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SEM and SCoT Markers Unveil New Taxonomic and Genetic Insights about Some Northern African *Triticum aestivum* L. Cultivars

Al-Safa H. Mohamed[1], Mohamed Ibrahim[1]*, S. S. Teleb[2] and M. E. Tantawy[1]

Abstract

Triticum aestivum L. is an annual grass of family Poaceae. The present study was concerned with the discrimination and Identification of some Northern African *T. aestivum* L. cultivars. Fourteen *T. aestivum* L. cultivars, representing seven Northern African countries, were presented in this study. High-resolution conventional SEM imaging was performed to survey on and to examine the grain surface sculpture (dorsal and ventral views) of the studied cultivars. SEM analysis revealed six grain surface sculpture for the ventral surface and four for the dorsal side. In addition, SCoT polymorphism analysis was performed using 14 primers to access and characterize the genetic diversity between *T. aestivum* L. cultivars under study. The relationship resulted from applied multidisciplinary analyses *viz*. (UPGMA, structural analysis, and principal coordinate analysis) via SEM and SCoT analyses were in complement and efficient in assessing the characterization of taxonomic and genetic diversity of studied *T. aestivum* L. cultivars. In conclusion, it is most likely speculated that breeding lines from *T. aestivum* cultivars range of North Africa and their rational comparison may emerge novel insights and give better understanding of the domestication of *T. aestivum* genetic diversity. Also, some cultivars could be used as important genetic resources for genetic improvement of *T. aestivum* in future breeding program.

Keywords

Poaceae; *Triticum aestivum* L; SEM; Genetic diversity; Molecular markers; SCoT polymorphism

Introduction

Triticum aestivum L. (common bread wheat) is an annual grass belonging to the family Poaceae [1]. Thousands of common wheat species cultivars have been developed that differ in number of chromosomes from the original diploid primitive types [2]. Classification of wheat grain has significant importance in taxonomic studies. Different cultivars are classified according to the horticultural demand (spring against winter wheat), food uses, texture (for pastry and food vs. hard one containing more gluten). Therefore, the discrimination and characterization of wheat cultivars are necessary for consumers, farmers and plant breeders to guess quality and yield [3]. The use of grain exomorphology is of great value in taxonomic discrimination of wheat varieties**.** Numerous exomorphological characters from grains are constant and reliable to be used for taxonomic purposes within cultivars [4]. Morphological characters and surface sculpture of grass grain have been used in several studies to compare taxa and genera [5,6,7]. Grain features, especially exomorphic characters provided through SEM, have been used in resolving several systematic problems [8]. SEM revealed fine differences in structural features to characterize the species of the same groups [9,10]. Seven gramineous genera, belonging to tribe Triticeae Dumort, were characterized by SEM to observe the grain surface features [11]. A considerable degree of similarity among genotype of four cereals was revealed based on SEM survey on the grain surface [12]. Also, nine different species of *Eragrostis* were differentiated by their grain exomorphic characters [13].

Detection of genetic variation and determination of genetic relationships between individuals and populations is an important consideration for the efficient conservation and utilization of plant genetic resources [14,15]. A significant restriction which usually limits the improvement programs of plant crops is the deficiency of plant materials triggering rich genetic variation [16]. However, genetic variation was evaluated to select the best crosses in common wheat (*Triticum aestivum* L.) cultivars in Isfahan province [17]. Previous investigations have elucidated the importance of integration of useful genetic diversity into cultivars or cultivars materials of *T. aestivum* L. and *Dactylis glomerata* L., respectively [16,18,19]. Therefore, the development and use of molecular markers for the detection and exploitation of DNA polymorphism is one of the most significant developments in the field of molecular genetics. Specifically, molecular markers have been used in cultivar identification and phylogenetic analysis [2,15,20,21,22]. These markers involve; i.e. Randomly Amplified Polymorphic DNA (RAPD) markers [23,24], Microsatellites or Simple Sequence Repeat (SSR) markers [25,26], Inter-Simple Sequence Repeat (ISSR) markers [20], and sequence-related amplified polymorphism (SRAP) markers [27]. Recently, improved and simpler with greater reliability markers were emerged. Start Codon Targeted (SCoT) Polymorphism marker concerned with the conserved flanking short region of the ATG translation initiation (or start) codon in plant genes [28]. SCoT analysis does not need any prior knowledge of the target sequence. SCoT markers are fractionated, stained, and visualized by low cost simple effective technique valid for vast majority of standard equipped plant research labs [28]. Being characterized by lower recombination levels between its markers and the gene/trait, SCoTs are more directly used in constructing marker-assisted breeding programs than RAPDs, ISSRs, and SSRs [29]. Discontinuous molecular markers have resolved the speciation and species separation in *Hordeum* L. - Poaceae [30]. SCoT markers have been efficiently used for DNA fingerprinting of *Tritordeum bergrothii* L. - Poaceae [31]. Furthermore, the potential of highly reproducible SCoT marker in accessing genetic diversity and relationships between Chinese *Elymus sibricus* L. - Poaceae accessions was shown [32]. Knowledge on the genetic diversity and relatedness between *T. aestivum* cultivars localized in Northern Africans countries is so limited, but necessary for maintenance and breeding programs that focusing on triggering high quality and productivity.

***Corresponding author:** Ibrahim M, Botany Department, Faculty of Science, Ain Shams University, Cairo, Egypt, E-mail: Mohamed.ibrahim591978@gmail.com

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The specific objective of the present study is to address some characteristics and/or evidences to evaluate the relationships among some of *Triticum aestivum* L. cultivars from different Northern African countries. For that, high-resolution imaging by means of conventional SEM was performed to survey on and to examine the grain surface sculpture (dorsal and ventral views) of the studied cultivars. Moreover, the short conserved locus flanking the ATG translation start codon was monitored using SCoT markers analysis to reveal the genetic diversity between *T. aestivum* cultivars in question. Provided, to access and to investigate how the applied taxonomic and molecular approaches most likely could help in the development of comprehensive and multidisciplinary characterization of the studied cultivars.

