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Association analysis of salt tolerance in sunflower (*Helianthus annuus* L.) using retrotransposon markers

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ABSTRACT

Salinity is a serious impediment to agricultural production in the world. Development of salt tolerant hybrid varieties using marker aided selection (MAS) is a pioneering strategy to combat salinity stress. Here we used retrotransposon based molecular markers and salinity tolerance related characters in a panel of eighty-four sunflower inbred lines for marker-trait association (MTA) analysis. Characters such as grain yield per plant, leaf relative water content (RWC), K^+, Na^+ and Cl^- concentration in leaf lamina and petiole were measured separately under normal and 8 ds/m salt stress conditions. The impact of salinity was significant on grain yield, RWC, Na^+ concentrations in lamina and petiole as well as on K^+ to Na^+ ratio. To study genomic variability and survey of LTR retrotransposons activity, the lines were fingerprinted with retrotransposon-based DNA markers; IRAP and REMAP. In hierarchical cluster analysis using IRAP+REMAP markers data, the 84 inbred lines were categorized in four main groups, whereas in Bayesian model-based cluster analysis, the lines were assigned into 2 subpopulations (K=2). A mixed linear model was implemented to detect marker–trait associations incorporating membership coefficient of individuals in the subpopulation (Q matrix) and relationship coefficient (kinship matrix) as covariates in the model. In association analysis by using IRAP+REMAP markers; 8 and 12 loci were identified to be significantly linked with the studied characters in normal and salt stress states, respectively. After developing specific primers for identified markers, they could be potentially applied in marker aided selection (MAS) programs to achieve suitable parental lines and also the improvement of traits of interest.

Key words: Abiotic stress, LTR, Molecular markers, QTL analysis, Sunflower.

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INTRODUCTION

Helianthus annuus L. commonly known as sunflower is classified in family of Compositae (Asteraceae). This family consists of more diverse members among flowering plants (Funk *et al.,* 2005). The cultivated sunflowers are grouped in ornamental, oilseed and confection classes (Luan *et al.,* 2014; Jannatdoust *et al.,* 2015). Cultivated sunflower is a dicotyledonous plant with 2n=2x=34 chromosomes. The genome size of plant is in the range of 2871 to 3189 mega base pairs (Bazin *et al.,* 2011; Rengel *et al.,* 2012; Fernandez *et al.,* 2012) with an abundance of repeated sequences (Vukich *et al.,* 2009). Sunflower is one of the most ancient oilseed crops from North America, initially commercialized in Russia. Its oil contains a high percentage of poly-un-saturated fatty acids and low cholesterol (Francois, 1996). In 2017, sunflower was planted on ~41119 ha in Iran with the production of ~39855 tons (FAO, 2016).

The constant salinization of agricultural land is a serious impediment to agricultural production and food security in the world. Over 831 million ha of lands worldwide and about 444 million ha of Asia, the Pacific and Australia lands are salt affected (Arora, 2017). As NaCl is a dominant component of saline soils, the high levels of Na^+ and Cl^- are accumulated in plants grown under saline conditions. The high amount of Na^+ and Cl^- ions produce toxic effects and reduce the capacity of photosynthetic apparatus in plants. In other hand, hyper osmolality happens in soils with higher concentrations of soluble salts leading to nutritional imbalances in most of plants. This is harmful for plants and decline their growth and developments (Kumar *et al.,* 2022). The high number of ions with toxic effects such as Na^+ and Cl^- accumulated in the plant chloroplasts under saline conditions (Munns, 2005; Munns *et al.,* 2006), damage to K^+ absorption; leading to a decrease in the ratio of K^+ to Na^+ (van Zelm *et al.,* 2020). Potassium plays essential role in many processes of cell; including the activation of enzymes, cell turgor, setting the conductance of stomata and keeping osmotic homeostasis (Srivastava *et al.,* 2020). Therefore, in the plants grown under saline stress, the amount of K^+ is very vital for establishing ionic homeostasis in the cytosol (Zhu, 2003). Indeed, the ability of plants to overcome the harmful effects of salinity stress largely depends on their potassium status. Because of this, the high amount of K^+ to Na^+ are considered as an essential selection criterion in plants community for salt tolerance (Ashraf and Wu, 1994; Tester and Davenport, 2003; Reynolds *et al.,* 2005). Although sunflower is a moderately salt tolerant plant, its production is heavily influenced by salinity stress (Khalifani *et al.,* 2023). Morphological characters and seed yield of sunflower reduce under salinity stress (Khalifani *et al.,* 2022). Han *et al.* (2022) studied the effect of salinity on oil seed sunflower. Their results showed that salinity decreased the plant height, biomass, and plant water content. In another research work, it was shown that the salinity caused a reduction in sunflower yield by reducing head diameter and 100-grain weight (Rehman and Hussain, 1998; Khatoon *et al.,* 2000). It is suggested that seed weight, seedling growth and K/Na ratio may be used as important criteria for early evaluation of sunflower genotypes to salt stress tolerance (Hussain and Rehman, 1993).

Genetic manipulation of complex quantitative characters needs to be investigated thoroughly. Understanding the genetic/molecular basis of complexly inherited characters like seed yield, tolerance against salinity stress, quality, etc. helps breeder to design and develop accurate selection programs. The availability of molecular markers plays a key role in deciphering the genetic mechanism of quantitative characters and these markers are considered very critical for increasing the efficiency of breeding programs via marker-aided selection (MAS). Association or linkage disequilibrium (LD) mapping/analysis is a current method for identifying molecular markers associated with characters that profit from all recombination events occurred in meiotic cycles during plants evolution (Ashwath *et al.,* 2023). It is an efficient approach in determining QTLs involved in controlling quantitative characters and it provides great opportunities to determine the pleiotropic effects exists in some regions of chromosomes (e.g. Zhu *et al.,* 2008; Huang *et al.,* 2010). Retrotransposons based REMAP (Retrotransposon Microsatellite Polymorphism) and IRAP (Inter Retrotransposon Polymorphism) molecular markers are reliable DNA markers for genetic investigations of complex characters (Kalendar and Schulman, 2006).

In the context of association mapping, surveying population structure is an essential step. If it exists and is not considered, it can lead to false associations between markers and characters (Zhao *et al.,* 2007). Population structure together with kinship between individuals are factors that produce linkage disequilibrium (LD) between genetic loci without any physical linkage between them (Yu *et al.,* 2006). Sunflower has many characters of interest for consumers and producers. Most of these characters are quantitatively controlled. Reports on the evaluation of salinity and alkali stresses tolerance in sunflower and the use of DNA markers in its resistance breeding programs are limited (Liu

and Baird, 2003). Considering literature review, retrotransposon markers were implemented effectively for the identification of the genomic regions controlling agro-morphological traits in confectionary sunflower (Jannatdoust *et al.,* 2016). For instance, Jannatdoust *et al.* (2016) utilized a panel of 48 confectionery sunflower cultivars and determined 131 and 117 retrotransposon markers using GLM and MLM algorithm which associated with agro-morphological traits. In other study, Rasoulzadeh Aghdam *et al.* (2021), determined that 17 and 19 retrotransposon markers had a significant correlation with agro-morphological characters in optimal and phosphorus deficient conditions using the MLM model. They also found marker cfcr8-1 as common genomic region between traits in two states (Rasoulzadeh Aghdam *et al.,* 2021).

The aim of this study was to identify retrotransposon-based DNA markers associated with some ionic and water relational characters in *Helianthus annuus* under normal and salt stress conditions on 84 oily sunflower lines from different origins. Introduction and determination of DNA markers linked with salt tolerance-associated characters will aide sunflower breeding scientists in choosing and selecting salt tolerant genotypes via maker aided selection (MAS) within its breeding programs.

