PIC) calculated based on Anderson (1987) equation (14 using the following formula: $PIC_i = 1-\Sigma P^2ij$, where P_{ij} is the frequency of the j^{th} allele in i^{th} marker (Table 2).

Population structure and relative kinship

The genetic structure of the evaluated populations was analyzed using three different methods: 1. Principal coordinate analysis (PCoA) on dissimilarity coefficients among natural populations and 2. Hierarchical cluster analysis of genotypes and also the populations using UPGMA algorithm.

PCoA based on the Nei's genetic distance (17) performed using the GenAlex 6.5 software (18). 3. Frequency pattern of amplified alleles by STRUCTURE software (19), with the length of the burn-in period of 100,000 followed by 10,000 Monte Carlo Markov Chain (MCMC) replicates. The admixture model and correlated alleles frequencies were considered in the analysis. The number of hypothetical subgroups (K) was set from 1 to 10 and three independent runs were made for each K. Optimal number of K determined by the log likelihood of the data [Ln P(D)] in STRUCTURE output and ΔK based on the rate of the change in Ln P(D) between successive K values (19); The pairwise kinship coefficients among the genotypes estimated by the TASSEL program (20).

Association analysis

Association between genotyping and phenotyping data implemented using GLM (General Linear Model)

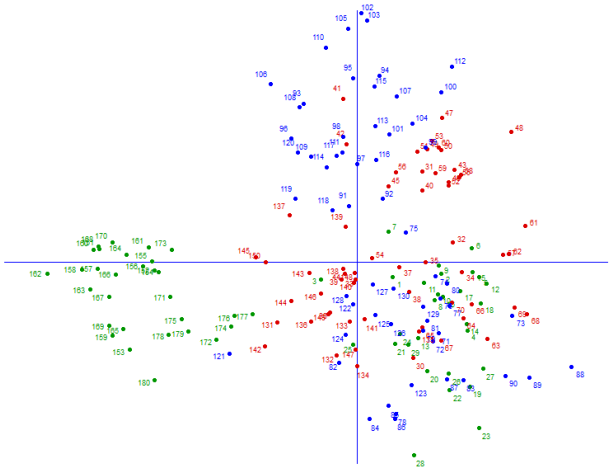

Figure 2. Bi-plot of 180 genotypes based on 157 alleles amplified for 15 ISSRs markers.

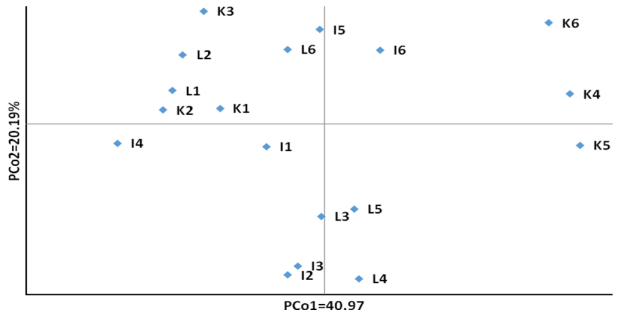

Figure 3. Bi-plot of 18 Persian oak stands based on 157 alleles amplified for 15 ISSRs markers.

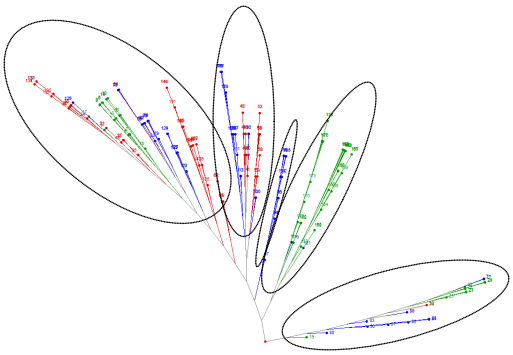

Figure 4. Grouping of genotypes by a UPGMA dendrogram constructed based on Nei distance matrix.

and MLM (Mixed Linear Model) procedures. All association analysis carried out by TASSEL program.