Material and Methods

Plant material

A total of fourteen *Triticum aestivum* L. cultivars representing Six Northern African countries were presented in the present study. Grains of thirteen *T. aestivum* cultivars representing Algeria, Egypt, Libya, Morocco, Sudan, and Tunisia were kindly provided and assessed by the International Center for Agricultural Research in the Dry Areas (ICARDA) research program of crop genetics established at the Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC), Giza, Egypt. While, the grains of the remainder cultivar representing Libyan taxa were kindly obtained from and accessed by Libyan Arab People's Socialist Jamahiriya's ARC as listed in Table 1.

Grains SEM

The detailed surface sculpture of mature wheat grains were examined by using SEM at different magnification levels. Briefly, the mature dry grain, of each cultivar, was mounted on stubs, attached with sticky tabs, and coated with gold sputter coater (SPI-module). Then, the grains were examined and studied in a JEOL-JSM 5600 LV scanning electron microscope at regional center of Mycology and Biotechnology, Al-Azhar Univeristy, Cairo, Egypt, using high vacuum mode. During the analysis of selected *T. aestivum* grains, the emphasis was focused on detailed views of three structural aspects (regions); ventral view, dorsal view near embryo, and dorsal view far from embryo. For the sake of consistency of studied taxonomic characters, several grains from each selected cultivar/variety were analyzed. Identification of grain sculpture was taken from the scheme adopted by Murley [33].

Extraction, purification, and quantification of genomic DNA from *T. aestivum* cultivars

All chemicals and reagents used in this study were purchased in analytical purity grade from Sigma Aldrich (Munich, Germany), VWR (Darmstadt, Germany), and Roth (Karlsruhe, Germany). High molecular weight plant Genomic DNA was isolated from 50-100 mg of freeze-dried and powered grains of *T. aestivum* L. cultivars by using DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) and 2% CTAB extraction procedure [34]. The quantity and purity of the extracted DNA were assessed by spectrophotometry using the ND-1000 system (NanoDrop Technologies, Thermo Fisher Scientific Inc.). DNA manipulation and analysis procedures in terms of quantification and purity analysis were performed according to the Molecular Cloning Laboratory Manual [35].

SCoT markers technique

The genetic differentiation and genetic diversity between the studied *T. aestivum* cultivars were analyzed by SCoT marker technique. *T. aestivum* samples were subjected to be differentiated using fourteen SCoT primers (Table 2). SCoT primers were designed as previously described by Collard and Mackill [28], while primers 47-48 were designed according to Luo et al. [36]. The primers were synthesized by HVD Vertriebs-Ges. m.b.H. (Vienna, Austria) in 10 nM stock concentration and delivered in a lyophilized form. They were dissolved in sterilized water to a final concentration of 100 µM and kept at -20°C. The underlined nucleotides sequence, representing ATG codon in the primers, was fixed (Table 2).

The polymerase chain reaction was carried out in 25 µl reaction volume containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 30 µM primer, 25-50 ng genomic DNA and 1 unit of Phusion® High-Fidelity DNA Polymerase (Espoo, Finland). PCR amplification was performed in a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems) programmed to fulfill 35 cycles after an initial denaturation cycle for 3 min at 94°C. Each cycle consisted of a denaturation step at 94°C for 1 min, an annealing step at 55°C for 1 min, and an elongation step at 72°C for 1.5 min. The primer extension segment was extended to 7 min at 72°C in the final cycle. The PCR amplicons of SCoT markers were subsequently fractionated on a 1.5% wt/vol agarose gel, stained with ethidium bromide (final

Table 1: Geographical distribution of *T. aestivum* L. studied cultivars of some Northern African countries.

Geographic Origin	Code*: Cultivar name	Source	Growth Habit	Status
Algeria (DZA)	DZA1 : Altar	ICARDA	Spring	Cultivar
	DZA2 : Attila	ICARDA	Spring	Cultivar
	DZA3 : Hidhab	ICARDA	Spring	Cultivar
Egypt (EGY)	EGY1: Gemmeiza-9	ICARDA	Spring	Cultivar
	EGY2 : Giza-168	ICARDA	Spring	Cultivar
	EGY3 : Sachah	ICARDA	Spring	Local Variety
Morocco (MAR)	MAR1 : Aguilal	ICARDA	Spring	Cultivar
	MAR2 : Arrehane	ICARDA	Spring	Cultivar
	MAR3 : Morocco	ICARDA	Spring	Cultivar
Sudan (SDN)	SDN1 : Debeira	ICARDA	Spring	Cultivar
	SDN2 : El-Nielain	ICARDA	Spring	Cultivar
	SDN3 : Sasaraib	ICARDA	Spring	Cultivar
Libya (LBY)	LBY1: Al Zerda	Libyan ARC	Winter	Cultivar
Tunisia (TUN)	TUN1 : Utique96	ICARDA	Spring	Cultivar
Total	14			

* Three digit codes of Northern African countries were according to (wheatatlas.org) website.

Table 2: Sequence of primers used in SCoT polymorphism analysis.

Sl. No.	Name	Sequence (5´› 3´) %GC	% GC	Application
1	ScoT1	CAACAATGGCTACCACCA	50%	
2	ScoT2	CAACAATGGCTACCACCC	56%	
3	ScoT3	CAACAATGGCTACCACCG	56%	
4	ScoT5	CAACAATGGCTACCACGA	50%	
5	ScoT8	CAACAATGGCTACCACGT	50%	SCoT Analysis
6	ScoT9	CAACAATGGCTACCAGCA	50%	
7	ScoT10	CAACAATGGCTACCAGCC	56%	
8	ScoT11	AAGCAATGGCTACCACCA	50%	
9	ScoT12	ACGACATGGCGACCAACG	61%	
10	ScoT13	ACGACATGGCGACCATCG	61%	
11	ScoT16	ACCATGGCTACCACCGAC	56%	
12	ScoT20	ACGACATGGCGACCCACA	67%	
13	ScoT47	ACAATGGCTACCACTGCC	56%	Sequencing of unique SC oT amplicons
14	ScoT48	ACAATGGCTACCACTGGC	56%	
15	M13 (fw.)	GTAAAACGACGGCCAGT	53%	

concentration 100 µM/L, Sigma-Aldrich®) in 1X TBE, visualized, and finally documented using a gel documentation and image analysis system according to the Molecular Cloning Laboratory Manual [35].

