GENETIC DIVERSITY AND POPULATION STRUCTURE OF QUINOA (*CHENOPODIUM QUINOA* WILLD.) USING IPBS-RETROTRANSPOSONS MARKERS

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Abstract. Quinoa (*Chenopodium quinoa* Willd.) is a nutritionally important plant with a good protein quality and a high concentration of vitamins and minerals. It has been cultured for several thousand years in South America. In this study, we investigated the use of inter-primer binding site (iPBS) for the molecular characterization of 17 quinoa genotypes (*Chenopodium quinoa* Willd.) cultivated in Turkey. For this purpose, 25 iPBS markers were employed, and six primers provided sufficient polymorphic data generating a total of 19 alleles with an average of 2.83 bands/primer. The number of iPBS bands per individual was calculated as 1.12. The rate of polymorphism information content ranged from 0.02 to 0.49 with an average of 0.20. Genetic associations were assessed using the Dice dissimilarity coefficient between different pairs of accessions and revealed an average value of 0.84 for the French population and Q-52 genotypes. Cluster analysis on the unweighted pair-group mean average divided the 17 quinoa genotypes into two major clusters. The results of the principal component analysis were in agreement with those of the cluster analysis. The highest number of alleles, Nei's genetic diversity, and Shannon's information index were obtained from the French Vanilla genotype at 1.99, 0.50 and 0.69, respectively, whereas the lowest values were observed in the Q–52 genotype at 1.10, 0.09 and 0.20, respectively. The expected heterozygosity ranged from 0.398 in the first sub-population to 0.140 in the second sub-population with an average of 0.269. The mean population differentiation measurement (F_{st}) values of the sub-populations were 0.048 and 0.676 for the first and second sub-populations, respectively. The results of this study provide useful information for the management of the quinoa germplasm and contribute to the improvement of existing breeding approaches. They also presented the iPBS marker system as a suitable tool for identification and genetic diversity analysis of quinoa genotypes.
Keywords: *Bayesian clustering, Chenopodium quinoa, genetic diversity, iPBS*

Introduction

In the region of Andean-South America, quinoa (*Chenopodium quinoa* Willd.) is considered to be one of the essential food crops. It is known as a member of the amaranth family (formerly *Chenopodiaceae*), which also contains other frugally essential species, such as spinach (*Spinacea oleracea* L.) and sugar beet (*Beta vulgaris* L.). Quinoa usually grows in saline and arid soils, frequent forests, and high altitudes of the Altiplano (Prado et al., 2000). Quinoa is traditionally widely consumed as a food crop by the people of the Altiplano region as part of their daily diet. Quinoa contains a perfect balance of lipids, protein, and carbohydrates, as well as amino acids essential for human nutrition (Chauhan et al., 1999). It is an allotetraploid (2n = 4x = 36) which has a domestic inheritance of most quality characters (Ward, 2000).

To simplify the application of molecular tools and enhance basic knowledge concerning quinoa, Fairbanks et al. (1990) and Ruas et al. (1999) reported DNA-based markers for this species based on the random amplified polymorphic DNA (RAPD) method. Microsatellite markers (SSR) have been developed to characterize the quinoa germplasm by Mason et al. (2005), Christensen (2007), Fuentes et al. (2009), Costa

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(2012) and Lu et al. (2015). Ana-Cruz et al. (2017) and Al-Naggar et al. (2017) also attempted to characterize the genetic diversity of a collection of quinoa using inter-simple sequence repeats. Employing markers, such as amplified fragment length polymorphism (AFLP), RAPD, and SSR, a genetic linkage map was established for quinoa (*C. quinoa*) (Maughan et al., 2004); furthermore, single nucleotide polymorphism (SNP) markers were developed (Coles et al., 2005; Maughan et al., 2012). Anabalon-Rodriguez and Thomet-Isla (2009) detected the level of polymorphism and the genetic relationships using the AFLP technique in quinoa (*C. quinoa*).

