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SRAP and ISSR genetic markers and seed protein electrophoresis of some quinoa (*Chenopodium quinoa* Willd.) genotypes

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Abstract

Egypt, with its growing population, suffers from a lack of self-sufficiency in important food crops such as wheat and depends on imports from abroad. Therefore, we urgently need to develop agriculture and introduce new crops that help reduce the food gap. Quinoa is a promising new crop introduced to Egypt through the few past years. Molecular and biochemical markers were used to identify the level of polymorphism and to study the genetic relationships among quinoa genotypes. Ten quinoa genotypes characterized by using ten genetic molecular primers (five ISSR and five SRAP markers) as well as protein electrophoresis pattern. The genetic polymorphism analysis revealed that the 10 primers combinations successfully amplified and generated 138 bands of which 88 (64%), were polymorphic across the 10 genotypes. The PIC values for ISSR and SRAP primers combination varied from 0.23 to 0.21 respectively, with an average of 0.22. The general average of MI values was 1.99 for ISSR and SRAP. While, general average of resolving power (RP) was 4.4% for all primer's combinations**.** Nei and Li/Dices similarity coefficients ranged from 0.75 to 0.91 for ISSR and SRAP primers combinations. Also**,** protein electrophoresis showed seven common bands among the ten quinoa genotypes. Egyptian and Black genotypes revealed unique bands at the molecular weights 58 and 44 kDa, respectively. Finally, five polymorphic protein bands produced 42% of polymorphism. Obtained results indicated that molecular and biochemical markers techniques are useful in the establishment of the genetic fingerprinting and estimation of genetic relationships among quinoa genotypes.

1. Introduction

Quinoa (*Chenopodium quinoa* Willd.) is a pseudocereal of the Amaranthace family which originated from the Andes of South America where it has been cultivated since more than 5,000 years ago [1]. Quinoa is an allotetraploid genus (2n=4x=36) thus exhibits disomic inheritance for most qualitative traits [2]. *Chenopodium quinoa* Willd., commonly known as quinoa, is a native crop of South America, which has been traditionally used as a staple food source by ancestral populations along the Andes region [3].

Over the past decade, quinoa has gained worldwide attention because of its nutritional value and functional features [4]. Where, quinoa is one of the Andean crops with little research in the area of genetics and plant breeding, although, it has a high variability in its characteristics [5]. In country like Egypt where the population is very high and the cultivated area is very limited, crop like quinoa (*Chenopodium quinoa* Willd.) can play an important role in food supply. This because quinoa can be cultivated, and give considerable yield, in new reclaimed poor sandy soil with saline water [6-7]. The Danish Company Eghøjgaard and the Egyptian Natural Oil Company (NATOIL) has been established a partnership since the year 2007 for promoting quinoa in Egypt [8]. Shams [9] showed successfully growing Quinoa under Egyptian condition, where, he tested 13 varieties and strains in field trials in the Fareast Sinai Peninsula (South Sinai governorate) which proved to be a success**.**

Hirich *et al.*, [10] reveled a high potential of adaptation of Quinoa in Morocco. Many research teams worldwide indicate that there is a great interest for this crop in developing countries, since it is considered as one of the most important future crops involved in feed conditions improvement of this century. In the last years, quinoa has begun to occupy areas of agricultural land and is integrated into the agricultural map of many countries of the worldwide due to the remarkable nutritional properties of its seeds, that include high protein content and essential amino acids (including lysine), fats, flavonoids, vitamins and minerals and as a gluten-free product [11-12].

Ruas *et al.*, [13] reported DNA-based markers based on the random amplified polymorphic DNA (RAPD) method to simplify the application of molecular tools and enhance basic knowledge concerning quinoa. Number of researchers e.g., Mason *et al.*, [14]; Al-Naggar *et al.*, [15] studied the genetic diversity in quinoa germplasm using microsatellite markers (SSR) and inter-simple sequence repeats (ISSR) to detect polymorphism. However, ISSR markers are simpler to use than

SSR technique [16]. Moreover, theoretically, ISSR markers are considered superior to RAPD [17]. ISSR markers have been used to characterize gene bank accessions [18], as well as to identify closely related cultivars [19]. ISSR markers show good agreement with morphological, biochemical, and other molecular markers. The use of ISSR does not require prior knowledge of the target sequences flanking the repeat regions, is not expensive and is relatively easy to score manually compared to SSR.