Oligonucleotide sequencing of selected SCoT amplicons

Fractionated and selected SCoT amplified fragments were recovered from agarose gel using QIAquik® PCR PURIFICATION KIT (Qiagen inc., Cat. no. 28106) according to the manufacturer's instructions. Selected and purified SCoT amplicons were then cloned into pGEM cloning vector prior to sequencing process. The DNA sequence was determined by automated DNA sequencing method. The automated DNA sequencing reactions was performed using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE applied Biosystems, USA) in conjunction with ABI PRISM (310 Genetic Analyzer). Cycle sequencing was performed using the Gene Amp 2400 Thermal Cycler, the reaction was conducted in a total volume of 20 µl containing 8 µl of terminator ready reaction mix, 1 µg of plasmid DNA, and 3.2 pM of M13 universal forward primer. The cycle sequencing program was 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. Repeated for 25 cycles with rapid thermal ramping, the nucleotide sequence was determined automatically by the electrophoresis of the cycle sequencing reaction product on 3100 Genetic Analyzer. The data were provided as fluorimetric scans from which the sequence was assembled using the sequence analysis software.

Data analysis

Grain exomorphic characters and the banding patterns generated by SCoT markers analysis were analyzed to determine the taxonomic and genetic relatedness of the fourteen examined *T. aestivum* samples. The amplified bands were scored as presence (1) or absence (0), and only reproducible bands were considered. Polymorphic information content (PIC) values were calculated for each SCoT primers according to the formula: $PIC = 1 - p2 - q2$ [32]; where p is frequency of present band and q is frequency of absent band. A dendrogram was constructed by Dice coefficient's genetic similarity matrix to display accession relationships using the unweighted pair group method with arithmetic mean (UPGMA) of NTSYS version 2.10 [36,37] The principal coordinate analysis (PCoA) was constructed based on Dice coefficient genetic similarity matrix using DCENTER module in NTSYS. Population structure of the fourteen *T. aestivum* L. cultivars was analyzed using STRUCTRE v2.3.4 software [15,38] as described by Zhang et al. [39]. Maximum likelihood and delta K (△K) values were used to determine the optimum number of groups [38,40]

Results and Discussion

Structural features as revealed by SEM

The grain exomorphic characters extracted from the fourteen examined *T. aestivum* L. cultivars, as revealed by SEM, were summarized in Table 3. The grain sculpture of *T. aestivum* L. cultivars has revealed high range of variation representing ventral and dorsal sides (Figures 1 and 2). SEM investigation has clarified that the grains surface has the same sculpture in both ventral and dorsal views *viz* (scalariform reticulate in DZA2: Attila and LBY1: Al Zerda, scalariform reticulate foveate in DZA3: Hidhab and SDN2: El-Nielain, and reticulate in SDN1: Debeira and TUN1: Utique96).

In addition, the ventral sculpture of the grain was on the different morphological plan as in dorsal one as follows: firstly, in ventral sculpture, six types of grain features were revealed by SEM *viz* (rugose sculpture in DZA1: Altar, scalariform in EGY1: Gemmeiza-9, reticulate in in SDN1: Debeira and TUN1: Utique96, scalariform reticulate in DZA2: Attila, MAR1: Aguilal, MAR2: Arrehane, and LBY1: Al Zerda, Scalariform reticulate foveate in DZA3: Hidhab, EGY2: Giza-168, SDN2: El-Nielain, and SDN3: Sasaraib, and scalariform foveate in EGY3: Sachah and MAR3: Morocco (Figure 1).

Secondly, in dorsal sculpture of the grain four types were scored as follows: reticulate scrobiculate in MAR2: Arrehane and MAR3: Morocco, scalariform reticulate in DZA1: Altar, DZA2: Attila, EGY1: Gemmeiza-9, EGY3: Sachah, and LBY1: Al Zerda, scalariform reticulate foveate in DZA3: Hidhab and SDN2: El-Nielain, reticulate in EGY2: Giza-168, MAR1: Aguilal, SDN1: Debeira, SDN3: Sasaraib, and TUN1: Utique96 (Figure 2). The grain surface sculpture provided diversity of diagnostic characters helped in separation between the studied cultivars. These results are in agreement with Barthlott [4,41] and Abou-Taleb et al. [12].

Taxonomic relationships between *T. aestivum* cultivars as revealed by SEM

The sharp and clear exomorphic characters were scored as presence (1) or absence (0). To investigate the taxonomic similarity among the fourteen studied *T. aestivum* L. cultivars, the scored data obtained from the grain exomorphic characters were analyzed using the Dice coefficient to compute the similarity matrix as shown in Table 4. The estimated taxonomic similarities ranged from 13 to 83 % revealing low to high level of taxonomic similarity among the studied *T. aestivum* L. cultivars. The highest detected similarity 83% was detected between MAR2 and LBY1. Then, relatively high similarity 73% was between LBY1 and EGY1 and MAR2 and EGY1. This was followed by 62% value between EGY1 and DZA1 and between SDN2 and DZA1. While, the lowest taxonomic similarity 13% was detected between TUN1 and SDN1. Notably, no level of similarity was recorded between EGY2 and DZA3, EGY3 and EGY2, and SDN1 and MAR3.Low similarity percentages 0 - 13 % between studied *T. aestivum* L. cultivars of different Northern African countries were probably accounted for by the alterations of several limiting factors; climatic changes, watering characteristics, preservation conditions, and geographical distribution. Furthermore, there is no report focused on the grain surface sculpture by SEM to address the taxonomic relatedness of studied *T. aestivum* taxa from different northern African countries so far.