The fundamentally predominant existence of the tRNA complement as a converse transcriptase primer binding site (PBS) in LTR retrotransposons is a source of polymerase chain reaction (PCR)-based iPBS amplification (inter-primer binding site - iPBS). Specifically, the iPBS amplification technique was developed as a remarkable DNA fingerprinting technology that does not require any primary sequence data. Therefore, for controlling the changes in the DNA profile of plants, the iPBS marker system is an easy and fast process. This technique has been successfully used in flax (Smykal et al., 2011), apricot (Baránek et al., 2012), latvia (*Saussurea esthonica* L.) (Gailite and Rungis, 2012), chickpea (Andeden et al., 2013), guava (Mehmood et al., 2016), grape (Guo et al., 2014), okra (Yildiz et al., 2015), rice (Comertpay et al., 2015), lentil and pea (Baloch et al., 2015), tea (Phong et al., 2016), saffron (Gedik et al., 2017) and motherwort (*Leonurus cardiaca* L.) (Borna et al., 2017). In this study, to simplify the application of molecular tools and offer a better understanding of the genetic diversity of quinoa genotypes, we utilized iPBS molecular markers for these species for the first time in the literature.

The purpose of this experiment was to investigate the genetic diversity of 17 quinoas genotypes using the iPBS marker system, to evaluate the structure of the diversity in the germplasm, and to generate useful information for future breeding programs on quinoa.

Materials and methods

Genetic material

The quinoa (*C. quinoa* Willd.) genotypes were collected from different countries as a work package of a project supported by The Scientific and Technological Research Council of Turkey (TUBITAK) (TOVAG 214 O 232). A total of 17 quinoas genotypes were used in the experiment (*Table 1*).

Genomic DNA isolation

Total genomic DNA from the quinoa genotypes was isolated from 300 mg young leaf tissue using the method described by Zeinalzadeh-Tabrizi et al. (2015). For determination of the concentration and quality of genomic DNA, a spectrophotometer (Thermo Fisher Scientific) was used and electrophoresis was performed in 0.8% (w/v) agarose gel.

iPBS-PCR amplification

Twenty-five primers were used in iPBS-PCR reactions (*Table 2*). The PCR amplifications were carried out in a thermal cycler (Labcycler). The PCR mixture consisted of 10x buffer, 2 μM (20 pmol) primer, 2 mM $MgCl_2$, 0.25 mM of each dNTP, 0.5 U *Taq* polymerase, and 50 ng/μl DNA template in a total volume of 20 μl. The

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amplification conditions were; an initial denaturation step of five min at 95 °C, 38 cycles of 60 s at 95 °C, 60 s at 44-60 °C and 60 s at 72 °C, and a final extension step of 10 min at 72 °C. The amplification products were resolved in 1.5% agarose gel in 1X SB buffer at 100 V/cm for 120 min, stained with ethidium bromide (0.2 ug/ml), and visualized under a UV light in a Universal Hood II (Bio-Rad, Hercules, CA, USA). The sizes of the base pairs were determined based on a DNA ladder between 50 and 1000 bp (Vivantis Product No: NM2421).

Table 1. *List of quinoa (Chenopodium quinoa Willd.) accessions used in the experiment*

Number	Genotype name	Country
1	Ecuador 7	Ecuador
2	Q haslala Blanca	Peru
3	Red population	Peru
4	Q-52	Denmark
5	White Population	United Kingdom
6	Titicaca	Peru
7	UK6	USA
8	French Vanilla	USA
9	Red Head	USA
10	Sandoval Mix	United Kingdom
11	Mint Valle	USA
12	Oro de Valle	USA
13	Chinese Population	China
14	France Population	France
15	Chery Vanilla	USA
16	Moqu Arrochilla	Peru
17	Rainbow	USA