MATERIALS AND METHODS

Plant materials and phenotyping

Eighty-four sunflower inbred lines coming from various countries (Table 1) were assessed in plastic pots using randomized complete block design (RCBD) with three replications. The pot size was 24×26 cm which filled with 3:1 of field soil and compost. Distances between rows (rows of pots) and between pots inside each rows were 50 cm and 30 cm, respectively. In the present study, the growth conditions of the plants, including the soil of the pots, were uniform; due to the fact that the number of genotypes and traits under investigation was large and their measurement was time-consuming, therefore, errors might occur during the measurement. To divide the work and minimize the possible errors and estimate these errors, a randomized complete block design was used. The lines were evaluated under normal (2 dS m^{-1}) and salt stress (8 dS m^{-1}) (Morsali Aghajari, 2015) conditions in an open-air area at Urmia University in 2015. Two seeds of each pure line was sown in pots and after plant establishment, one plant per pot was retained. Salinity stress was applied with NaCl. To achieve 8 dS m^{-1} salinity, based on the primary amount of soil salinity of each pot, 12 grams of NaCl was disbanded in 500 ml of water and added to each pot after plants reached the 8-leaf-stage. To avoid osmotic stress, salinity was applied in two steps, 250 ml of salt solution was added in the morning and the rest 250 ml was applied in the afternoon of the same day. Soil salinity was controlled by EC-meter during experiments. The irrigation was carried out by dripping and the complete fertilizer was added (three times) during the vegetative growth. During irrigation times, care was taken to not exceed watering from the drainage of the pots. After flowering, some characters including leaf relative water content (RWC), Cl^-, Na^+ and K^+ concentrations were determined in leaf lamina and petiole. Crop harvest was made at physiological maturity stage and seed yield per plant was measured. Characters measuring were performed in both normal and salt stress conditions, separately (Morsali Aghajari, 2015).

Inorganic ions

One hundred mg ground samples of leaf lamina and petiole were weighed and transferred to 15 ml plastic centrifuge tubes containing 10 ml of deionized water. Then, the tubes were posited in the boiling water bath, for approximately 1 h and then centrifuged at 5000 rpm at 4 °C for 20 min. The supernatants were transferred into new tubes, and the final volume was set to 10 ml with deionized water. Half a ml aliquots were used for determining chloride concentration by utilizing a Chloride Analyzer (Model 926, Corning). Sodium and potassium concentrations were determined by utilizing a flame photometer (Fater 405) (Morsali Aghajari, 2015).

Relative water content (RWC)

From the top of each plant, the third youngest leaf with full expansion was sampled and four 1.0 cm diameter leaf discs per leaf were taken and immediately weighed and recorded as fresh weight (FW). Then, they were plunged in distilled water in Petri dishes for 24 hat 4 °C in the dark and the turgid weight (TW) was measured. The discs then were desiccated in an oven at 70 °C for 24 h and the dry weight (DW) was measured. Then, RWC was computed with formula as follows (Boyer *et al.,* 2008):

$$\text{RWC}\ (\%) = \frac{(FW - DW)}{(TW - DW)} \times 100 \quad (1)$$

Morphological data analysis

Summery statistics, such as average, standard error, coefficient of variation as well as two-way analysis of variance (Factorial Randomized Complete Block Design) for each one of the studied characters were computed using SAS software version 9.4 (PROC UNIVARIAT and GLM).

Table 1. The studied oily sunflower lines and their origin.

Code	Line name	Origin	Research center	IRAP Q value		REMAP Q Value	
				Q1	Q2	Q1	Q2
1	H100A/83HR4	France	ASGROW	0.598	0.402	0.094	0.906
2	H209A/LC1064	France	ASGROW	0.669	0.331	0.44	0.56
3	H205A/H543R	France	ASGROW	0.707	0.293	0.151	0.849
4	AS5306	France	ENSAT	0.186	0.814	0.724	0.276
5	RHA858	America	USDA	0.976	0.024	0.172	0.828
6	H209A/83HR4	France	ASGROW	0.427	0.573	0.91	0.09
7	As3211	France	ENSAT	0.481	0.519	0.704	0.296
8	254-ENSAT	France	ENSAT	0.429	0.571	0.677	0.323
9	AS5304	France	ASGROW	0.679	0.321	0.334	0.666
10	1009329.2(100K)	France	ENSAT	0.028	0.972	0.793	0.207
11	270-ENSAT	France	ENSAT	0.346	0.654	0.583	0.417
12	AS613	France	ASGROW	0.886	0.114	0.296	0.704
13	A-F1POPA	France	NOVARTIS	0.68	0.32	0.552	0.448
14	OES	France	INRAMONT	0.034	0.966	0.445	0.555
15	H100A/LC1064	France	ASGROW	0.025	0.975	0.632	0.368
16	RHA266	America	USDA	0.719	0.281	0.557	0.443
17	PAC2	France	ENSAT	0.554	0.446	0.87	0.13
18	H157A/LC1064	France	ASGROW	0.205	0.795	0.504	0.496
19	5DES20QR	France	BRN	0.223	0.777	0.461	0.539
20	1009337(100K)	France	ENSAT	0.238	0.762	0.226	0.774
21	AS3232	France	ENSAT	0.106	0.894	0.932	0.068
22	12ASB3	France	ASGROW	0.415	0.585	0.655	0.345
23	8ASB2	France	ASGROW	0.375	0.625	0.441	0.559
24	9CSA3	France	Caussade	0.342	0.658	0.777	0.223
25	H049+FSB	France	-	0.662	0.338	0.826	0.174
26	5AS-F1/A2×R2	France	ASGROW	0.379	0.621	0.867	0.133
27	7CR16-PRH6	France	ASGROW	0.11	0.89	0.253	0.747
28	ENSAT699	France	C.F	0.823	0.177	0.589	0.411
29	SSD-581	France	ENSAT	0.9	0.1	0.057	0.943
30	TMB-51	France	ASGROW	0.017	0.983	0.496	0.504
31	110	Iran	INRAMONT	0.881	0.119	0.126	0.874
32	H603R	France	SPII	0.408	0.592	0.681	0.319
33	4	Iran	SPII	0.803	0.197	0.449	0.551
34	703-CHLORINA	France	INRAMONT	0.343	0.657	0.38	0.62
35	NSF1-A4×R5	France	SPII	0.984	0.016	0.036	0.964
36	28	Iran	ENSAT	0.268	0.732	0.773	0.227
37	30	Iran	NOVARTIS	0.761	0.239	0.209	0.791
38	F1250/03	Hungary	SPII	0.357	0.643	0.602	0.398
39	SDR18	America	SPII	0.443	0.557	0.901	0.099
40	LP-CSYB	France	-	0.077	0.923	0.917	0.083
41	803-1	Serbia	USDA	0.068	0.932	0.729	0.271
42	1009370-1(100K)	France	ENSAT	0.628	0.372	0.058	0.942
43	CSWW2X	France	IFVC	0.236	0.764	0.81	0.19
44	1009370-3(100K)	France	ENSAT	0.236	0.764	0.137	0.863
45	H158A/H543R	France	ASGROW	0.969	0.031	0.377	0.623
46	H100A	France	ASGROW	0.075	0.925	0.518	0.482
47	15031	France	ASGROW	0.159	0.841	0.793	0.207
48	H205A/83HR4	France	ASGROW	0.46	0.54	0.408	0.592
49	RHA265	France	ASGROW	0.121	0.879	0.661	0.339
50	PM1-3	America	USDA	0.976	0.024	0.056	0.944
51	RT948	France	RUSTICA	0.504	0.496	0.71	0.29
52	283-ENSAT	France	-	0.153	0.847	0.927	0.073
53	QHP-1	France	INRAMONT	0.401	0.599	0.559	0.441
54	SDR19	America	USDA	0.451	0.549	0.824	0.176

Table 1 (Continued). The studied oily sunflower lines and their origin.