Cluster analysis of studied *T. aestivum* L. cultivars was generated using the UPGMA method (Figure 3). The resulted dendrogram has revealed the level of similarity and the relationships among the studied cultivars. *T. aestivum* L. cultivars were grouped into two series I and II. Series I included EGY2: Giza-168, while series II comprised the rest of the studied cultivars. Series II subdivided into two subseries; I and II. Subseries I in turn subdivided into two clusters; C1 and C2. The former included EGY1: Gemmeiza-9, while the latter include MAR2: Arrehane and LBY1: Al Zerda together. Subseries II subdivided into two clusters; C3 and C4. C3 included two subclusters; sub CI and sub CII. Subcluster CI included two groups; G1 and G2. The group G1 comprised of SDN1: Debeira, DZA2: Attila, TUN1: Utique, and DZA1: Altar. The group G2 collected SDN3: Sasaraib, and MAR1: Aguilal. Subcluster CII had one group G3. The group G3 put DZA3: Hidhab and MAR3: Morocco together. Cluster C4 included SDN1: Debeira and EGY3: Sachah.

In conclusion, grouping of Egyptian cultivar (EGY2: Giza168) in one series has prompted us to further analyze *T. aestivum* L. studied

Table 3: Grain exomorphic characters of *T. aestivum* L. cultivars as revealed by SEM.

Cultivar code		Surface pattern		Anticlinal wall				Periclinal wall			
		Ventral	Dorsal	Thickness		Surface		Surface		Elevation	
				Ventral	Dorsal	Ventral	Dorsal	Ventral	Dorsal	Ventral	Dorsal
Algeria	DZA1	Rugose	Scalariform reticulate	Thick *Epicuticular wax	Thick	Smooth	Smooth	Fine stria	Foveate stria	Shallow elevated	Shallow elevated
	DZA2	Salariform reticulate	Salariform reticulate	Broad *Epicuticular wax	Broad	Smooth	Smooth	Fine stria	Constricted	Depressed	Shallow depressed
	DZA3	Scalariform reticulate foveate	Scalariform reticulate foveate	Thick	Thick	Smooth	Smooth	Foveate stria	Foveate stria	Shallow elevated	Shallow elevated
Egypt	EGY1	Scalariform	Scalariform reticulate	Thick	Broad	Smooth	Smooth	Fine stria foveate	Fine stria foveate	Depressed	Shallow elevated
	EGY2	Scalariform reticulate foveate	Reticulate	Thick	Thick	Striated	Striated	Foveate stria	Fine stria	Elevated	Shallow elevated
	EGY3	Scalariform foviate	Scalariform reticulate	Broad	Broad	Smooth	Smooth	Foveate stria	Fine stria	Elevated	Shallow elevated
Libya	LBY1	Scalariform reticulate	Scalariform reticulate	Thick *Epicuticular wax	Thick	Smooth	Smooth	Foveate stria	Transverse stria	Depressed	Depressed
Morocco	MAR1	Scalariform reticulate	Reticulate	Thick	Thick	Smooth	Smooth	Fine transverse stria	Fine transverse stria	Shallow depressed	Elevated
	MAR2	Scalariform reticulate	reticulate scrobiculate	Broad	Thick	Striated	Striated	Striated longitudinal	Transverse stria	shallow depressed	shallow depressed
	MAR3	Scalariform foveate	Scalariform reticulate	Thick *Epicuticular wax	Thick	Smooth	Striated	Foveate stria	Fine stria	Shallow elevated	Shallow elevated
Sudan	SDN1	Reticulate	Reticulate	Thick	Thick	Smooth	Smooth	Striated longitudinal	Transverse stria	Shallow depressed	Shallow depressed
	SDN2	Scalariform reticulate foveate	Salariform reticulate foveate	Thick	Thick	Striated	Striated	Foveate stria	Foveate stria	Shallow depressed	Shallow depressed
	SDN3	Scalariform reticulate foveate	Reticulate	Thick *Epicuticular wax	Thick	Smooth	Smooth	Fine stria constricted	Fine stria constricted	Shallow depressed	Shallow depressed
Tunisia	TUN1	Reticulate	Reticulate	Thick	Thick	Smooth	Smooth	Transverse stria	Transverse stria	Shallow depressed	Elevated

cultivars on the genetic level, by means of SCoT analysis, to proof/disproof unique characteristics of Egyptian-localized wheat cultivars and to decipher genetic diversity among *T. aestivum* L. studied cultivars.

SCoT-PCR amplification profiling

The fourteen *T. aestivum* L. cultivars were subjected to be genetically distinguished by SCoT polymorphism analysis approach. Hereby, genetic relatedness/diversity among the *T. aestivum* L. studied cultivars was further analyzed by SCoT markers. Polymorphism revealed by some representative SCoT primers (Figure 4) was shown.

Genetic aiversity among studied *T. aestivum* cultivars as revealed by SCoT markers

The amplification products produced from 14 SCoT primers in terms of the percentage of PCR products appeared in the studied genotypes (Table 5). A total of 176 bands were generated, among which 66 bands were polymorphic. The percentage of polymorphic bands ranged between 8 % for SCoT-9 and 57 % for SCoT-2. The polymorphic informative content (PIC) values varied from 0.031 (SCoT-9) to 0.176 (SCoT- 2) with an average of 0.105 for these cultivars. The amplification profile (Figure 4) revealed by the three SCoT primers; SCoT-2, SCoT-1, and SCoT-5 yielded highly informative patterns based on calculated PIC (Table 5). These primers, with higher PIC values, have more potential for further study, allowing investigating more cultivars or sampling sites using a reduced number of primers.

To investigate the genetic similarity of *T. aestivum* cultivars in question based on SCoT results, the scored data obtained from fourteen primers were analyzed using the Dice coefficient to compute the similarity matrix. This similarity matrix was used to generate a dendrogram using the UPGMA method. As shown in Table 6, the estimated genetic similarities ranged from 84 % to 95 % revealing high levels of genetic similarity among the studied cultivars. The highest genetic similarity 95% was detected between TUN1 and DZA3, followed by 94 % among EGY1/EGY2 and DZA3. On the other hand, the lowest genetic similarity 84 % was detected between MAR1 and LBY1. Genetic similarities percentages (as revealed by SCoT analysis) were concomitant with their corresponding percentages (as revealed by SEM) showing close relatedness between Egyptian/ Sudanese and Algerian cultivars The higher investigated similarity percentages of 84-95% observed by the SCoT analysis might be speculated due to the conservation of *T. aestivum* L. genome organization regardless of the close/distant geographic distribution. Furthermore, there is no report that covered the use of SCoT markers for characterization of genetic diversity of studied *T. aestivum* cultivars from Northern African countries so far.