Data analysis

For each primer, the presence and absence of a strong and sharp polymorphic band were scored as 1 and 0, respectively using TotalLab TL120 software package (Germany). The association between the genetic dissimilarity was evaluated with the Numerical Taxonomy and Multiware Analysis System (NTSYSpc version 2.0) according to the Dice similarity matrix (Dice, 1945). Using the same software, an unweighted pair-group mean average (UPGMA) tree was constructed and a principle component analysis (PCA) was undertaken (Rohlf, 1998). The diversity of each iPBS marker was calculated using polymorphism information content (PIC) according to the following equation: $PIC = 1 - \Sigma pi2$, where P_{ij} is the frequency of the patterns (j) for each marker (i) (Anderson et al., 1993). To determine the genetic parameters, the number of alleles (ne), Nei's genetic diversity (h), and Shannon's information index (I) were calculated by POPGEN 1.32 (Yeh et al., 1997).

The genetic structure datasets were formed to be determined by a model-based cluster analysis using STRUCTURE software version 2.2 (Pritchard et al., 2000a, b). The number of populations (K) was presented for a run of ten times for each population, which varied from 2 to 10, characterized by a set of distinctive allele frequencies at each locus, and individuals were situated in K clusters. In this process, the posterior

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probabilities were estimated using the Markov Chain Monte Carlo (MCMC) method. The MCMC chains were run with a 100,000-iteration burn-in period, followed by 100.000 iterations using a model allowing for admixture and correlated allele frequencies. The most expected value for K was predicted using Evanno's ΔK method (Evanno et al., 2005) using STRUCTURE HARVESTER. Furthermore, the expected heterozygosity (gene diversity) and population differentiation (Fst) between the individuals in a sub-population were determined using STRUCTURE (Earl, 2012).

Table 2. *Primers used in iPBS -PCR and retrotransposon, amplified fragment length*

Primer no.	Primer name	Primer sequence (5′→3′)
1	iPBS-2074	GCTCTGATACCA
2	iPBS-2075	CTCATGATGCCA
3	iPBS-2076	GCTCCGATGCCA
4	iPBS-2077	CTCACGATGCCA
5	iPBS-2080	CAGACGGCGCCA
6	iPBS-2221	ACCTAGCTCACGATGCCA
7	iPBS-2222	ACTTGGATGCCGATACCA
8	iPBS-2224	ATCCTGGCAATGGAACCA
9	iPBS-2225	AGCATAGCTTTGATACCA
10	iPBS-2270	ACCTGGCGTGCCA
11	iPBS-2279	AATGAAAGCACCA
12	iPBS-2375	TCGCATCAACCA
13	iPBS-2376	TAGATGGCACCA
14	iPBS-2377	ACGAAGGGACCA
15	iPBS-2378	GGTCCTCATCCA
16	iPBS-2379	TCCAGAGATCCA
17	iPBS-2380	CAACCTGATCCA
18	iPBS-2381	GTCCATCTTCCA
19	iPBS-2390	GCAACAACCCCA
20	iPBS-2391	ATCTGTCAGCCA
21	iPBS-2392	TAGATGGTGCCA
22	iPBS-2400	CCCCTCCTTCTAGCGCCA
23	iPBS-2401	AGTTAAGCTTTGATACCA
24	iPBS-2402	TCTAAGCTCTTGATACCA
25	iPBS-2415	CATCGTAGGTGGGCGCCA

Results and discussion

Polymorphism revealed by iPBS primers

In this experiment, 25 iPBS primers were used to evaluate 17 quinoa genotypes, and only six primers generated a sufficient number of polymorphisms (*Fig. 1*) with four primers providing more than one polymorphic band. The average of polymorphic bands was calculated as 2.83, and that of monomorphic bands was 0.33. Based on these values, the number of iPBS bands per individual was calculated as 1.12 (*Table 3*).