Results

The most important molecular diversity criteria calculated based on 157 polymorph fragments amplified at 15 ISSR marker loci across all 180 genotypes. Shannon index varied of 0.203 to 0.319. Genetic heterogeneity ranged from 0.136 to 0.219. Also total polymorphism per population was different from 45.86% to 68.15% (Table 3). Mean of all calculated criteria was high for Ilam population, old natural oak forests scattered across north and north-eastern regions of this province (Figure 1, Table 1).

Genetic distance among all evaluated stands, presented in table 4. Average genetic distances within and

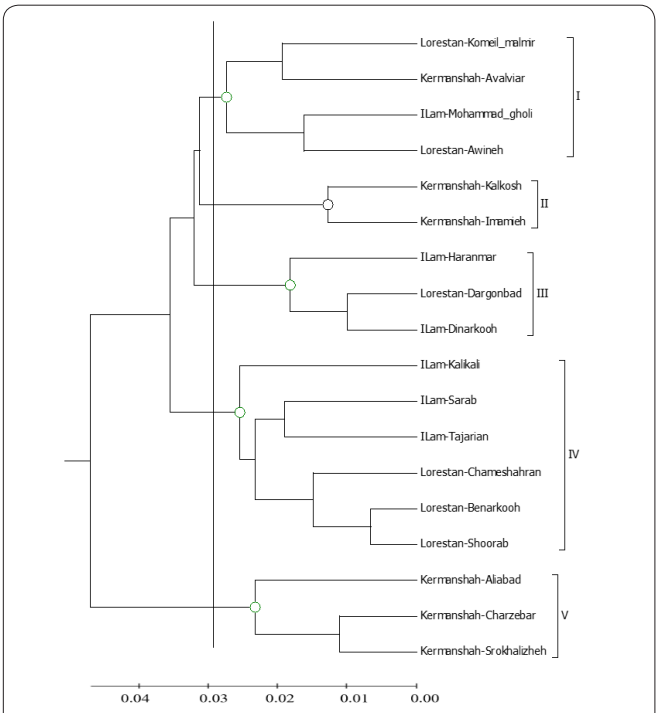

Figure 5. Grouping 18 populations evaluated from three central Zagros geographical regions using the UPGMA algorithm (Five groups are distinguished by cut off line).

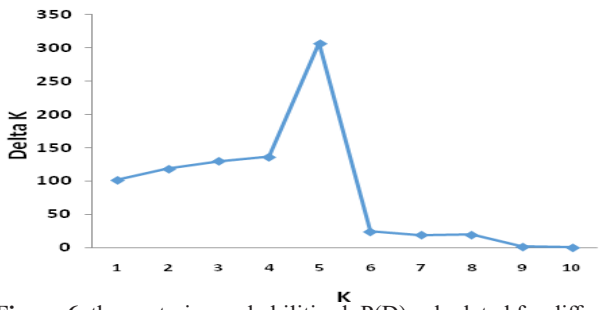

Figure 6. the posterior probabilities lnP(D) calculated for different Ks and determining the optimum K by calculating DeltaK from Dividing Mean lnP(D) to mean of SD[lnP(D)].

between the studied regions has been calculated (Table 5). The most genetic distance obtained for Kermanshah stands. Since the low heterogeneous stand (K4) and highest one (K2) are in this region, this result was expectable (Table 3). Despite the low rate of genetic heterogeny (He) within stands, there is a noticeable differentiation between stands (Fig 4 and Table 4). Analysis of molecular variance showed a significant variation between 18 assessed stands. The amount of this specific variation was 22 percent and the rest one was shared in all stands (Table 6). Also here the molecular variance among different geographical regions calculated and, the highest variation obtained for Ilam province (Table 7).

Principle coordinate analysis for all genotypes (Figure 2) as well as stands (Figure 3) applied to find out the pattern of distribution of them and presence of structure and sub-population in these oak forests. Based on illustrated plots there is distinct subpopulations across evaluated materials. Although, make a decision on number of subpopulations based on the biplot of genotype was no possible, but five subpopulations could be recognized based on the biplot of stands.

Hierarchical clustering carried to determine more exact number of sub-populations in studied regions.

Table 4 Genetic similarity (above the diagonal) and genetic distance (below the diagonal) calculated based on 157 detected alleles for 15 ISSR primer (Nei, 1987).