Code	Line name	Origin	Research center	IRAP Q value		REMAP Q Value	
				Q1	Q2	Q1	Q2
55	HA337B	America	USDA	0.025	0.975	0.607	0.393
56	H100B	France	ASGROW	0.252	0.748	0.608	0.392
57	B454/03	Hungary	-	0.515	0.485	0.198	0.802
58	HA304	America	USDA	0.186	0.814	0.786	0.214
59	RT931	France	RUSTICA	0.711	0.289	0.755	0.245
60	HA335B	America	USDA	0.023	0.977	0.523	0.477
61	SDB3	America	USDA	0.596	0.404	0.753	0.247
62	LC1064C	France	ASGROW	0.579	0.421	0.199	0.801
63	NS-R5	France	NOVARTIS	0.141	0.859	0.918	0.082
64	H156A/RHA274	France	ASGROW	0.26	0.74	0.653	0.347
65	SDB1	America	USDA	0.264	0.736	0.836	0.164
66	HAR-4	America	USDA	0.427	0.573	0.737	0.263
67	AS5305	France	ASGROW	0.088	0.912	0.932	0.068
68	RHA274	America	USDA	0.133	0.867	0.83	0.17
69	H100A/RHA274	France	ASGROW	0.135	0.865	0.713	0.287
70	H209A/H566R	France	ASGROW	0.782	0.218	0.446	0.554
71	ASO-1-POP-A	France	ENSAT	0.383	0.617	0.342	0.658
72	AS6305	France	ENSAT	0.615	0.385	0.535	0.465
73	D34	America	USDA	0.222	0.778	0.545	0.455
74	CAY	France	ENSAT	0.176	0.824	0.315	0.685
75	346	Iran	SPII	0.115	0.885	0.471	0.529
76	NS-F1-A5×R5	France	NOVARTIS	0.461	0.539	0.928	0.072
77	36	Iran	SPII	0.441	0.559	0.772	0.228
78	38	Iran	SPII	0.597	0.403	0.247	0.753
79	SDB2	America	INRAMONT	0.095	0.905	0.304	0.696
80	H158A/LC1064	France	-	0.505	0.495	0.681	0.319
81	H156A/H543R	France	ASGROW	0.969	0.031	0.081	0.919
82	H543R/H543R	France	ASGROW	0.669	0.331	0.29	0.71
83	H543R	France	-	0.822	0.178	0.19	0.81
84	15038	France	ASGROW	0.883	0.117	0.124	0.876

IRAP and REMAP data analyses

The marker data were kindly provided by Basirnia *et al.* (2014a). Briefly, molecular profiles of sunflower lines were prepared by 14 IRAP and 14 REMAP pair primers through PCR reaction descried in detail by Basirnia *et al.* (2014a). A total of 118 out of 128 and 113 out of 120 amplified bands using 14 IRAP and 14 REMAP primers, were polymorphic, respectively. A minimum evolution algorithm (ME) dendrogram based on number of differences coefficient was drawn in distance-based cluster analysis using MEGA 4 software package (Tamura *et al.,* 2007). Population structure was analyzed using Bayesian model-based clustering algorithm (Wade, 2023) implemented in the Structure 2.3.4 software package (Pritchard *et al.,* 2000). For this, the number of sub-populations (K) was set from 1 to 20; with 10 replications for each K. Burn-in period length and Markov Chain Monte Carlo replication numbers were fixed to 100,000. Correlated allele frequencies and model of admixture were selected in analysis. The optimum number of sub-populations was obtained by: (1) Ln P (D)=L (K) which is the logarithm of likelihood for each K (Rosenberg *et al.,* 2002), and (2) the Delta K (ΔK) statistic which is calculated based on the secondary rate of variations in likelihood; ΔK=[L"(K)]/Stdev (Evanno *et al.,* 2005). In this approach, the probability of slope fracture at the point, where the value of hypothetical K is at the maximum rate of likelihood. Association analysis was run in TASSEL 2.1 using ancestry coefficients (Q values), and kinship (K-values) matrices as covariates in the mixed linear model (MLM). In general, two methods, GLM and MLM, have been proposed for association analysis, and today MLM is widely used (Ghavami *et al.,* 2011). In the general linear model (GLM), only the population structure is considered in the association analysis, but in the mixed linear model (MLM), in addition to the matrix

Table 2. Analysis of variance for the studied characters in oily sunflower lines under normal and salt stress conditions.

Source of variation	df	Mean of square									
		Na_L	K_L	Cl_L	K/Na_L	Na_P	K_P	Cl_P	K/Na_P	RWC	GYP
Salt stress	1	163.26*	1.41^{ns}	0.39^{ns}	1585.24*	802**	21.65^{ns}	1.79^{ns}	539**	18375*	765.4*
Rep	2	3.8**	6.55^{ns}	5.41^{ns}	43.22**	0.55^{ns}	9.64^{ns}	11.11^{ns}	2.89**	3265**	78.24^{ns}
Line	83	1.14**	18.19**	12.81**	7.9**	14.65**	73.88**	34.03**	4.69**	577^{ns}	272.85**
Lines×Salt	83	0.2^{ns}	4.45^{ns}	2.91^{ns}	3.59^{ns}	1.21**	11.55^{ns}	8.78^{ns}	2.34**	569^{ns}	45.04^{ns}
Error	334	0.43	3.95	3.56	2.98	0.25	13.96	8.52	0.28	555.6	51.5
Coefficient of variation (%)		18.45	14.58	16.09	15.97	9.15	17.96	12.05	20.6	12.59	18.77

df: Degree of freedom, Rep: replication, Na_L: Sodium concentration in lamina, K_L: Potassium concentration in lamina, Cl_L: Chloride concentration in lamina, Na_P: Sodium concentration in petiole, K_P: Potassium concentration in petiole, Cl_P: Chloride concentration in petiole, RWC: Leaf relative water content, GYP: Grain yield per plant.
*, **: Significant at 5% and 1% probability levels, respectively.

of the population structure (Q), the matrix of kinship relations (K) between the individuals of the population are considered as covariates in association model. Therefore, the false associations between markers and characters are minimized. Yu and Buckler (2006) in their association mapping studies in maize showed a significant improvement in the reduction of false positive results in association mapping of traits such as flowering time, spike weight and spike diameter by using the MLM with Q+K compared to single K or Q linear models.

RESULTS AND DISCUSSION

Phenotypic diversity

Analysis of variance showed significant differences among sunflower lines for all the studied traits, except for leaf relative water content (RWC) suggesting the existence of potentially useful genetic variability. The impact of salinity was significant on grain yield, leaf relative water content (RWC), sodium concentrations in lamina and petiole as well as K^+/Na^+ ratio (Table 2). The detrimental effects of salinity are commonly thought to rise from scant water potential and ion toxicity (Munns, 2002).

The descriptive statistics including mean, standard deviation, range and coefficient of variation for the assessed characters were summarized in Tables 3 and 4. The highest heritability value under normal conditions was observed for sodium and potassium concentration in petiole (98%, 95%), and the lowest one observed for sodium concentration in lamina (Table 3). In salt stress conditions, the highest and lowest heritability values were observed for potassium concentration in petiole (0.97) and for leaf relative water content (0.21) (Table 4). However, due to genotype×environment interaction, it is better to estimate heritability based on several years' data.

Heritability estimates for Na^+, K^+, Cl^- and K^+/Na^+ ratio in petiole were generally higher than those in lamina under natural and salt stress conditions. Calculated heritabilities (H^2) revealed that the most of studied characters (4/6) show high a heritability (H^2>65%) in both normal and salinity stress conditions (Tables 3 and 4). Therefore, it is worth to navigating association analyses between characters studied and retrotransposons based molecular markers. Characters with a higher heritability value can be easily varied via selection (Abd El-Aty *et al.,* 2011). Overall, the heritability values were lower under salt stress conditions than that in normal ones. Arraouadi *et al.* (2011) announced the high broad sense heritability (H^2) value for leaf and stem dried weight in diploid alfalfa (*Medicago truncatula*) under normal and salinity conditions illustrating that these characters are managed by genes with additive gene effects and effective selection could be applied for their improvement.

Potassium contents were alleviated in salt stress conditions. However, a higher decline was evident in lamina. Chloride accumulation was more in petiole and lamina of the plants grown under salinity conditions compared to those in plants grown under normal conditions. However, petioles showed higher Cl^- concentrations than lamina. Sodium as a counterpart cation for chloride, considerably ($P<0.05$) showed higher values in leaf and petiole of all lines grown under salinity conditions compared to those in the plants grown under normal condition.

Chloride as a micronutrient with essential roles in plants, functions as an osmotically active solute in

Table 3. Descriptive statistics for agronomic traits in sunflower inbred lines evaluated under normal condition.