On the other side, Li and Quiros, [20] informed that SRAP is based on two-primer amplification of open reading frames (ORFs) by targeting the exonic regions, intronic regions and regions with promoters and the primers are 17 or 18 nucleotides long. SRAPs amplify several reproducible and polymorphic loci and alleles, and they may amplify functional genes since they are sequence related. Compared with the other molecular marker systems, SRAP markers are more reproducible and not complex. SRAP had been applied in various studies such as genetic linkage map construction [20-21], genetic diversity [22] and evolutionary study [23]. Proteins and isozymes as simple cheap techniques have been successfully used to identify wild Chenopodiaceae species [24-25]. Maughan *et al.,* [2] was used the data of isozymes in quinoa for confirming the genetic difference between ecotypes of the plateau and valleys for help to made a genetic map in order to establish genotypic differences between quinoa from the North and the South of Chile.

Wilckens *et al.,* [26] reported that successful use of stored proteins of seed (isoenzymes) as biochemical markers. Seed protein electrophoresis has been utilized as a powerful tool in solving taxonomic problems and explaining the origin and evolution of a number of cultivated plants [27-28]. Only few lines of investigation exist to establish applied genetics and molecular characteristics of this crop. Up to now, only a few researchers have reported the development and use of breeding, biochemical and molecular markers in quinoa.

The main objectives of the present study were to investigate the molecular diversity among and within ten quinoa genotypes growing successfully in Egypt and to compare the seed protein electrophoresis under climatic conditions in El Minia, Egypt to use this information in future breeding programs.

2. MATERIALS AND METHODS

2.1. Experimental site and set up:

Seeds of ten quinoa (*Chenopodium quinoa* Willd.) genotypes were kindly offered by Agricultural Research Centre (ARC, Giza) and Desert Research Centre (DRC, Cairo), Egypt. Those genotypes are one quinoa commercial cultivar (Rainbow), one official variety (Regalona Bar) and 8 other genotypes (i.e., Egyptian, Misr-1, Q-37, Black, KVL-SRA3, KVL-SRA2, KVL-5204 and Q-52) were used in this study. The field trials were carried out in the Experimental Farm of Faculty of Agriculture, Minia University, El-Minia, Egypt; under irrigated conditions, with no fertilization during the 2017-2019 two growing seasons. Sowing date was conducted at the 3rd week of November, all agricultural practices and the experimental design were mentioned in details in our previous published paper [29].

2.2. Molecular analyses:

2.2.1. DNA isolation: Genomic DNA was extracted from young leaf tissues (2-week-old seedlings) of ten quinoa genotypes following the CTAB method described by Doyle and Doyle [30]. The quality of DNA was checked on 0.8 % agarose gel and the concentration were measured using UV spectrophotometer at 260 nm.

2.2.2. ISSR and SRAP detection and analyses.

A total of 5 ISSR primers and 5 SRAP primers combinations **(Table 1)** obtained from Metabion International AG Company (Germany) were used. The reaction conditions were optimized and mixtures (25 µL total volume) were composed of 11.7 µL dH_2O, 3.0 µL 10X reaction buffer, 3.0 µL dNTP's mix (2.5 mM each dNTP; Promega), 2.0 µL primer (2.5 µM) for ISSR. While for SRAP analysis, 1.0 µL forward primer, 1.0 µL reverse primer, 4.0 µl $MgCl_2$ (25 mM), 0.3 µL *Taq* DNA polymerase (5 U per µL; Promega) and 1 µL template DNA (50 ng per µL). PCR procedures were carried out in a Lab Cycler (Model Senso Quest, GmbH, Germany).