	I1	I2	I3	I4	I5	I6	K1	K2	K3	K4	K5	K6	L1	L2	L3	L4	L5	L6
I1	…	0.963	0.942	0.939	0.968	0.935	0.056	0.049	0.042	0.921	0.929	0.912	0.953	0.958	0.966	0.956	0.941	0.951
I2	0.038	…	0.958	0.937	0.923	0.910	0.079	0.067	0.084	0.911	0.922	0.899	0.937	0.915	0.953	0.965	0.946	0.934
I3	0.06	0.043	…	0.930	0.918	0.925	0.059	0.063	0.089	0.907	0.913	0.907	0.918	0.918	0.953	0.955	0.945	0.917
I4	0.062	0.065	0.073	…	0.926	0.913	0.066	0.049	0.063	0.873	0.883	0.888	0.968	0.936	0.929	0.92	0.926	0.942
I5	0.032	0.080	0.086	0.077	…	0.973	0.062	0.062	0.052	0.929	0.930	0.937	0.045	0.044	0.052	0.066	0.062	0.020
I6	0.067	0.094	0.078	0.091	0.027	…	0.066	0.08	0.088	0.928	0.93	0.934	0.071	0.079	0.069	0.074	0.066	0.046
K1	0.923	0.923	0.923	0.923	0.923	0.923	…	0.923	0.923	0.923	0.923	0.923	0.923	0.923	0.923	0.923	0.923	0.923
K2	0.953	0.935	0.939	0.952	0.940	0.923	0.026	…	0.954	0.903	0.889	0.889	0.947	0.94	0.93	0.921	0.918	0.944
K3	0.959	0.920	0.915	0.939	0.949	0.916	0.073	0.047	…	0.903	0.896	0.907	0.954	0.962	0.919	0.903	0.928	0.941
K4	0.082	0.093	0.097	0.136	0.074	0.075	0.096	0.102	0.101	…	0.978	0.942	0.122	0.114	0.088	0.077	0.079	0.081
K5	0.074	0.081	0.091	0.125	0.072	0.073	0.113	0.118	0.11	0.022	…	0.967	0.104	0.114	0.073	0.063	0.071	0.085
K6	0.092	0.106	0.098	0.119	0.065	0.068	0.109	0.118	0.098	0.059	0.034	…	0.105	0.111	0.084	0.1	0.083	0.076
L1	0.048	0.065	0.086	0.033	0.956	0.931	0.069	0.055	0.047	0.885	0.901	0.901	…	0.958	0.936	0.93	0.941	0.958
L2	0.043	0.089	0.085	0.066	0.956	0.924	0.079	0.062	0.039	0.892	0.892	0.895	0.043	…	0.942	0.922	0.92	0.943
L3	0.034	0.048	0.048	0.074	0.950	0.934	0.074	0.073	0.085	0.915	0.929	0.92	0.066	0.06	…	0.987	0.965	0.943
L4	0.045	0.035	0.047	0.084	0.936	0.929	0.08	0.082	0.102	0.926	0.939	0.905	0.073	0.081	0.013	…	0.976	0.932
L5	0.061	0.056	0.057	0.077	0.940	0.936	0.085	0.085	0.075	0.924	0.931	0.921	0.061	0.083	0.035	0.025	…	0.933
L6	0.050	0.068	0.087	0.060	0.980	0.955	0.056	0.058	0.060	0.922	0.919	0.927	0.043	0.058	0.058	0.070	0.069	…

Table 5 The average genetic distances within and between the studied regions.

Region	Average distance within populations	Average distance between populations		
		Kermanshah	Ilam	Lorestan
Kermanshah	0.082	-		
Ilam	0.065	0.078	-	
Lorestan	0.056	0.080	0.059	-

Table 6. Analysis of molecular variance for 18 natural stands of Persian oak.

Variance Percentage	Variance Percentage	Variance Percentage	Variance Percentage	Variance Percentage
22	22	22	22	22
78	78	78	78	78
100	100	100	100	100

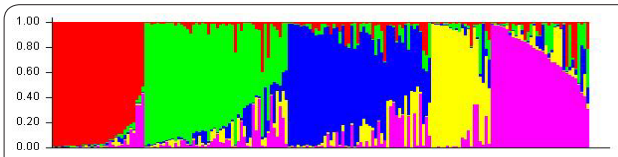

Figure 7. Q-Plot of evaluated genotypes for pattern of allele frequencies across 15 multi-locus ISSR markers.