Trait	Mean	Sd	Max	Min	Range	Vg	Vphe	h^2	CVg	CVphe
Na_L	1.67	0.70	5.76	0.66	5.1	0.08	0.48	0.36±0.16	0.17	0.41
K_L	9.92	2.32	17	5	12	2.15	5.40	0.65±0.07	0.15	0.23
Cl_L	3.83	1.83	16.6	1.02	15.58	1.74	3.33	0.76±0.05	0.34	0.48
K/Na_L	6.79	2.83	15.71	1.52	14.19	2.40	7.69	0.56±0.1	0.23	0.41
Na_P	4.13	1.98	12.6	0.82	11.78	3.72	3.91	0.98±0.00	0.47	0.48
K_P	13.57	5.42	42	1.2	40.8	25.69	29.47	0.95±0.00	0.37	0.40
Cl_P	8.62	3.34	26.2	0	26.2	9.25	11.17	0.93±0.00	0.35	0.39
K/Na_P	3.73	1.85	15.2	0.24	14.96	2.91	3.40	0.85±0.01	0.46	0.49
RWC	78.03	31.10	711.11	32.14	678.97	2.00	87.90	0.06±0.09	0.05	0.36
GY	25.92	9.48	69.4	9.04	60.36	34.14	88.68	0.64±0.05	0.09	0.14

Sd: Standard deviation, Max: Maximum value, Min: Minimum value, Vg: Genotypic variance, Vphe: Phenotypic variance, H^2: Heritability, CVg: Genotypic coefficient of variation, CVphe: Phenotypic coefficient of variation, Na_L: Sodium concentration in lamina, K_L: Potassium concentration in lamina, CL_L: Chloride concentration in lamina, Na_P: Sodium concentration in petiole, K_P: Potassium concentration in petiole, CL_P: Chloride concentration in petiole, RWC: Relative water content, GYP: Grain yield per plant.

Table 4. Descriptive statistics for agronomic traits in sunflower inbred lines evaluated under salt stress.

Trait	Mean	Sd	Max	Min	Range	Vg	Vphe	h2	CVg	CVphe
Na_L	2.96	0.82	6.68	1.50	5.18	0.15	0.64	0.47±0.13	0.20	0.41
K_L	7.86	2.49	16.80	2.20	14.60	1.84	6.16	0.55±0.1	0.17	0.32
Cl_L	5.80	2.65	19.80	2.16	17.64	2.67	7.02	0.64±0.08	0.28	0.46
K/Na_L	2.85	1.13	5.79	0.40	5.39	0.42	1.19	0.61±0.09	0.14	0.23
Na_P	6.95	2.13	15.08	4.10	10.98	4.17	4.55	0.96±0.006	0.41	0.43
K_P	9.15	3.61	21.33	0.58	20.75	12.00	13.07	0.97±0.006	0.38	0.40
Cl_P	11.99	3.10	20.98	2.83	18.15	8.76	9.64	0.96±0.002	0.25	0.26
K/Na_P	1.41	0.63	4.07	0.06	4.00	1.01	0.40	0.94±0.01	0.27	0.30
RWC	66.60	11.49	136	4.88	131.12	0.14	0.85	0.21±0.002	0.005	0.42
GYP	23.96	8.10	49.52	9.18	40.34	17.70	65.45	0.51±0.06	0.05	0.10

Sd: Standard deviation, Max: Maximum value, Min: Minimum value, Vg: Genotypic variance, Vphe: Phenotypic variance, H^2: Heritability, CVg: Genotypic coefficient of variation, CVphe: Phenotypic coefficient of variation, Na_L: Sodium concentration in lamina, K_L: Potassium concentration in lamina, CL_L: Chloride concentration in lamina, Na_P: Sodium concentration in petiole, K_P: Potassium concentration in petiole, CL_P: Chloride concentration in petiole, RWC: Relative water content, GYP: Grain yield per plant.

vacuoles of plants and implicates in both turgor and osmoregulation (White and Broadley, 2001). However, at high concentrations, it can be toxic for plants. In fact, Na^+ and Cl^- both are toxic for plants at high concentrations, but some plant species can manage Na^+ transport predominantly better than Cl^- and vice versa (Munns and Tester, 2008). Salt tolerance is indeed related to the capability of plant genotypes to adjust both Cl^- and Na^+ transports in order to reduce ion toxicity. Some genotypes are high efficacious at adjusting Na^+ or Cl^- transports (or both) than others, due to different mechanisms of ion balance (Teakle and Tyerman, 2010). There is general agreement that high Cl^- accumulation in certain genotypes can cause growth reduction (Wu and Li, 2019).

Potassium is a macro element that plays a serious role in offsetting membrane potential and turgor, prompting enzymes, and adjusting osmotic pressure, stomatal action and tropisms (Cherel, 2004). When membrane depolarization is induced by NaCl under saline conditions, potassium leakage routinely happens (Shabala *et al.,* 2003). As intracellular K^+/Na^+ homeostasis is obligate for cell metabolism, so it is considered a key index to salinity tolerance in plants (Chen *et al.,* 2007).

Genomic diversity and population structure

IRAP markers

Out of 25 LTR primers examined in IRAP analysis, six single primers including Cf, Cr, 1062, 1065, Ur1 and Uf and eight primers combinations generated a distinguishable and polymorphic banding pattern. Cf, Cr, Uf, and Ur1 primers existed in the combinations of

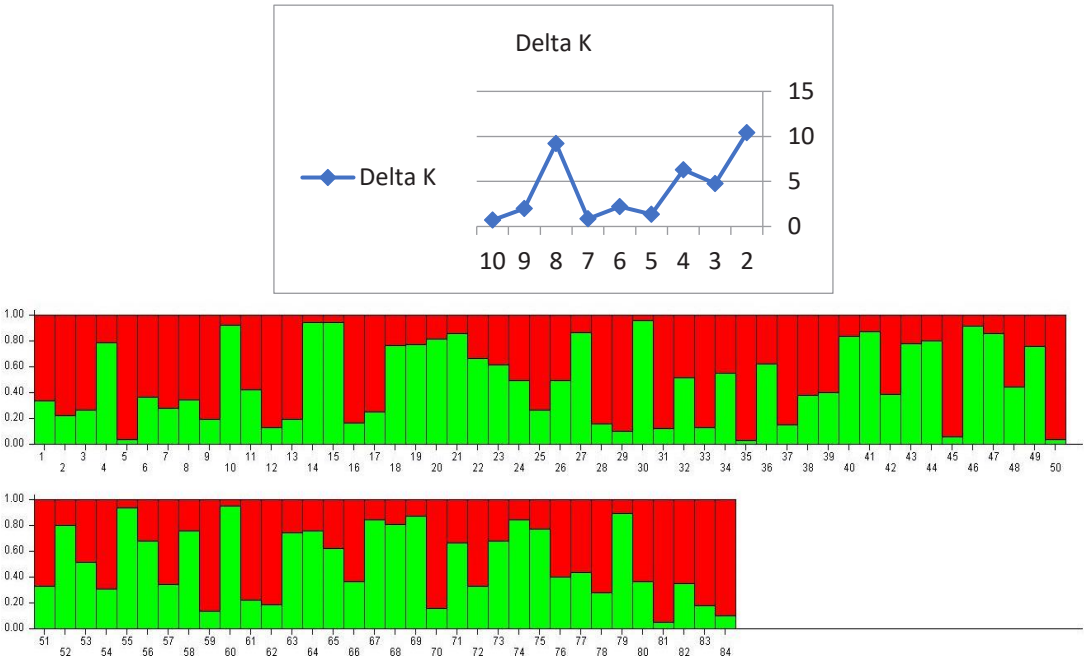

Figure 1. Bar plot of structure analysis of the studied oily sunflower germplasm according to 118 loci of IRAP using Bayesian model. Each color shows one subpopulation or cluster. The numbers on horizontal and vertical axes correspond to the number and the member.

three out of eight primer combinations, whereas 1061, 1062, 1063, 1064, and 1065 primers were present in five other primers combinations. A hundred and twenty eight bands were generated by 14 primer pairs (six single and eight primer combinations), out of which 118 markers were polymorphic (92.18%) (Basirnia *et al.,* 2014a).