2.2.3. The PCR amplification conditions:

For ISSR; protocol was as follows: initial denaturation for 5 min at 94ºC, 45 cycles of 1 min denaturation at 92ºC, 1 min annealing at 38ºC - 44ºC and 2 min extension at 72ºC, 10 min final extension at 72ºC, then followed by a final hold at 4ºC. While, for SRAP; was as follows initial denaturation for 4 min at 94ºC, 10 cycles of 1min denaturation at 92ºC, 1 min annealing at 35ºC and 2 min extension at 72ºC, 35 cycles of 1 min denaturation at 92ºC, 1 min annealing at 50-55ºC and 2 min extension at 72ºC, 10 min final extension at 72ºC, then followed by a final hold at 4ºC. Amplification products were separated on agarose 2% and 2.5% for ISSR and SRAP, respectively. Gels were stained with ethidium bromide (EB; 0.5 µg/ml) and DNA fragments were visualized using GelDoc-It®2 Imager.

Table 1: List of 5 ISSR and 5 SRAP primers combinations used to check the polymorphism of 10 deferent quinoa genotypes (*Chenopodium quinoa* Willd.) in the present study.

	Primers	Sequences (5´to 3´)	
ISSR	HB06	GACAGACAGACAGACA	
	HB10	GAG AGA GAGAGA CC	
	HB13	GACGACGAC GC	
	HB14	GTGGTGGTG GC	
	HB09	GTGTGTGTGTGT GG	
SRAP	SRAP-1	F	TGA GTC CAA ACC GGA TA
		R	GAC TGC GTA CGA ATT AAT
	SRAP-2	F	TGA GTC CAA ACC GGA TA
		R	GAC TGC GTA CGA ATT GAC
	SRAP-3	F	TGA GTC CAA ACC GGA TA
		R	GAC TGC GTA CGA ATT CAA
	SRAP-4	F	TGA GTC CAA ACC GGA GC
		R	GAC TGC GTA CGA ATT TGA
	SRAP-5	F	TGA GTC CAA ACC GGA GC
		R	GAC TGC GTA CGA ATT CAA

2.2.4. SDS PAGE Protein:

One dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted according to the method of Laemmli, [31]. Soluble seed proteins were extracted

from mature and healthy seeds of each genotype/accession using 20 mM Tris-Cl extraction buffer (pH 8.0) containing 2 mM EDTA and 1 mM PMSF. Protein concentration in each sample was determined according to Bradford *et al.*, [32]. SDS-PAGE of the extracted seed protein was carried out on 15% polyacrylamide gel. The electrophoretic profile of seed proteins of each accession was recorded as presence (1) or absence (0) of a band of a particular molecular weight.

2.2.5. Statistical Analyses.

DNA and protein generated bands were counted and their molecular sizes were compared with protein and DNA markers. The presence or absence of DNA and protein, isozymes bands were entered into SPSS-10 computer program**.**

3. RESULTS AND DISCUSSION

3.1. Molecular markers:

3.1.1. Polymorphism based on ISSR and SRAP:

Molecular markers are highly heritable, are available in high numbers, and often exhibit enough polymorphism to discriminate closely related genotypes. In the present investigation, analysis of inter-simple sequence repeats (ISSR) and sequence related amplified polymorphisms (SRAP) were conducted to characterize the genetic markers and differences on a molecular level among the 10 quinoa genotypes and assay the genetic relationships among commercial cultivar (Rainbow), one official variety (Regalona Bear) and 8 others genotypes (Egyptian, Misr-1, Q-37, Black, KVL-SRA3, KVL-SRA2, KVL-5204 and Q-52). Five ISSR primers and five SRAP primers **(Table 1),** were tested to amplify the template DNA profiles of those ten quinoa genotypes (*Chenopodium quinoa* Willd.).

Total number of ISSR and SRAP generated fragment.

Data in **(Table 2)** showed the summary of ISSR and SRAP primer combination characteristics representing 10 quinoa (*Chenopodium quinoa* Willd.) genotypes using five primers of ISSR and five primers of SRAP.