UPGMA algorithm on Nei's pair-wise distance matrix (15) of genotypes and also the stands showed the presence of five separate groups (potentially subpopulations). The obtained dendrogram is representing these five groups. Analysis of genetic structure was conducted to demonstrate these findings based on the frequency of alleles across all genotypes (whole population).

The pattern of genetic structure has been assessed in the set of sampled trees. The maximum value of Delta K Genotypes as the number of distinct subpopulations in oak forests in central Zagros was five (Figure 6). These subpopulations are presented in the presented Q-plot (Figure 7) which obtained of pattern of allele frequency distributed across the genotypes. The Q-matrix as cofactor used in latter association analyses.

All sampled trees were evaluated for age (Diameter at Breast Height, DBH), growth type (High tree or Coppice form), dieback rate and also geographic coordinates of tree place. This information is presented at table 8 and also used as phenotypic data in association analysis process.

General linear model (GLM) using genotyping and phenotyping data sets along with Q-matrix (genetic structure of evaluated stands) carried out to detect linked markers to dieback trait and growth type of oak trees. Since the GLM procedure has some false positive results, the obtained results reconsidered by mixed linear model (MLM) procedure. All association results with at least 95 percent of confidence (p-value<0.05) have been reported for tree tolerance to dieback (Table 9) and tree growing type (Table 10). It is necessary to mention that calculation of K- matrix (Kinship data derived from general similarity in genetic background arising from shared kinship) was the pre-request of running MLM association analysis (supplementary data).

The result of association study for dieback trait shows that seven of markers linked to dieback tolerance. In these marker loci there were 14 alleles (PCR fragments with different size) which determine 55 percent of dieback trait by GLM model. R^2 value in tables 9 & 10 is an estimate for the impact of recognized genomic fragments (QTLs) on the studied traits. This technique (ISSR) is just confirmed genetic control of these traits.

Table 7 Amount of molecular variance observed within 18 oak stands.

Stand	df	SS	MS	Variance
K1	9	138.4	15.38	97.0
K2	9	176.1	19.57	
K3	9	165.1	18.34	
K4	9	123.1	13.68	
K5	9	129.9	14.43	
K6	9	140.4	15.60	
I1	9	190.0	21.11	117.3
I2	9	172.4	19.16	
I3	9	181.3	20.14	
I4	9	181.6	20.18	
I5	9	165.4	18.38	
I6	9	164.8	18.31	
L1	9	175.9	19.54	113.7
L2	9	173.6	19.29	
L3	9	182.2	20.24	
L4	9	174.5	19.39	
L5	9	152.1	16.90	
L6	9	165.4	18.38	

Although all these loci were confirmed in MLM model but just 10 of produced alleles were significantly associated to evaluated trait. All details of this association analysis have been presented in table 9.

Association of trunk shape of stem in Persian oak investigated as another important trait. All high trees have one of cylindrical non-fork shape or non-cylindrical forked (old coppice like) shape. None forked and forked trees scored by 1 and 0, respectively. Association of trait with genotyping data of trees carried out again using both GLM and MLM models. Eight of markers have amplified fragments linked to genetic factors which control growth type in this oak species. In these marker loci there were 11 alleles (PCR fragments with different size) which determine 43 percent of growth type trait by GLM model. All these eight loci and their linked alleles were confirmed in MLM model. Details about these significantly associate markers and their association has been come in table 10. Partial r^2 for all associate markers as the contribution or determination coefficient of them has been presented.

Discussion

The objectives of present study were analyzing the

Table 8. Sampling from 18 stands of Persian oak with known affected by dieback phenomena in central Zagros area, Iran (K=Kermanshah, I= Ilam, L=Lorestan).