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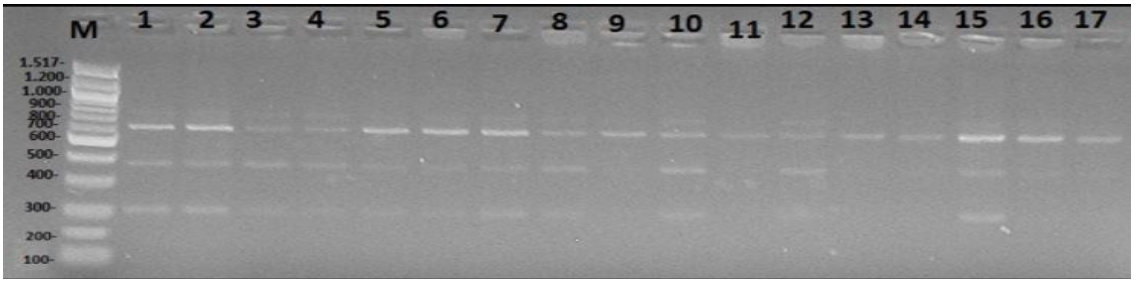

Figure 1. *iPBS profiles of 17 quinoa accessions obtained with the primer iPBS 2391*

Table 3. *Diversity statistics for Quinoa (Chenopodium quinoa Willd.) with iPBS*

Primer name	Allele number	Percentage of polymorphism	PIC value
iPBS-2080	7	100.00%	0.49
iPBS-2270	1	100.00%	0.03
iPBS-2279	3	66.66%	0.14
iPBS-2390	1	100.00%	0.02
iPBS-2391	4	75.00%	0.23
iPBS-2392	3	100.00%	0.18

Similar to our results, Mehmood et al. (2013) selected 6 primers out of 83 iPBS primer set in guava genotypes and Guo et al. (2014) selected 15 primers out of 41 iPBS primers in grape varieties, for further analysis. This is also in agreement with reports on other plants, such as grape (Guo et al., 2014), guava (Mehmood et al., 2016), and those investigated by Kalendar et al. (2010). The total number of polymorphic bands was 19 for iPBS markers, of which 17 were polymorphic and the remainder were considered as monomorphic. In this research, the number of alleles/polymorphic loci ranged from one to seven with an average of 3.16 for the iPBS analysis. Furthermore, the highest number of polymorphisms was obtained from iPBS-2080 with seven bands. In a study that aimed to develop molecular markers for the Fusarium wilt resistance gene in eggplant, Mutlu et al. (2008) found an average number of 1.5 bands per primer. The PIC value varied between 0.02 (iPBS 2390) and 0.49 (iPBS 2080), with an average of 0.20 (*Table 3*). These results are in agreement with earlier research into different plants, such as guava (*Psidium guajava* L.) cultivars with an average PIC of 0.28 (Mehmood et al., 2016) and tea (*Camellia sinensis*) cultivars with an average PIC of 0.30 (Phong et al., 2016). The percentage of polymorphism (%) value ranged from 66% (iPBS 2279) to 100% (iPBS 2080, 2390 and 2392) with an average of 90.27% (*Table 3*).

Genetic diversity in quinoa

Table 4 presents a summary of the statistical results for each of the 17 quinoa genotypes. The highest number of alleles (ne), Nei's genetic diversity (h), and Shannon's information index (I) were obtained from the French Vanilla genotype at 1.99, 0.50 and 0.69, respectively, whereas the lowest values were observed in the Q–52 genotype at 1.10, 0.09 and 0.20, respectively. In addition, the total average number of alleles (ne), Nei's genetic diversity (h), and Shannon's information index (I) were 1.52, 0.032 and 0.49, respectively. Fuentes et al. (2009) used 20 SSR loci and calculated

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Shannon's index as 2.582 for the Highland genotype and 3.714 for the coastal genotypes Furthermore, the average value of Shannon's information index was reported as 0.12 in the Turkish okra germplasm (Yildiz et al., 2015), 0.29 for the *Crocus sativus* genotypes (Gedik et al., 2017), and 0.27 for guava (Mehmood et al., 2013) using iPBS-retrotransposon markers.