Population structure and cluster analysis

The population structure of the fourteen cultivars was estimated by using the Hardy-Weinberg Equilibrium using STRUCTURE V2.3.4 software. Based on maximum likelihood and delta K (ΔK) values, the number of optimum groups was five (Figure 5). Group one

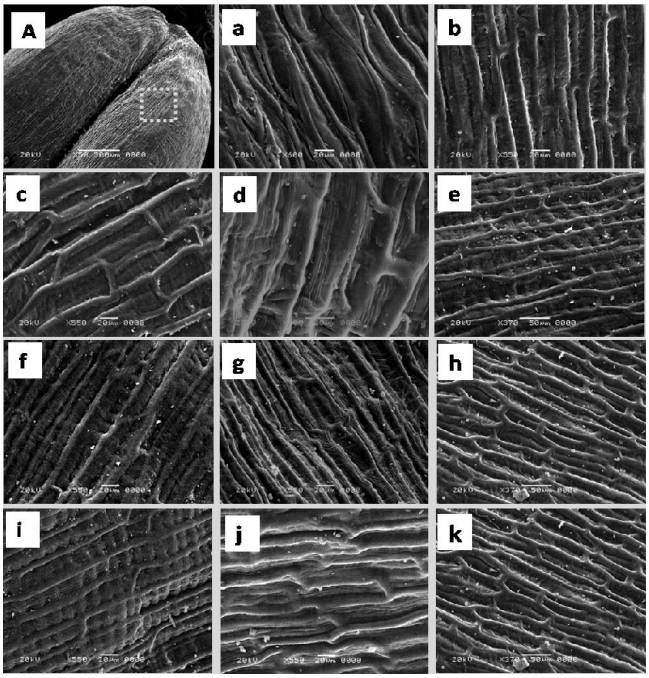

Figure 1: SEM microphotographs showing grain surface sculpture (ventral side). A. whole ventral side of wheat grain; a. Rugose (DZA1 : Altar); b. Scalariform (EGY1:Gemmeiza9); c. Reticulate (SDN1 : Debeira); d. Scalariform reticulate (MAR2 : Arrehane); e. Scalariform reticulate foveate (DZA3 : Hidhab); f. Scalariform foveate (EGY2 : Giza-168; g. Stariated anticinal wall (SDN2 : El-Nielain); h. Smooth anticilal wall ; i. faoveate stria of periclinal wall (EGY2 : Giza-168); j. longitudinal striation (SDN1 : Debeira); and k. transvers striation (SDN3: sasaraib) sculptures. The white dotted rectangle in panel (A) emphasizes the region that was analyzed by SEM in the ventral side of studied grains.

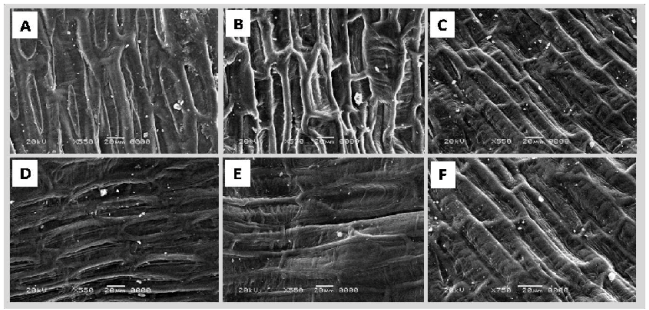

Figure 2: SEM microphotographs showing grain surface sculpture (dorsal side). A. Reticulate scrobiculate (MAR2 : Arrehane); B. Scalariform reticulate (DZA1: Altar); C. Scalariform reticulate foveate (DZA3: Hidhab); D. Reticulate (EGY2: Giza-168); E. Transvers Striated pericilanl wall (LBY1: Al zerda); F. foveat striation (DZA3 : Hidhab) sculptures.

(G1) has included DZA3, EGY2, MAR1, TUN1, EGY1, and SDN3; group two (G2) has contained DZA1 and MAR2; group three (G3) has enclosed SDN2; group four (G4) has comprised EGY3, MAR3, and DZA2; group five (G5) has involved LBY1 and SDN1. To summarize, high degrees of genetic differentiation between *T. aestivum* L. studied cultivars was investigated by population structure analysis [42,43].

Based on 66 polymorphic SCoT fragments, a dendrogram was constructed based on the genetic similarity matrix of the studied cultivars (Figure 6). The fourteen studied cultivars were grouped into two series; SI and SII (with a similarity index of 0.88). Series I divided into two subseries; SubSI and SubSII. Subseries I is further subdivided into three clusters; C1, C2, and C3. Cluster C1 included SDN1: Debeira and DZA2: Attila. Cluster C2 is subdivided into two subclusters; SubCI and SubCII. Subcluster I collected SDN2: El-Nielain and LBY1: Al Zerda, while subcluster II comprised MAR2: Arrehane and DZA1: Altar. Cluster C3 is further subdivided into two subclusters; SubCIII and SubCIV. Subcluster III included SDN3: Sasaraib and MAR1: Aguilal. Subcluster IV divided into two groups G1 and G2. The group G1 collected TUN1: Utique and DZA3: Hidhab, whereas the group G2 collected EGY1: Gemmeiza and EGY2: Giza-168. Subseries II had only one cluster C4 which included MAR3: Morocco. On the other hand, Series II included EGY3: Sachah [44].

To conclude, the SCoT-based dendrogram was almost in accordance with SEM-based results (Figure 3), for example Egyptian cultivar EGY3: Sachah was clustered away from the other Egyptian cultivars EGY1: Gemmeiza and EGY2: Giza-168. On the same context, Ma et al. [26] has evaluated eight *E. sibiricus* L. populations from the eastern Qinghai-Tibet, China using ISSR markers and has showed that there was no distinct geographical tendency in the distribution of the genetic diversity. The same investigation was shown by Zhang et al. [32] using SCoT marker to estimate genetic diversity and relatedness among Chinese *E. sibiricus* L. cultivars. L. cultivars constructed from the SCoT polymorphsim data using Unweighed Pair-group Arithmetic (UPGMA) and similarity matrices computed according to Dice coefficient.