Stand	Sample Size	Longitude (N) Latitude (E)	forked Trees%	none-forked Trees%	DBH		Dieback%		Elevation(m)	
					Range	Mean	Range	Mean	Range	Mean
Kalkosh (K1)	10	34° 08′ 46 10	77.78	22.22	5-70	20.67	0-75	31.67	1215-1255	1233
Imamieh (K2)	10	34 14 46 02	50	50	8-20	14.2	0-30	16.5	1094-1108	1102.2
Avalviar (K3)	10	34 06 46 03	40	60	11-50	28.5	0-20	10	1304-1326	1314.6
Charzebar(K4)	10	34 13 46 40	100	0	4-15	7.2	0-40	15.5	1586-1617	1598.1
Srokhalizheh(K5)	10	34 21 46 06	80	20	10-38	19.4	10-40	17.5	1386-1423	1408.2
Aliabad(K6)	10	34 06 46 27	70	30	8-20	13.7	0-20	5.3	1378-1395	1387.5
Bwineh (L1)	10	33 39 47 07	70	30	10-55	34.2	0-20	9.5	917-968	946.3
Komeil malmir(L2)	10	33 30 47 31	90	10	5-30	18.6	0-50	23	1277-1310	1299.2
Benarkooh(L3)	10	33 31 47 52	90	10	5-25	15	0-30	12.5	1286-1294	1291.7
Shoorab(L4)	10	33 26 48 11	80	20	10-30	17.2	15-70	41.5	1203-1214	1208.8
Chameshahran(L5)	10	33 20 47 55	60	40	5-50	23.1	10-80	44	903-953	929
Dargonbad(L6)	10	33 40 47 05	90	10	10-20	14.4	5-40	19	943-950	945.5
Sarab (I1)	10	34 44 46 21	54.55	45.45	18-90	43.64	0-10	6.82	1318-1364	1352.3
Tajarian (I2)	10	34 42 46 19	0	100	50-90	76.1	0-40	20	1149-1156	1151.6
Kalilali (I3)	10	33 42 46 33	50	50	25-40	33.8	5-30	14	1256-1302	1281.6
Mohammad-Gholi (I4)	10	33 48 46 30	90	10	10-45	23.1	5-60	26.5	1183-1197	1190.6
Dinarkooh(I5)	10	33 05 47 20	100	0	5-20	15	0-40	22.5	1008-1041	1021.6
Haranmar(I6)	10	33 16 47 07	80	20	15-40	27.4	20-40	30	947-960	954.4

Table 9 The results of association analysis for tree tolerance to dieback using GLM and MLM procedures.

Marker	Allele	Fragment Size	GLM Model (Genotype + Q + Phenotype)	MLM Model (Genotype + K+ Q + Phenotype)	Partial r^2
ISSR008	4	550	0.000	0.01130	0.0736
ISSR016	9	750	0.001	0.02880	0.0608
	12	900	0.009	-	0.0357
	17	1400	0.019	0.01440	0.0288
ISSR017	2	500	0.041	0.02540	0.0217
	3	600	0.017	-	0.0295
ISSR155	8	800	0.000	0.01730	0.0622
	9	900	0.001	0.01960	0.052
	2	400	0.021	0.03200	0.0276
ISSR165	3	450	0.020	0.02620	0.028
	9	800	0.021	-	0.0278
UBC810	1	350	0.034	0.03790	0.0234
	5	600	0.005	0.02780	0.0402
UBC842	2	250	0.006	-	0.0393
Determination Coefficient (r^2)			**0.5506**	**0.4183**	

GLM: General linear model. Q: Population structure data or Inferred ancestry of individuals. MLM: Mixed linear model. K: Kinship data derived from general similarity in genetic background arising from shared kinship.

Table 10 The results of association analysis for growing type trait using MLM and GLM procedures.

Marker	Allele	Fragment Size	GLM Model (Genotype + Q + Phenotype)	MLM Model (Genotype + K+ Q + Phenotype)	Partial r^2
ISSR 008	4	550	0.013	0.02350	0.0335
ISSR 016	1	300	0.022	0.02340	0.0284
	8	700	0.026	0.03150	0.0269
ISSR 017	2	500	0.001	0.00170	0.0632
	4	700	0.000	0.00048	0.0831
ISSR 165	9	800	0.021	0.03540	0.0288
UBC 810	1	350	0.017	0.02630	0.0305
	7	700	0.006	0.01780	0.0401
UBC 836	6	600	0.020	0.02410	0.0295
UBC 895	6	650	0.044	0.04680	0.022
UBC 841	1	250	0.005	0.00860	0.0422
Determination Coefficient (r^2)			**0.43**	**0.43**	

GLM: General linear model. Q: Population structure data or Inferred ancestry of individuals. MLM: Mixed linear model. K: Kinship data derived from general similarity in genetic background arising from shared kinship.

population structure and identification of molecular markers associated with the level of tolerance to oak dieback phenomena and also the growth type of Persian oak. Site specific DNA markers have not been developed on this species and polymorphic ISSR markers recognized in our work can be used for marker assistant selection in Persian oak and potentially other oak species.