Table 4. *Summary statistics for 17 for Quinoa genotypes assessed with 10 iPBS primers*

Number	Genotype name	(ne)*	(h)	(I)
1	Ecuador 7	1.36	0.27	0.44
2	Q haslala Blanca	1.63	0.39	0.58
3	Red Population	1.63	0.39	0.58
4	Q - 52	1.10	0.09	0.20
5	White Population	1.63	0.39	0.58
6	Titicaca	1.23	0.19	0.34
7	UK6	1.23	0.19	0.34
8	French Vanilla	1.99	0.50	0.69
9	Red Head	1.50	0.33	0.51
10	Sandoval Mix	1.11	0.10	0.21
11	Mint Valle	1.50	0.33	0.51
12	Oro de Valle	1.36	0.27	0.44
13	Chinese Population	1.95	0.49	0.68
14	France Population	1.36	0.27	0.44
15	Chery Vanilla	1.50	0.33	0.51
16	Moqu Arrochilla	1.87	0.47	0.66
17	Rainbow	1.87	0.47	0.66
Mean		**1.52**	**0.32**	**0.49**

*ne = Effective number of alleles, h = Nei gene diversity, I = Shanon information index

Cluster analysis and principal component analysis for iPBS-retrotransposon markers

The Dice genetic similarity coefficient was used for the diversity estimation of the genotypes. This coefficient is commonly utilized to estimate genetic distances. The genetic similarity between the accessions based on the iPBS markers ranged from 11% to 100% with an average value of 55.5%. In this study, the 100% similarity value was found between the two most closely related accessions, Titicaca and UK6, and Q haslala Blanca and Red (*Table 5*). Nemli et al. (2014) reported similar findings in a study that explored the genetic similarity between the accessions of common bean (*Phaseolus vulgaris* L.) by peroxidase gene-based markers. The authors found the similarity coefficients to vary between 0.7 and 1.

The UPGMA tree constructed using the Jaccard genetic distance coefficient is presented in *Figure 2*. The analysis divided the quinoa genotypes into two main groups: Group A containing three genotypes (17.65%) and group B containing 14 genotypes (82.35%). Group A had two sub-groups: the first containing French Population and the second comprising the genotypes Chinese Population and Mint Valle. Similar to group A, group B had two sub-groups; the first containing Rainbow, Moqu Arochilla, and Red Head and the second comprising French Vanilla, USA 4, Red Population, Q Blanca, Sandoval Mix, Q-52, Chery Vanilla, Oro de Volle, UK6, Titicaca, and Ikwadur 7 (*Fig. 2*).

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Table 5. *Dice genetic similarity coefficient among 17 quinoas genotypes based iPBS*

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)
(1) Ecuador 7	1																
(2) Qhaslala Blanca	0.11	1															
(3) Red Population	0.11	1.00	1														
(4) Q-52	0.16	0.26	0.26	1													
(5) White Population	0.11	0.21	0.21	0.26	1												
(6) Titicaca	0.05	0.16	0.16	0.11	0.16	1											
(7) UK6	0.05	0.16	0.16	0.11	0.16	1.00	1										
(8) French Vanilla	0.32	0.21	0.21	0.47	0.32	0.37	0.37	1									
(9) Red Head	0.37	0.47	0.47	0.21	0.37	0.32	0.32	0.58	1								
(10) Sandoval Mix	0.11	0.21	0.21	0.05	0.21	0.16	0.16	0.42	0.26	1							
(11) Mint Valle	0.63	0.53	0.53	0.79	0.53	0.68	0.68	0.32	0.58	0.74	1						
(12) Oro de Valle	0.11	0.21	0.21	0.16	0.21	0.05	0.05	0.42	0.37	0.21	0.63	1					
(13) Chinese Population	0.42	0.42	0.42	0.58	0.32	0.47	0.47	0.32	0.37	0.53	0.21	0.42	1				
(14) France Population	0.68	0.58	0.58	0.84	0.58	0.74	0.74	0.37	0.63	0.79	0.16	0.68	0.26	1			
(15) Chery Vanilla	0.16	0.26	0.26	0.21	0.26	0.11	0.11	0.47	0.42	0.26	0.68	0.05	0.47	0.63	1		
(16) Moqu Arrochilla	0.42	0.53	0.53	0.37	0.42	0.47	0.47	0.63	0.26	0.32	0.53	0.42	0.32	0.47	0.37	1	
(17) Rainbow	0.53	0.63	0.63	0.37	0.53	0.47	0.47	0.74	0.26	0.42	0.63	0.42	0.42	0.47	0.37	0.11	1