3.1.1.1. ISSR primers:

A total of 60 amplified fragments (amplicons) with an average 12% DNA ranged from 141 bp to 1.6 kp was recorded (**Figure 1).** The number of polymorphic bands was 39 with an average 7.8/primer, while, the percentage of polymorphic bands was 64.77% when polymorphic information content as average 0.23% and marker index 1.86% as well as resolving power was 4% **(Table 2).** These results in agreement with Al-Naggar *et al*., [15] who detected 10 ISSR primers produced 53 amplicons, out of them 33 were polymorphic and the average percentage of polymorphism was 61.83%. The number of amplicons per primer ranged from 3 to 10 with an average of 5.3 fragments/primer across the different quinoa genotypes, on the other hand these data are suitable for estimating genetic diversity when compared with others species that used ISSR markers [33-34]. ISSRs are polymorphic markers that are useful for the discrimination of closely related quinoa individuals [35-16].

3.1.1.2. SRAP primers.

A total of 78 amplified fragments (amplicons) with an average 15.6% DNA ranged from 56 bp to 1.25 kp was recorded (**Figure 2)**. The number of polymorphic bands was 49 with an average 9.8/primer, while, the percentage of polymorphic bands was 63.42% when polymorphic information content as average 0.21% and marker index 2.12% as well as resolving power was 4.8% **(Table 2).** These results consist with that the result of Khaled *et al.*, [36]. They mentioned that SRAP analysis showed that the average of the percentage of polymorphism band (PPB%) was 62.59%, as well as the average of polymorphic information content (PIC) was 0.23. Moreover, the means of marker index (MI) was 1.49. In eight wheat genotypes, the ME1F-EM5R and ME9F-EM3R primers combination showed higher range of polymorphism of 88.89% and 90.91%, respectively. The 10 primers combinations successfully amplified and generated 138 bands of which 88 (64%) were polymorphic across the eight genotypes. The polymorphism information content (PIC) index has been used extensively in many genetic diversity studies [37-38]. Moreover, the PIC values indicate the usefulness of DNA markers for gene mapping, molecular breeding and germplasm evaluation [39].

In our study, the PIC values for ISSR and SRAP primers combination varied from 0.23 to 0.21 respectively, with an average of 0.22. The ISSR and SRAP primers combinations showed that the marker index (MI) values ranged from 0.75 to 3.37 with an average of 1.86 and 2.12 for ISSR and SRAP, respectively and general average 1.99 **(Table 2).** Khaled and Hamam [40] found among 36 bread wheat genotypes that the average of polymorphic information content ranged from 0.15 to 0.16, also the marker index was 1.03 reflecting that SRAP marker are more efficient in genetic diversity assessment. In our data, the primers showed resolving power (RP) with an average 4% for ISSR primers and 4.8% for SRAP primers with a general average 4.4% for all primer's combinations. High level of diversity was obtained similar to result of Alghamdi *et al.*, [41]. by the other hand These results agreement with Polat, *et al.*, [42] mentioned that the amplified fragments ranged in size from 390 bp (primer 112) to 1.6 Kb (primer 809). A low level of genetic diversity among the Czech strains were obtained (h=0.0203, I=0.0367), whereas the results underlined a moderate variability in the Israeli genetic background.

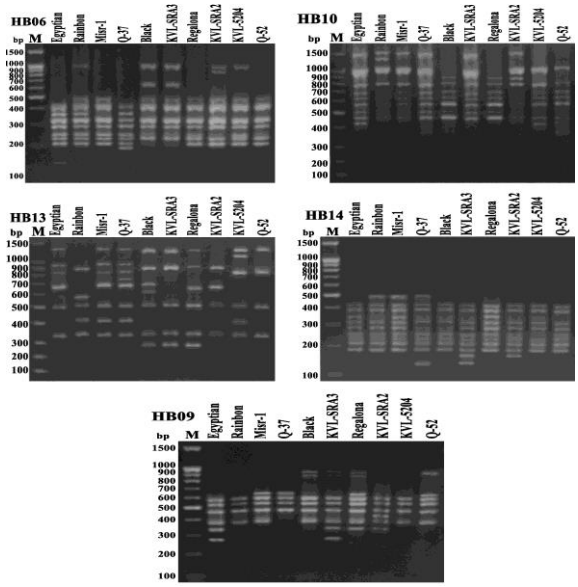

Figure (1): Agarose gel electrophoresis of amplification products obtained with ISSR primers in ten Quinoa (*Chenopodium quinoa* Willd.) genotypes.