Therefore, some geographically close cultivars were clustered in different groups and geographically distant ones were clustered in the same groups. Diverse selection forces tend to exhibit genetic heterogeneity under the different small niches [26]. Furthermore, the role of ecological conditions in determining the extent and distribution of genetic diversity has been well documented [45]. The relationship observed in the principal coordinate analysis (PCoA) was in agreement with the UPGMA analysis: about 48.48 % of the total variation was described by the first three PCo percentages; with PCo1 accounting for 20.00 %, PCo2 for 14.96 % and PCo3 for 13.96 % (Figure 7). The PCoA multivariate approach was performed to complement the cluster analysis outcomes, because cluster analysis shows a higher resolution for analysis of closely related populations. On the other hand the PCoA is more informative regarding distances among major groups.

First implications elucidated from the sequenced SCoT amplicons

SCoT-PCR amplification profile has shown a significant polymorphism among the studied *T. aestivum* L. cultivars. Sequence analysis of highly pronounced unique polymorphic SCoT amplicons (Figure 4), generated by SCoT-2, SCoT-3, SCoT-11, SCoT-47, and SCoT-48 primers were performed. Also, the same procedure was followed to analyze unique monomorphic SCoT amplicon obtained with SCoT-8 (Figure 4). Forward sequences of the latter SCoT amplicons were aligned and compared by their BLAST scores to published available sequences via GenBank (Table 7). Generally, sequencing results of both mono- and some polymorphic amplicons have shown high identity between examined cultivars and the American *T. aestivum* L. cultivar Chinese spring (query cover 81-95%). These results most likely strengthened the implication of genetic diversity of *T. aestivum* L. genome to the level of cultivars. Moreover, SCoT amplicon produced by SCoT-2 has scored a hit against *T. aestivum* gene for TaAP2-D (determines the cleistogamous/non-

Table 4: Similarity matrix among studied *T. aestivum* cultivars as computed according to Dice coefficient as revealed by SEM.

Taxa	DZA1	DZA2	DZA3	EGY1	EGY2	EGY3	LBY1	MAR1	MAR2	MAR3	SDN1	SDN2	SDN3	TUN1
DZA1	100													
DZA2	53	100												
DZA3	31	43	100											
EGY1	50	62	36	100										
EGY2	14	27	**00**	33	100									
EGY3	43	53	46	33	**00**	100								
LBY1	46	43	33	**73**	15	31	100							
MAR1	53	50	43	31	27	40	29	100						
MAR2	46	43	33	**73**	31	31	**83**	29	100					
MAR3	17	31	55	20	17	33	18	31	18	100				
SDN1	43	53	15	33	29	57	15	27	15	**00**	100			
SDN2	50	62	55	40	00	33	36	46	36	60	17	100		
SDN3	14	40	46	17	14	43	15	53	15	33	43	33	100	
TUN1	53	50	29	31	27	27	29	50	29	46	**13**	46	40	100

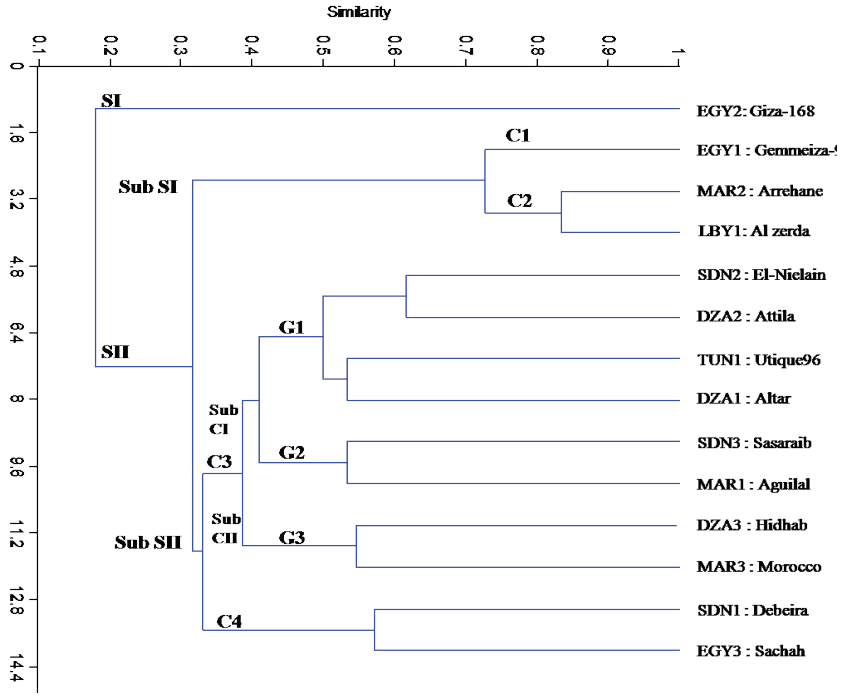

Figure 3: Cluster analysis resulted from *T. aestivum* cultivars. A dendrogram for the fourteen examined *T. aestivum* cultivars was constructed using scored exomorphic characters resulted by SEM data using Unweighed Pair-group Arithmetic (UPGMA) and similarity matrices computed according to Dice coefficient.

cleistogamous flowering) of cultivar Chinese Spring (query cover 36%) [46].

Furthermore, another hit against *T. aestivum* L. MADS-box transcription factor TaAGL29 mRNA complete coding sequence (query cover 89.7%) was obtained from SCoT-8 primer. Another remarkable hit was found with *T. aestivum* L. is ammonium transporter Amt1 mRNA complete coding sequence (query cover 69.8%) which was generated by SCoT-48. No hits were found to SCoT amplicon of SCoT- 47. Although the SCoT analysis in this study was performed on isolated genomic DNA from *T. aestivum* L. grains, some mRNA sequences were successfully shown to be amplified by genomic ATG- designed primers.