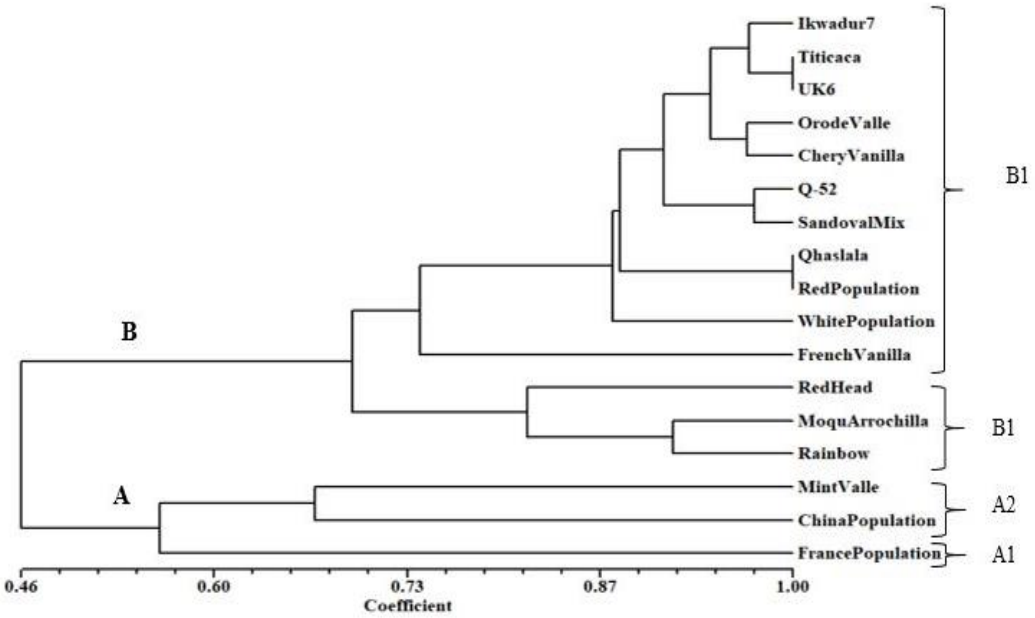

Figure 2. *Dendrogram of 17 Quinoa genotypes based on iPBS markers according to UPGMA with the Dice similarity index*

As a one-dimensional reduction technique, PCA can be used to review molecular marker profiles into some uncorrelated components (Hotelling, 1933). The pattern of cluster analysis was further confirmed by PCA because this method provides a better-defined structure than a dendrogram. In the current study, PCA was used to determine the variations between the two populations (*Fig. 3*). The clustering of varieties based on a dendrogram and a PCA plot was similar with no discrepancy being observed. This result is supported by Belaj et al. (2002) and Zargar et al. (2014), who reported similar

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findings using RAPD and SSR markers obtained similar findings related to the diversity of 32 olive cultivars based on the dendrogram topologies.

Christensen et al. (2007) evaluated the hereditary mutation in the USDA and CIP-FAO international nursery collections of quinoa (*Chenopodium quinoa* Willd.) by means of microsatellite markers and found that both UPGMA and PCA analyses divided the quinoa accessions into two key groups. Fuentes et al. (2009) investigated the genetic variety patterns in the Chilean quinoa (*Chenopodium quinoa* Willd.) germplasm using multiplex fluorescent microsatellite markers and reported that PCA divided PC1 into two major branches, conforming the clusters and groups of the highland and coastal quinoa accessions of the UPGMA analysis.