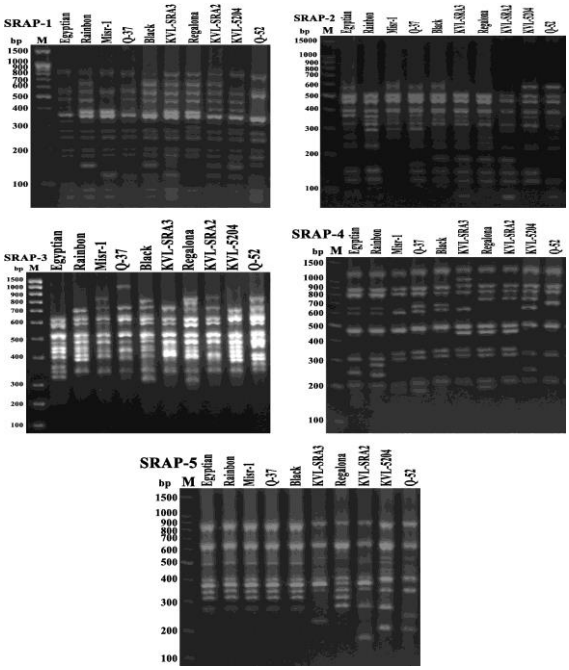

Figure (2): Agarose gel electrophoresis of amplification products obtained with SRAP primers in ten Quinoa (*Chenopodium quinoa* Willd.) genotypes.

Table (2): Summary of ISSR and SRAP primer combination characteristics.

Markers	TB*	NPB	PPB%	PIC	MI	RP%
HB06	13	9	69.23	0.22	2.01	4.2
HB10	12	7	58.33	0.20	1.41	3.4
HB13	13	11	84.62	0.31	3.37	6.2
HB14	12	5	41.67	0.15	0.75	2.4
HB09	10	7	70.00	0.25	1.78	3.8
Total	60	39				
Average	12	7.8	64.77	0.23	1.86	4
SRAP-1	17	7	41.18	0.17	1.18	4.6
SRAP-2	16	12	75.00	0.23	2.76	5.2
SRAP-3	15	9	60.00	0.21	1.86	4.6
SRAP-4	15	9	60.00	0.23	2.05	5
SRAP-5	15	12	80.00	0.23	2.77	4.6
Total	78	49				
Average	15.6	9.8	63.24	0.21	2.12	4.8
Total	138	88				
Average	13.8	8.8	64.00	0.22	1.99	4.4

***TB: total bands; NPB: polymorphic bands; PPB: percentage of polymorphic bands: PIC: polymorphic information content: MI: marker index; RP: percentage of resolving power.**

3.1.1.3. Genetic Similarity:

3.1.1.3.1. Genetic similarity and cluster analysis based on ISSR and SRAP markers.

The obtained data were used to estimate the genetic similarity among the tested ten quinoa genotypes shown in **Table (3)** and **Figure (3).** The Unweighted Pair Group Method with Arithmetic mean (UPGMA) cluster analysis based on ISSR and SRAP markers separated the studied quinoa genotypes into three groups **(Figure 3).** Group (A); consist of 4 genotypes (Egyptian, Rainbow, Miser-1 and Q-37); group (B); consist of 2 genotypes only (KVL-5204 and Q-52), while group (C) consist of 4 genotypes (Black, Regalona Bar, KVL-SRA3 and KVL-SRA2). Nei and Li/Dices similarity coefficients ranged from 0.75 to 0.91 for ISSR and SRAP primers combinations. Both primers indicated the highest similarity coefficient between Q-37 and Misr-1 (91%) and the lowest between KVL-SRA2 and Egyptian 75% **(Table 3).** Similar results were found by Said *et al.*, [43] and El-Sherbeny *et al.*, [44]. They showed that each group in the UPGMA cluster analysis includes the most relative genotypes according to their response to drought stress. This result is consistent with the existence of common ancestral genes in the crop. The commercial variety Regalona Bar included in the study is close to Andean eco-types from the molecular point of view, confirming the existence of parental genes originating in Andean material [45]. However, genetic molecular markers (ISSR, SRAP) and biochemical markers allowed the determination of the genetic variability in quinoa materials by grouping them according to the geographical location of origin as reveled by our former published paper with agronomical and seed color studies (29). Furthermore, the use of these results in the future is important for quinoa germplasm management and improvement as well as for the selection strategies of parental lines that facilitate the prediction of crosses in order to produce hybrids with higher performance as was indicated by several investigators [46-47].