In conclusion, high level of genetic diversity and distinct taxonomic differentiation of *T. aestivum* L. had been monitored in this study. Taxonomic characterization of observed grain exomorphic characters of *T. aestivum* L. cultivars, as revealed by SEM, has shown that the grain sculpture as well as aspects of anticlinal and periclinal walls were able to verify the main diagnostic criteria at the cultivar level and also provides an important tool for more precise characterization of grain surface. On the other hand, this study emphasized and accessed the invalidity of notion that the genetic diversity of studied cultivars is obeyed by the rule of geographical distribution tendency. Furthermore, performed SCoT polymorphism analysis was significant to monitor the genetic differentiation and to generate unique genomic loci at *T. aestivum* L. cultivars that encode functional mRNA. Hereby, it is most likely speculated that breeding lines from *T. aestivum* L. cultivars range of North Africa

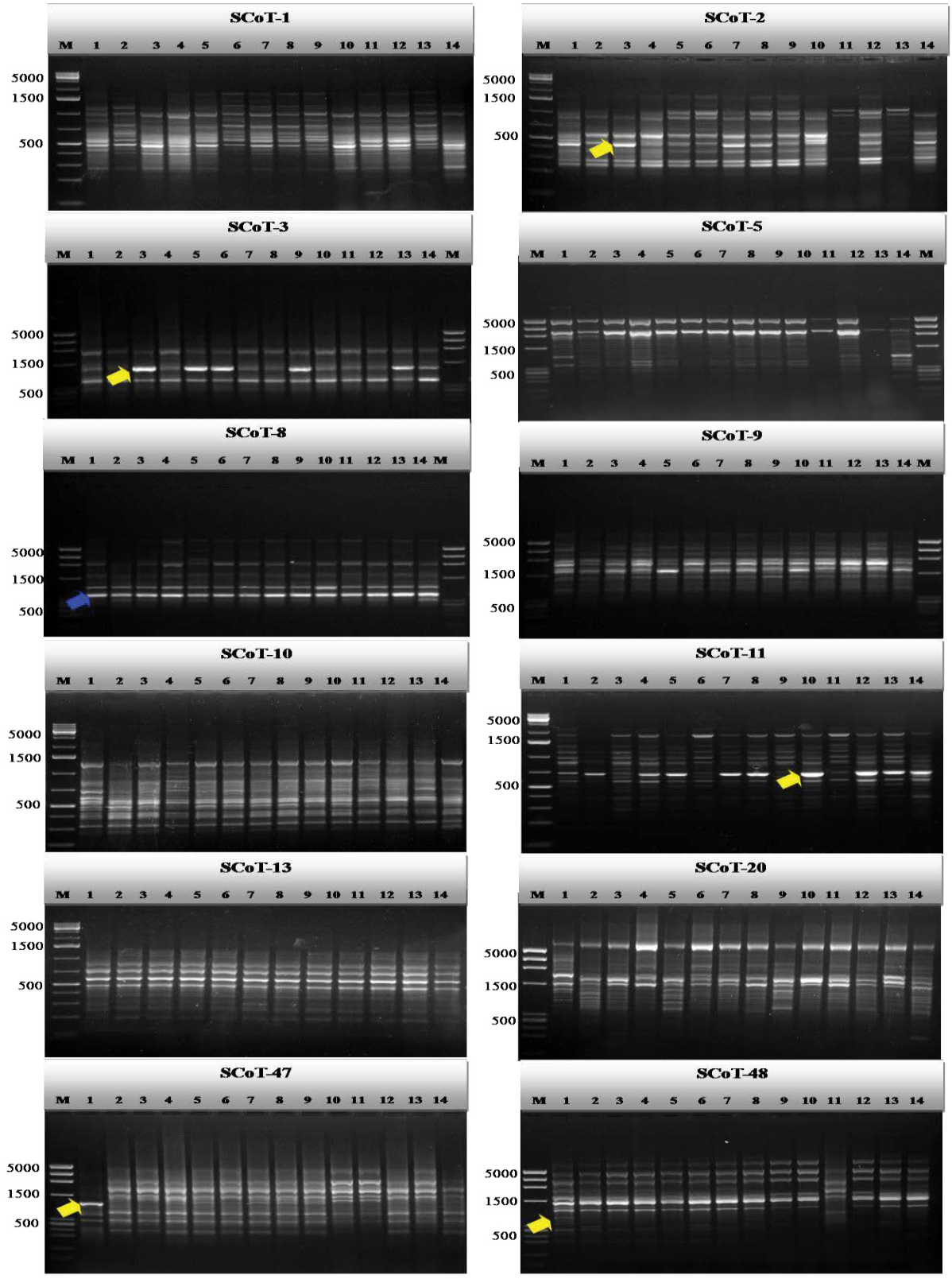

Figure 4: Polymorphism resultant from SCoT markers. Agarose gel electrophoresis of PCR amplicons some representative SCoT primers. DNA size marker GeneRuler 1 Kb plus DNA ladder (lane M) was loaded and denoted by numbers left-handed of the figure indicating molecular size standards in kbp. Yellow and blue arrows refers to sequenced SCoT amplicons.

Table 5: Total number of amplified fragments, monomorphic amplicons, polymorphic fragments, and percentage of polymorphism as revealed by SCoT markers between the examined *T. aestivum* L. cultivars.

Primer	Total of amplicons	Monomorphic amplicons	Polymorphic amplicons	Percentage of polymorphism (%)	PIC
SCoT-1	22	10	12	54	**0.171**
SCoT-2	14	6	8	**57**	**0.176**
SCoT-3	6	5	1	16	0.059
SCoT-5	12	7	5	41	**0.148**
SCoT-8	11	9	2	18	0.039
SCoT-9	12	11	1	**8**	0.031
SCoT-10	18	12	6	33	0.101
SCoT-11	18	10	8	44	0.133
SCoT-12	16	13	3	18	0.064
SCoT-13	12	8	4	33	0.095
SCoT-16	11	7	4	36	0.107
SCoT-20	15	8	7	46	0.124
SCoT-47	15	10	5	33	0.088
SCoT-48	17	9	8	47	0.127
Total	176	110	66	34	-
Average	14.7	9.2	5.5		0.105