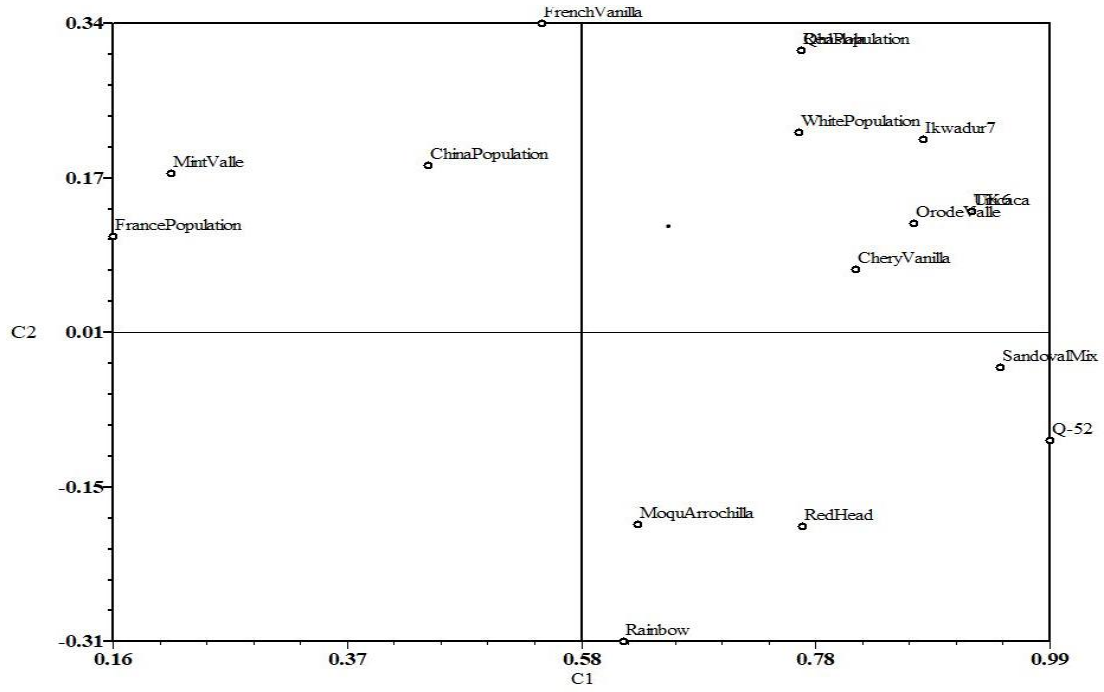

Figure 3. *PCA of 17 Quinoa genotypes based on 10 iPBS markers*

Population genetic structure analysis for iPBS-retrotransposon markers

Crop improvement is based on the understanding of the population assembly of germplasm collections. Before performing an association mapping study, it is crucial to first set the population structure within the germplasm to avoid spurious associations (Flint-Garcia et al., 2005). In this research, the population structure of the 17 quinoa accessions was categorized according to the iPBS data using STRUCTURE version 2.2 (*Fig. 4*) and two sub populations were found. Population 1 (POP 1) contained three accessions (French Population, Chinese Population and Mint Valle) and Population 2 (POP 2) consisted of the following 14 accessions (82.35% with membership probability of <0.7): Rainbow, Moqu Arochilla, Red Head, French Vanilla, USA 4, Red Population, Q Blanca, Sandoval Mix, Q-52, Chery Vanilla, Oro de Volle, UK6, Titicaca, and Ikwadur 7). The membership coefficient of the genotypes to specific sub-populations was very high and no possible admixture was detected in a reduced number of landraces. This may be due to the high rate of self-pollination of quinoa. Similar results were reported by Zhang et al. (2017) investigating the development of novel InDel

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markers and genetic diversity in *Chenopodium quinoa* through whole-genome re-sequencing. The authors found that according to both L (K) and △K values, the two groups presented the optimal classification for these quinoa accessions. Parallel results were reported with high degrees of genetic variation as detected by AFLP in *Sideritis tmolea* by Nemli et al. (2014), who revealed the presence of a model-based structural analysis of two populations. Yoon et al. (2012) reported genetic diversity and population structure analysis of two populations of strawberry (Fragaria x ananassa Duch.) using SSR markers.