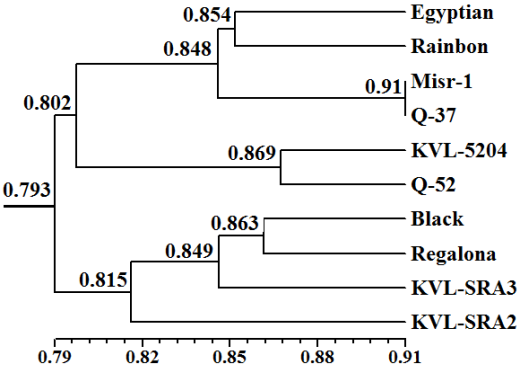

Figure (3): Dendrogram of ten Quinoa (*Chenopodium quinoa* Willd.) genotypes developed from ISSR and SRAP combined data using UPGMA analysis. The scale is based on Nei and Li coefficients of similarity.

Table (3): Genetic similarity values calculated from 138 DNA fragments generated with 10 primers (5 ISSR and 5 SARP) in ten Quinoa (*Chenopodium quinoa* Willd.) genotypes.

	Egyptian	Rainbow	Misr-1	Q-37	Black	KVL-SRA3	Regalona	KVL-SRA2	KVL-5204	Q-52
Egyptian	1									
Rainbow	0.854	1								
Misr-1	0.851	0.841	1							
Q-37	0.856	0.846	0.91	1						
Black	0.814	0.838	0.811	0.807	1					
KVL-SRA3	0.787	0.811	0.784	0.78	0.86	1				
Regalona	0.802	0.793	0.822	0.785	0.863	0.837	1			
KVL-SRA2	0.757	0.827	0.777	0.762	0.809	0.827	0.809	1		
KVL-5204	0.836	0.849	0.811	0.796	0.809	0.782	0.787	0.798	1	
Q-52	0.778	0.781	0.8	0.76	0.786	0.781	0.775	0.762	0.869	1

3.2. Biochemical markers

3.2.1. Protein marker:

Biochemical markers are used to detect genetic variability within and among populations. [48]. In our study**,** protein electrophoretic banding patterns of seed protein analysis for ten Quinoa (*Chenopodium quinoa* Willd.) genotypes presented in **Figure (4)** and **Table (4)**. Number, types and polymorphism percentage of seed storage protein for ten quinoa genotypes were seven monomorphic bands and five polymorphic bands, with total bands 12 and the percentage of polymorphism 42%. The obtained data showed that 12 bands distributed in all genotypes with molecular weights ranging from 25 kDa to 120 kDa **(Figure 4).** The results showed seven common bands among the ten tested quinoa genotypes. Egyptian genotype produced positive marker band at the molecular weight 120 kDa. However, Egyptian and Black genotypes revealed unique bands at the molecular weights 58 and 44 kDa, respectively. Finally, five polymorphic bands produced 42% of polymorphism. The same conclusion was found by Bhargava *et al.,* [25], who separated forty cultivated and wild taxa of *Chenopodium* by SDS-PAGE and found that seventy-two unique polypeptide bands were identified in the taxa studied. Maximum number of bands (30) was present in *C. berlandieri* ssp. *nuttalliae* PI 568156 and minimum (9) in *C. polyspermum* CHEN 52/75. Their results showed that the total protein electrophoresis was useful for genetic identification of genotypes. On the same side, Biochemical and molecular markers were used to identify the level of polymorphism and to study the genetic relationships among the five quinoas genotypes. Seventeen polymorphic protein bands produced (59%) of polymorphism. Regalona cultivar and Q-37 revealed that highest number of protein unique bands which could be considered as marker for salinity tolerance. reported by Omar *et al.,* [49].