In population structure analysis, a maximum value of ΔK was observed in K=2, so the studied association panel probably consists of 2 sub-populations (Figure 1). Out of 84 studied sunflower lines, 33 lines showed mixed structure and 51 lines belonged to one of the first (33) and the second (18) sub-groups (Table 1). Cluster analysis using IRAP markers grouped 84 inbred lines in seven main clusters (Figure 2). In each cluster, lines with different geographical origins were observed. Cluster A contained 27 inbred lines (20 from France, three from USA, three from Iran, and one from Hungary) and was represented mainly by the France inbred lines. Clusters B, C and D were dominated by the lines originated mainly from France. Cluster E contained of six inbred lines; three from France, one from USA, one from Iran, and one from Hungary. Cluster F included three lines; one from France and two from Iran. Cluster G consisted of nine inbred lines, five from France and four inbred lines from USA (Figure 2). However, the classification did not distinctly separate the inbred lines from each other according to their geographical regions.

REMAP markers

Out of 72 LTR and ISSR primer combinations tested in REMAP analysis, 14 combinations generated distinguishable polymorphic banding pattern, producing 120 markers out of which 113 were polymorphic. All single retro element tracer primers except for 1062 in combination with ISSR primers produced distinguishable and polymorphic banding pattern, with the scale of amplified fragments varying from 250 to 2000 bp (Basirnia *et al.,* 2014a).

In population structure analysis, the maximum value for ΔK was observed in K equal 2, so the investigated panel probably include 2 sub-populations (Figure 3). Out of 84 studied sunflower lines, 33 lines showed a mixed structure and 51 lines belonged to one of the first (37) and the second (14) subgroups (Table 1). Cluster analysis of individuals using REMAP markers, classified 84 inbred lines in seven main clusters (Figure 4). In each cluster, lines with different geographical origins were observed. Cluster A contained 20 lines; 13 from France, five from USA, one from Iran, and one from Hungary and it was represented mainly by the France inbred lines. Clusters B, C, E, F and G were dominated by the inbred lines originated from France. Cluster D contained 18 inbred lines; 12 from France, two from USA, two from Iran, one from Serbia, and one from Hungary (Figure 4).

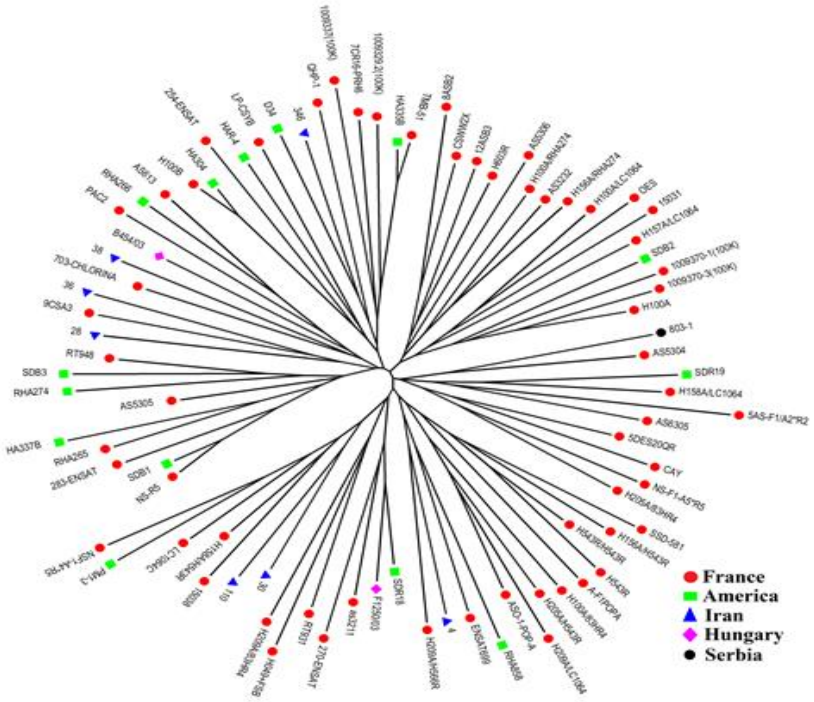

Figure 2. IRAP dendrogram of 84 sunflower germplasm using Minimum Evolution algorithm.

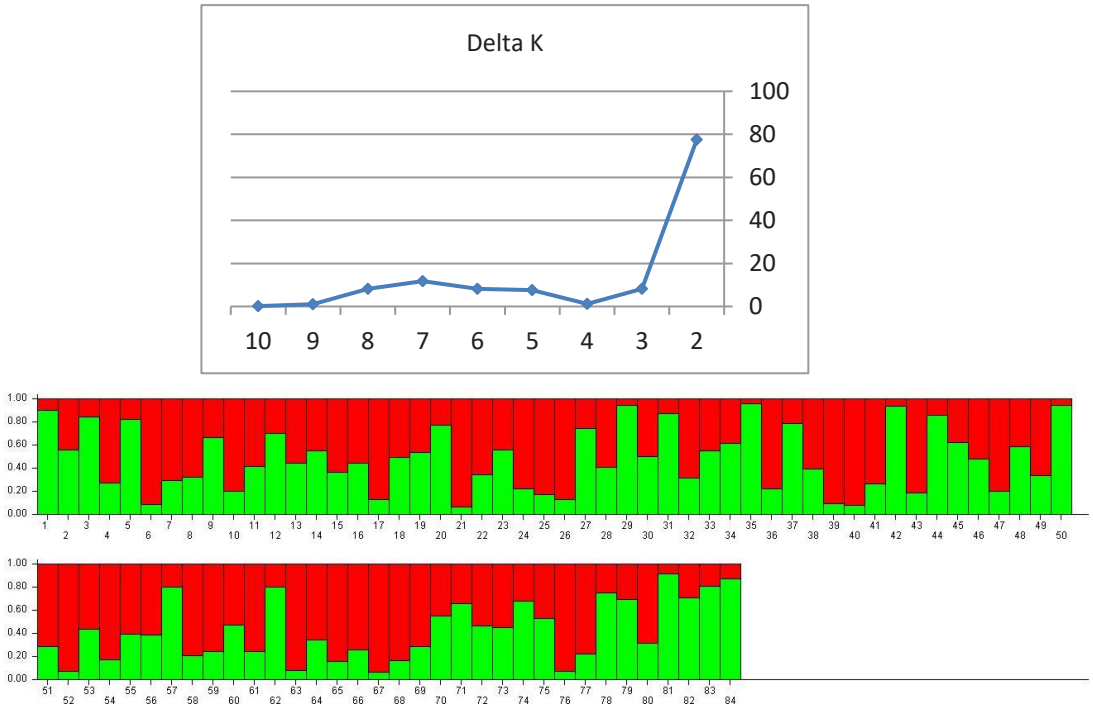

Figure 3. Bar plot of structure analysis of the studied oily sunflower germplasm according to 113 loci of REMAP using Bayesian model. Numbers on the y-axis show the membership coefficient to sub-populations and numbers on the x-axis show the individual code belonging to sunflower populations.