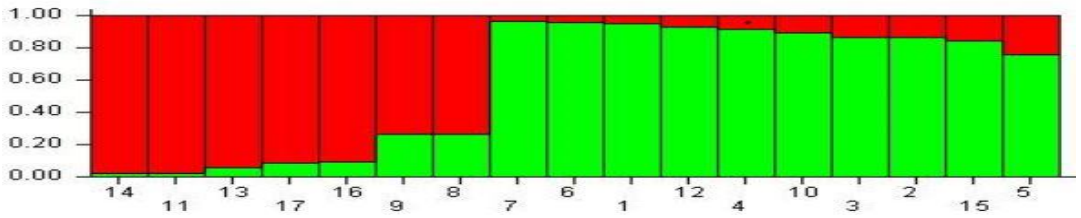

Figure 4. *Genetic structure of 17 quinoas genotypes as inferred by STRUCTURE software with 10 iPBS marker data sets. Single vertical line represents an individual accession and different colors represent genetic stocks/gene pools. Segments of each vertical line show extent of admixture in an individual*

In the current study, the population structure analysis confirmed the grouping of the genotypes, as observed by PCA and UPGMA clustering analyses. Similarly, Chen et al. (2015), who developed SSR markers to assess the genetic diversity of adzuki bean in the Chinese germplasm collection, showed that the structure and cluster analyses were usually consistent.

These results support the idea that dissimilar elevations in a topographical area might result in various levels of selection pressure for modification and could increase the differences within a population (Lopez-Gartner et al., 2009). The variation between gene pools suggests that cross breeding among these diverse areas will accelerate the process of diversifying germplasm creation and widen germplasm resources of quinoa. Meanwhile, efforts are being made to gather samples from different regions and produce the most effective markers to clarify the genetic diversity, population structure, and other details of population changeability in this ergonomically important genotype.

The expected heterozygosity which measures the probability of two randomly chosen individuals being different (heterozygous) in a given locus ranged from 0.398 in population 1 to 0.140 in the population 2 with an average of 0.269. The mean population differentiation measurement (F_{st}) values of the sub-populations were 0.048 and 0.676 (*Table 6*) for the first and second sub-populations, which was relatively high confirming the separation of the two sub-populations and their diversity in iPBS alleles. Similar results were reported by Zargar et al. (2016) who found that according to the population differentiation measurements (Fst), there were two distinct clusters or populations with an average Fst of 0.3301, indicating a clear separation of the sub-populations and their diversity in RAPD and SSR alleles. Blair et al. (2012) analyzed 108 common bean genotypes using 36 fluorescently labeled SSRs and also observed a high Fst value (0.203) for the genetic differentiation between all the five populations. In the current study, we obtained an even higher Fst value as a result of using the iPBS marker system.

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Table 6. *Heterozygosity and Fst value calculated for 2 quinoas sub-populations*

Sub-population (K)	Expected heterozygosity	Fst value
1	0.398	0.048
2	0.140	0.676
Average	0.269	0.362

Conclusion

Molecular markers are effectively used to explore genetic variation to enhance breeding efficiency. This study was undertaken using iPBS molecular markers for quinoa genotypes to simplify the application of this method and provide essential data on these genotypes. We also effectively categorized the population structure of 17 quinoa genotypes cultivated in Turkey using iPBS markers and model-based clustering. Moreover, the data obtained from the population structure analysis is valuable to perform association mapping on quinoa genotypes for various traits. The results obtained during the study can assist in the decision-making process concerning the selecting of markers for future experiments, as well as further characterization, breeding and management of the quinoa germplasm.

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