4. CONCLUSION:

The results of molecular and biochemical markers showed that ten primers combinations successfully amplified and generated 138 bands of which 88 (64%), were polymorphic across the 10 quinoa genotypes. The polymorphism information content (PIC) values for ISSR and SRAP primers combination varied from 0.23 to 0.21 respectively, with an average of 0.22. Marker index (MI) values ranged from 0.75 to 3.37 with an average of 1.86 and 2.12 for ISSR and SRAP, respectively and general average 1.99. While, general average of resolving power (RP) was 4.4% for all primer's combinations; with an average 4% for ISSR and 4.8% for SRAP primers**.** Nei and Li/Dices similarity coefficients ranged from 0.91 to 0.75 for ISSR and SRAP primers combinations.

On other hand**,** protein electrophoresis showed seven common bands among the ten quinoa genotypes. Egyptian genotype produced positive marker. Moreover, it was observed that Egyptian and Black genotypes revealed unique bands. Finally, five polymorphic protein bands produced 42% of polymorphism. ISSR, SRAP and biochemical markers allowed the determination of the genetic variability in quinoa materials by grouping them according to the geographical location of origin. On the same side, we were able to identify unique bands associated with quinoa genotypes. These bands might also be used in breeding programs for differentiating among *Chinopodium* quinoa genotypes**.** The results of this study can be used as a starting point for future researches with the aims of defining the level of genetic diversity of ten quinoa genotypes. And given the important clues in understanding the relationships of quinoa genotypes, which may further assist in developing and planning breeding strategies to select the most promising genotype**.**

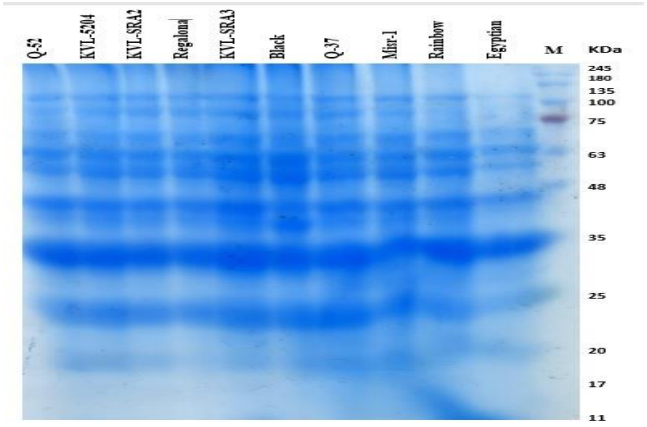

Figure (4): SDS-PAGE protein banding pattern for ten Quinoa (*Chenopodium quinoa* Willd.) genotypes.

Table 4: SDS-PAGE protein analysis for ten quinoa genotypes.

Band No.	MW	Egyptian	Rainbow	Misr-1	Q-37	Black	KVL-SRA3	Regalona	KVL.SRA2	KVL-5204	Q-52	Polymorphism
1	120	0	1	1	1	1	1	1	1	1	1	Positive
2	85	0	0	0	1	1	1	1	1	0	0	Polymorphic
3	68	1	1	1	1	1	1	0	0	0	0	Polymorphic
4	65	1	1	1	1	1	1	1	1	1	1	Monomorphic
5	63	1	1	1	1	1	1	1	1	1	1	monomorphic
6	58	1	0	0	0	0	0	0	0	0	0	Unique
7	55	1	1	1	1	1	1	1	1	1	1	Monomorphic
8	48	1	1	1	1	1	1	1	1	1	1	monomorphic
9	44	0	0	0	0	1	0	0	0	0	0	Unique
10	35	1	1	1	1	1	1	1	1	1	1	monomorphic
11	30	1	1	1	1	1	1	1	1	1	1	Monomorphic
12	25	1	1	1	1	1	1	1	1	1	1	monomorphic
Total		9	9	9	10	11	10	9	9	8	8	

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