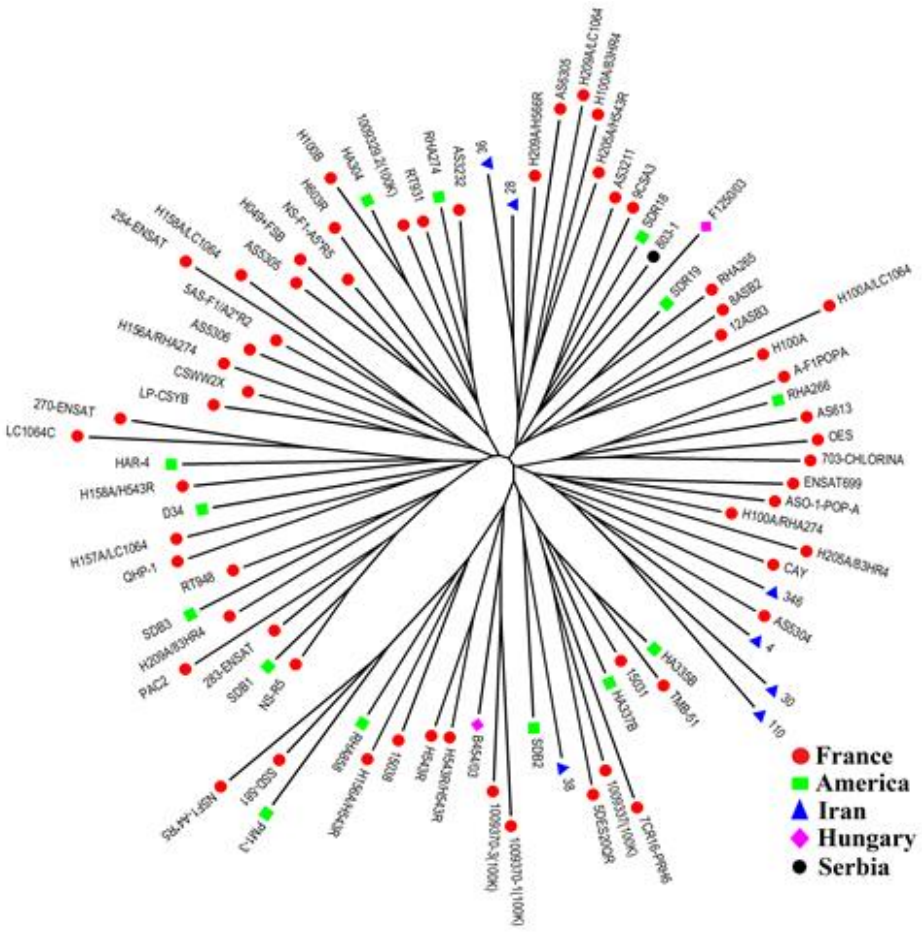

Figure 4. REMAP dendrogram of sunflower germplasm using Minimum Evolution algorithm.

IRAP+REMAP data

In population structure analysis, maximum value for ΔK was observed in K equal 2, so the investigated panel probably include 2 sub-populations (Figure 5). Cluster analysis of individuals using IRAP+REMAP markers data, classified 84 inbred lines in four main groups (Figure 6).

Association analysis

IRAP markers

Out of 8128 IRAP pairs markers, 2.09 percent showed significant r^2 value ($r^2 \geq 0.1$, P-value$\leq$0.01) (data not shown). Several factors are effective in establishing haplotype LD blocks in plants genome (Gupta *et al.,* 2005; Stich *et al.,* 2005, 2006, 2007; Oraguzie *et al.,* 2007), from them mutation and recombination rate are the essential factors that affect LD, significantly. Several IRAP loci associated with genes controlling characters were identified. Thirteen and 12 significant (P<0.01) marker-trait associations (MTA) were identified by MLM in natural and salt stress conditions, respectively (Table 5). Some markers associated with special characters such as; '63641' and '623' markers were also associated with sodium concentration in lamina under natural conditions, whereas '61658', '655' and 'crur12' markers associated with this character under salt stress conditions. 'Crur11', 'Cfcr8' and '61651' markers were associated with potassium concentration in lamina under natural conditions whereas crur12 marker was associated with this character under salt stress conditions. Marker '63641' was associated with chloride concentration in lamina under natural conditions, whereas '61651' marker was associated with this character in salt stress conditions. Marker '64651' was associated with potassium to sodium ratio in the lamina under natural conditions. Marker '61652' was associated with sodium concentration in petiole under natural conditions whereas marker '61656' was associated with this character under salt stress conditions. Marker 'Cfcr3' was associated with potassium concentration in petiole in both natural and salt stress conditions whereas '653' and '63641' markers were associated with this character in natural and salt stress conditions, respectively. Marker '63641' was associated with chloride concentration

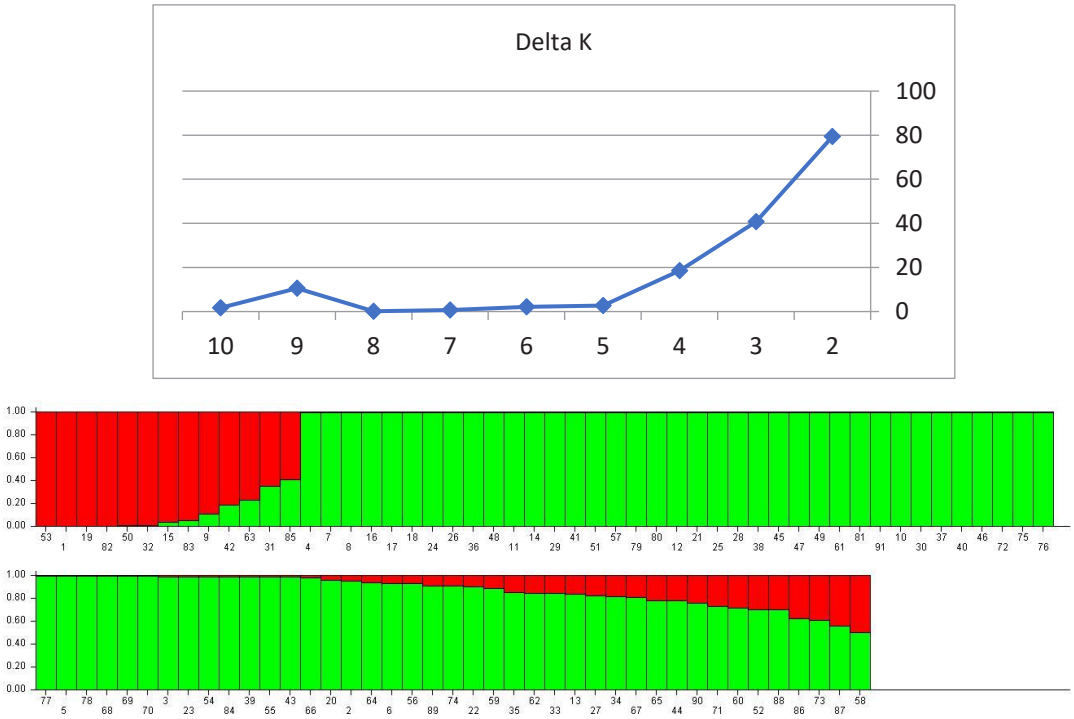

Figure 5. Bar plot of structure analysis of the studied oily sunflower germplasm according to REMAP+IRAP markers data using Bayesian model. Numbers on the y-axis show the membership coefficient to sub-populations and numbers on the x-axis show the individual code belonging to sunflower populations.
67: DM-2; 43: 803-1; 54: 283-ENSAT; 8: 254-ENSAT; 23: 8ASB2; 88: H543R/H543R; 13: A-F1POPA; 77: AS6305; 65: LC1064C; 40: F1250/03; 79: D34; 11: 270-ENSAT; 22: 12ASB3; 68: H156A/RHA274; 14: OES; 1: H100A/83HR4; 66: NS-R5; 62: HA335B; 57: HA337B; 10: 1009329.2(100K); 21: AS3232; 87: H156A/H543R; 36: 703-CHLORINA; 3: H205A/H543R; 70: HAR-4; 31: TMB-51; 59: B454/03; 20: 1009337(100K); 25: H049+FSB; 89: H543R; 16: RHA266; 2: H209A/LC1064; 69: SDB1; 42: LP-CSYB; 58: H100B; 19: 5DES20QR; 90: 15038; 74: H100A/RHA274; 17: PAC2; 76: ASO-1-POP-A; 71: AS5305; 52: PM1-3; 60: HA304; 28: 7CR16=PRH6; 49: 15031; 50: H205A/83HR4; 12: AS613; 80: CAY; 72: RHA274; 56: SDR19; 9: AS5304; 30: SSD-581; 34: H603R; 47: H158A/H543R; 81: 346; 41: SDR18; 51; RHA265: 5: RHA858: 29: ENSAT699; 37: NSF1-A4×R5; 6: H209A/83HR4; 83: 36; 61: RT931; 55: QHP-1; 7: AS3211; 24: 9CSA3; 82: NS-F1-A5×R5; 18: H157A/LC1064: 39: 30; 64: SDB3; 53: RT948; 4: AS5306; 27: 5AS-F1/A2×R2; 86: H158A/LC1064; 15: H100A/LC1064; 33: 110.