Based on the genetic variation measures, the level of genetic diversity in *Q. brantii* is low in the natural populations of this forest tree in central Zagros area. Same situation, (He=0.15-0.22) for this spices in northern regions of Zagros oak forest has been reported (21). Also, other Quercus species have low levels of gene diversity in a report from Denmark (22) on *Q. rubor* (He= 0.248) and *Q. Petraea* (He= 0.258). In contrary to these reports, a high level of molecular heterogeneity has been measured for *Q. rubra* and *Q. ellipsoidalis* in Peninsila, Mishigan (Mean He= 0.73). The significance of variance between populations (stands) in AMOVA means populations are subdivided in some way. So, here we are faced with a structured population.

As genetic diversity is a key factor in survival and adoptability of a species to adapt to changing environments, this low rate of genetic variation in natural stands of Persian oak displays vulnerability of this oak forests to the current climate change that Zagros regions have during recent decades (3). The mountainous topo-geography of these regions probably limits gene flow and wide cross pollination. Another reason or homogeneity in oak forests probably is the limited acorn dispersal from maternal trees and a small amount of cloning by root sprouts. Other researchers previously have reported all these observations in Quercus genera (22-24).

Large phenotypic variability was observed for the dieback symptoms in studied genotypes indicates suitability of the genotypes for association study (Table 8). Regard to the results of structure analysis, there were five different subpopulations among evaluated materials. In other words, the population consists of five genetically different subpopulations. It is necessary to account for population genetic structure and also any relatedness due to non-random mating must be accounted for in analysis of genetic association between phenotyping and genotyping data to avoid false positive (spurious) associations. Good sampling and by using appropriate algorithms to detect groupings in a population can accounting for these issues in an association Genetic analysis (Figure 7; 13, 21, 23, 27).

Mixed linear model (MLM) methods have proven useful in controlling for population structure and relatedness within genome-wide association studies (26, 27).

Here, the validation of detected markers in General Linear Model implemented by calculating the Kinship matrice of genotypes and running the mixed linear model. There were a noticeable number of markers linked to both evaluated traits (Table 9 and 10). Seven ISSR markers are informative for dieback phenomena and eight ISSR markers for growth form of oak tree. Regards to the determination coefficient of these markers, some share markers associate to both traits indicating a molecular correlation (indirect relationship) between rates of dieback with the growth type of oak tree. As the most affected stands have dominantly coppice form that is a surprising result. Because all Persian oak coppice stands are old growing and probably they are root sprouts from previously declined genotypes.

The results of the present study showed that genotyping data which obtained from low throughput DNA marker systems like Inter simple sequence repeats can generate reliable polymorphism patterns applicable for investigation of genetic structure of diversity within tree species such as *Q. bantii* that there is no previous information about their genome sequences. Probably, site specific markers like simple sequence repeats (SSR and EST-SSR markers) are the next nearest option that their transferability from other oak relatives should be tested on Persian oak. The low levels of genetic diversity detected in natural stands of Persian oak are another point must be considered in restoration of declined areas of these forests. The measured genetic distance of stands would be helpful for restoring heterogeneity to these natural forests. Attention population structure and differentiation in evaluated genotypes and using association study by MLM model was another achievement of present research. The significance of some of

amplified fragments in some of ISSR markers revealed genetic control for tolerance to dieback in *Q. brantii*. Noticeable contribution of associated markers to investigated traits can be useful for marker assisted selection (MAS) and pre-screening of Persian oak seedlings in early growth stages.

Conflict of interest

The authors declare that they have no conflict of interest.

Author's contribution

The authors declare that they all have made an important scientific contribution to the study and have assisted with the drafting or revising of the manuscript.

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