in petiole under salt stress conditions. Ufur18 marker was associated with potassium to sodium ratio in the petiole under natural conditions, whereas '62651', 'Cfcr1' and '63653' markers were associated with this character under salt stress conditions. Markers '655' and '62653' associate with grain yield per plant in natural condition (Table 5). Some markers were associated with more than one character such as '63641' that was associated with sodium and chloride concentration in lamina, potassium and chloride concentration in petiole. '61651' was associated with potassium and chloride concentration in lamina, '63641' was associated with chloride concentration in lamina, potassium and chloride concentration in petiole. '63653' was associated with potassium to sodium ratio in the petiole and grain yield per plant (Table 5). Similarly, Darvishzadeh (2016) in sunflower, Yagcioglu (2016) in watermelon and Abdollahi Mandoulakani *et al.* (2016) in wheat showed that some markers were associated with more than one character. Common markers between characters can be due to pleiotropic effects or linkage between genomic regions involved in controlling characters (Jun *et al.,* 2008). Identifying common markers is of great importance in plant breeding because it makes possible simultaneous selection of multiple characters (Tuberosa *et al.,* 2002).

REMAP markers

Out of 7140 REMAP pairs of markers, 2.39 percent showed significant r^2 ($r^2 \geq 0.1$, P-value≤ 0.01) (data not shown). Ten and 12 significant ($P<0.01$) DNA marker-character associations were detected by MLM in natural and salt stress conditions, respectively (Table 6). Some REMAP markers were associated with special traits such as '638262' that was associated with sodium concentration in lamina under salt stress conditions. 'Cf8181' was associated with potassium concentration in lamina in natural whereas '64a133', '638261' markers were associated with this trait under salt stress

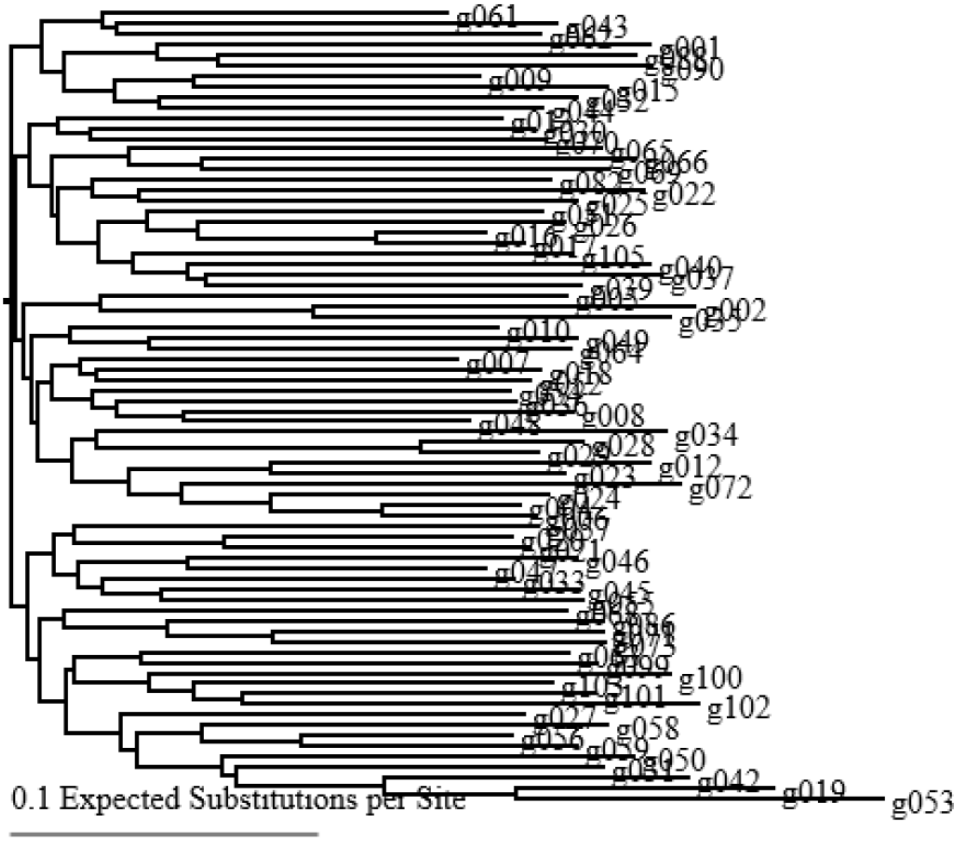

Figure 6. Dendrogram of sunflower germplasm using combined REMAP+IRAP markers data.
g001: DM-2; g002: LC1064C; g004: NS-R5; g005: HAR-4; g006: SDB1; g007: AS5305; g008: RHA274; g009: SDR18; g010: RT931; g012: SDB3; g013: 803-1: g015: F1250/03; g016: HA335B; g017: TMB-51; g018: LP-CSYB; g019: PM1-3; g020: SDR19; g021: RHA265: g022: QHP-1; g023: RT948; g024: 283-ENSAT; g025: D34; g026: HA337B; g027: B454/03; g028: H100B; g029: HA304; g030: AS5304; g031: RHA858; g032: as3211; g033; AS5306; g034; 254-ENSAT; g035: 270-ENSAT; g036: 1009329.2(100K); g037: 1009337(100K); g039: 5DES20QR; g040: 7CR16=PRH6; g042: SSD-581; g043: ENSAT699; g044: 9CSA3; g045: 5AS-F1/A2×R2; g046: 8ASB2; g047: 12ASB3; g048: AS3232; g049: H049+FSB; g050: 15038; g051: 15031; g052: H603R; g053: NSF1-A4×R5; g054: NS-F1-A5×R5; g055: H158A/LC1064; g056: H543R/H543R; g057: H156A/RHA274; g058: H156A/H543R; g059: H543R; g061: H100A/RHA274; g062: H205A/83HR4; g063: H158A/H543R; g064: H209A/83HR4; g065: H157A/LC1064; g066: H100A/LC1064; g068: A-F1POPA; g069: OES; g070: 703-CHLORINA; g071; RHA266; g072: PAC2; g073: AS613; g082; 346; g086: 36; g088: 30; g090: 110; g099: AS6305; g0100: H100A/83HR4; g101: H205A/H543R; g102: H209A/LC1064; g0103: ASO-1-POP-A; g105: CAY.

condition. '64a1310' was associated with chloride concentration in lamina in natural condition whereas '658261', '638264', 'cf8187' markers were associated with this trait in salt stress condition. '618182' was found to be linked with potassium to sodium ratio in the lamina under natural condition, whereas '618184' and '6581810' markers were associated with this trait in salt stress condition. '618402' was associated with sodium concentration in petiole in salt stress condition. '648405' was associated with potassium concentration in petiole under natural condition whereas '618575' was associated with this character in salt stress condition. '658187' was associated with chloride concentration in petiole in natural condition whereas '618185' and '638267' were associated with this trait in salt stress condition. Marker 'Cf8268' was associated with potassium to sodium ratio in the petiole in salt stress condition. '618404' and 'cf8183' markers were associated with leaf relative water content and seed yield per plant, respectively under normal condition. '618184' is a common marker between potassium concentration and potassium to sodium ratio in the lamina.

IRAP+REMAP data

Out of 11125 pairs IRAP+REMAP markers, 2.28 percent showed remarkable r^2 value ($r^2 \geq 0.1$, P-value$\leq$0.01) (data not shown). Using combined IRAP+REMAP data, eight and 12 significant ($P<0.01$) marker-trait associations (MTA) were identified by MLM for studied characters under normal and salt stress conditions, respectively (Table 7). '63641 and 64653' markers were associated

Table 5. IRAP loci identified for studied characters in oily sunflower inbred line under normal and salt stress conditions using MLM procedure.

Character	Marker	Normal condition		Salt condition	
		F-value	P-value	F-value	P-value
Sodium concentration in lamina	63641	7.55	0.0074	-	-
	623	7.22	0.0091	-	-
	61658	-	-	11.168	0.0013
	655	-	-	8.9189	0.0038
	Crur12	-	-	8.4851	0.0046
Potassium concentration in lamina	Crur11	8.78	0.004	-	-
	Cfcr8	8.86	0.0041	-	-
	61651	7.2	0.008	-	-
	Cr12	-	-	8.1792	0.0054
Chloride concentration in lamina	63641	31.29	0.0003	-	-
	61651	-	-	14.6464	0.0003
Potassium to sodium ratio in the lamina	64651	7.51	0.007	-	-
Sodium concentration in petiole	61652	10.29	0.002	-	-
	61656	-	-	7.0976	0.0094
Potassium concentration in petiole	653	8.93	0.004	-	-
	Cfcr3	7.165	0.009	7.4193	0.0082
	63641	-	-	7.3623	0.0082
Chloride concentration in petiole	63641	-	-	8.17	0.0054
Potassium to sodium ratio in the petiole	Ufur18	8.32	0.005	-	-
	62651	-	-	17.51	0.0001
	Cfcr1	-	-	13.9	0.0004
	63653	-	-	10.143	0.002
Leaf relative water content	-	-	-	-	-
Grain yield per plant	655	11.16	0.0013	-	-
	62653	7.24	0.009	-	-

with chloride concentration in lamina under normal condition. Under salt stress condition, '61651' was associated with this character. 'Cf8181', 'Crur11' and '618184' markers were associated with potassium concentration in lamina under normal condition. Under salt stress condition, 618182 was associated with this character. '61652' marker was associated with sodium concentration in petiole under normal condition. Under salt stress condition, 'Cf8185' was associated with this character. Marker '658187' was associated with chloride concentration in petiole under normal condition. Under salt stress condition, '638267' was associated with this character. Marker 64652 was associated with potassium to sodium ratio in the petiole under normal condition. Under salt stress condition, 'Cfcr1', '62651' and '63653' markers were associated with this character. Ur12 and Ur14 markers were associated with RWC and grain yield per plant under salt stress condition (Table 7).

Combined data affect the estimation of Q and kinship matrices, and therefore, the identification of markers in association analysis. The combined data provide more genomic coverage compared to single data in association analysis and therefore, their results are potentially more valuable.

Association analysis has proven to be a powerful method to dissect the complexity of quantitative characters in plants (Nordborg and Tavare, 2002; Flint-Garcia *et al.,* 2003; Mackay and Powell, 2007). Sahranavard Azartamar *et al.* (2015) using GLM and MLM association models identified some microsatellite markers associated with agro-morphological characters in 106 sunflower lines in normal conditions. Basirnia *et al.* (2014b) using a mixed linear model (MLM) procedure determined the simple sequence repeat (SSR) associated with chloride accumulation rate in oriental-type tobacco genotypes. Long *et al.* (2013) found quantitative traits loci for Na^+, K^+ and Cl- content on chromosome 4H for salt stress tolerance in *Hordeum vulgare*. Association analysis has been used in other crops such as wheat (Liu *et al.,* 2010), barley (Wang *et al.,* 2012), sorghum (Shehzad *et al.,* 2009) and corn (Anderson *et al.,* 2007). To some extent, these

Table 6. REMAP loci identified for the studied characters in oily sunflower inbred line under normal and salt stress conditions using MLM procedure.

Character	Marker	Normal condition		Salt condition	
		F-value	P-value	F-value	P-value
Sodium concentration in lamina	Cf8181	-	-	-	-
	638262	-	-	7.38	0.008
Potassium concentration in lamina	618184	12.38	0.0007	7.1581	0.009
	Cf8181	7.54	0.0076	-	-
	64a133	-	-	7.9826	0.0059
	638261	-	-	7.3299	0.0083
Chloride concentration in lamina	64a1310	7.2561	0.0086	-	-
	658261	-	-	10.6311	0.0016
	638264	-	-	8.741	0.0041
	Cf8187	-	-	7.2155	0.009
Potassium to sodium ratio in the lamina	618182	7.5321	0.0075	-	-
	618184	-	-	9.2293	0.0032
	6581810	-	-	7.7063	0.0068
Sodium concentration in petiole	618402	8.1486	0.0055	-	-
Potassium concentration in petiole	648405	9.204	0.0032	-	-
	618575	-	-	8.6932	0.0042
Chloride concentration in petiole	658187	13.0888	0.0005	-	-
	618185	-	-	10.3286	0.0019
	638267	-	-	8.8889	0.0038
Potassium to sodium ratio in the petiole	Cf8268	7.0636	0.0095	-	-
Leaf relative water content	618404	7.3844	0.008	-	-
Grain yield per plant	Cf8183	7.3482	0.0084	-	-

Table 7. Retrotransposon markers (IRAP+REMAP markers) identified for the studied characters in oily sunflower inbred lines under normal and salt stress conditions using MLM procedure.

Character	Marker	Normal condition		Salt condition	
		F-value	P-value	F-value	P-value
Sodium concentration in lamina	618189	-	-	7.68	0.007
Potassium concentration in lamina	Cf8181	10.90	0.0015	-	-
	618184	8.96	0.0037	-	-
	Crur11	7.19	0.0090	-	-
	618182	-	-	7.32	0.0084
Chloride concentration in lamina	64653	9.03	.0037	-	-
	63641	14.59	.0000	-	-
Potassium to sodium ratio in the lamina	61651	-	-	9.75	.0025
Sodium concentration in petiole	61652	7.94	.006388	-	-
Potassium concentration in petiole	618575	-	-	7.55	0.0075
Chloride concentration in petiole	638267	-	-	7.27	0.0086
Potassium to sodium ratio in the petiole	64652	9.73	0.0027	-	-
	Cfcr1	-	-	13.28	0.0000
	62651	-	-	11.84	0.0000
	63652	-	-	11.66	0.001
Leaf relative water content	Ur12	-	-	7.82	0.0065
Grain yield per plant	Ur14	-	-	7.95	0.0062

studies will accelerate applying DNA markers in plant improvement programs. Arraouadi *et al.* (2011) performed QTL analysis on physiological characters in normal and salt stress conditions in *Medicago truncatula* using recombinant inbred lines (RILs) and Simple Sequence Repeat markers. They showed that identified quantitative trait loci for characters attributed to leaves and roots were not contributed on the same map positions. Therefore, they concluded that the genomic regions involved in the transport of Na^+ and K^+ between the leaves and roots probably to be different or induced uncoordinatedly by salt stress.

Our study revealed a vast genetic variation for salt tolerance that can be exploited in sunflower improvement programs. Structure analysis partitioned the association panel in two sub-populations. We detected numerous remarkable DNA maker-trait associations over the whole sunflower genome. After validation of identified markers, they can be utilized in sunflower salt improvement programs.

CONCLUSION

Tolerance against salt stress is a quantitative character controlled by multiple genes. Our investigation revealed the existence of genetic variation for tolerance to salt stress in sunflower. Hierarchical cluster analysis based on minimum evolution (ME) algorithm and the number of different coefficients using IRAP+REMAP markers data assigned the 84 inbred lines into four main groups that could be useful for selecting parents in sunflower breeding programs. Bayesian model-based cluster analysis divided the studied inbred lines of sunflower into two sub-populations. The difference in grouping sunflower lines to different clusters is probably due to the nature of the models used in various reports. Several IRAP and REMAP markers have been identified for characters related to tolerance against salt stress that can be potentially applied in sunflower resistance improvement programs *via* marker aided selection (MAS). Some markers are linked with characters in both normal and salt stress conditions. The presence of genetic relationships between characters under various conditions indicate the presence of common QTL, which further proposes that improving a character in one environmental condition may result in the improved character in other conditions in progenies. Identified markers after validation and converting to SCAR markers can be used in marker assisted programs in sunflower for developing salt stress tolerant cultivars.

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Declarations

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Conflict of interest

The authors declare that they have no competing interests.

Availability of data and material

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability

Not applicable.

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