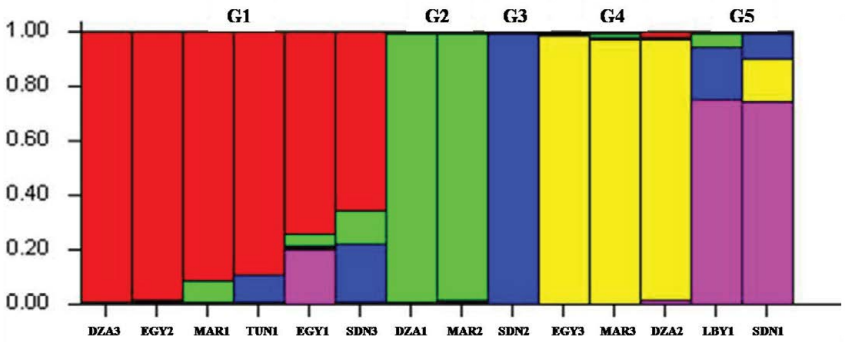

Figure 5: Five groups of the fourteen *T. aestivum* cultivars inferred from STRUCTURE V2.3.4 analysis. The vertical coordinate of each presented subgroup indicates the membership coefficients for each cultivar. Red zone: G1; Green zone: G2; Blue zone: G3; Yellow zone: G4; Pink zone: G5.

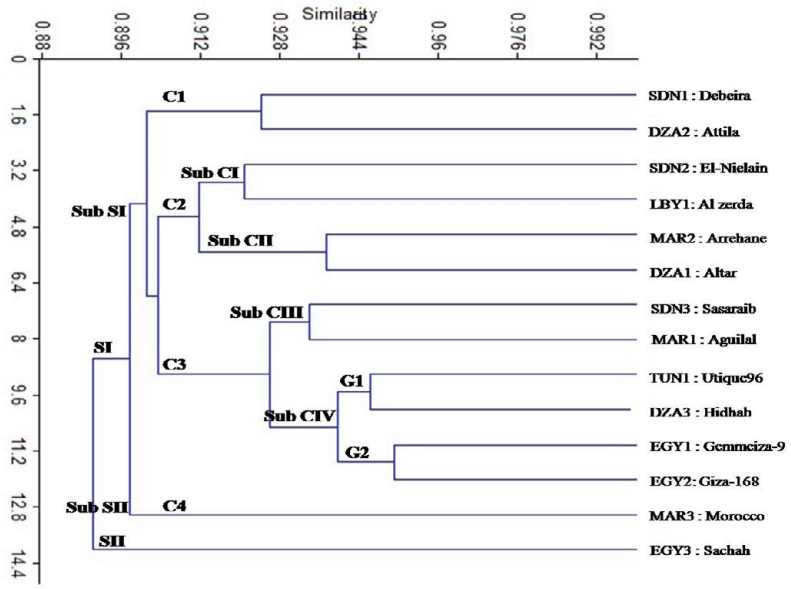

Figure 6: Cluster analysis resulted from *T. aestivum* cultivars. Dendrogram for the fourteen examined *T. aestivum* L. cultivars constructed from the SCoT polymorphsim data using Unweighed Pair-group Arithmetic (UPGMA) and similarity matrices computed according to Dice coefficient.

Table 6: Similarity matrix among studied *T. aestivum* L. cultivars as computed according to Dice coefficient as revealed by SCoT markers.

Cult.	DZA1	DZA2	DZA3	EGY1	EGY2	EGY3	LBY1	MAR1	MAR2	MAR3	SDN1	SDN2	SDN3	TUN1
DZA1	100													
DZA2	90	100												
DZA3	91	94	100											
EGY1	93	92	**94**	100										
EGY2	90	90	**94**	95	100									
EGY3	91	90	88	90	88	100								
LBY1	91	87	87	91	89	90	100							
MAR1	92	90	94	91	93	88	**84**	100						
MAR2	94	89	89	91	90	90	90	91	100					
MAR3	90	92	90	89	89	88	86	92	91	100				
SDN1	90	92	90	92	91	89	92	87	89	88	100			
SDN2	92	90	91	90	89	89	92	90	91	90	92	100		
SDN3	93	88	92	91	94	89	90	93	91	90	88	93	100	
TUN1	91	91	**95**	94	94	88	89	93	91	89	91	91	92	100

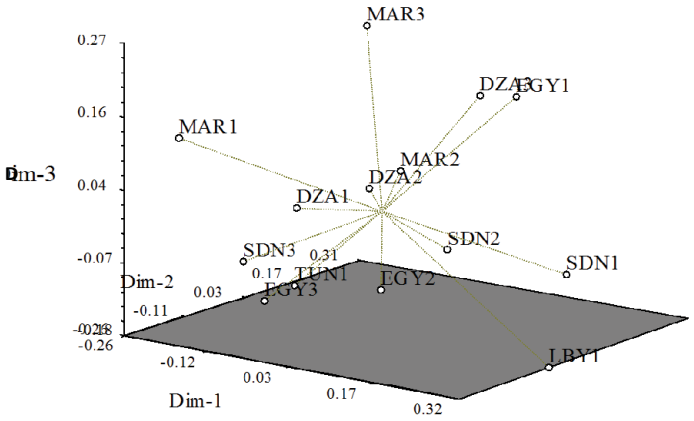

Figure 7: Principal coordinates analysis for the first three coordinates calculated for SCoT markers of the studied fourteen *T. aestivum* L. cultivars.

Table 7: Identification, size, and identity of sequenced SCoT amplicons from *T. aestivum* cultivars, which were cloned into pGEM cloning vector.

Spot ID / cultivar code	SCoT primer	Size (Nucleotides)	Description	Max score	Total score	Query cover	e-value	Identity (%)	Accession
Sfa-2/ SDN2: El-Nielain	SCoT-2	450	1- *T. aestivum* chromosome 3B, cultivar Chinese Spring. 2- *T. aestivum* gene for TaAP2-D (determines the cleistogamous/non-cleistogamous flowering), cultivar Chinese Spring. 3- *T. monococcum* (FR-Am2 locus), frost resistance-2 (Fr-Am2).	737 161 150	1.461e+05 161 150	89% 36% 36%	0.0 1e-35 2e-32	89% 76% 75%	HG670306.1 AB749310.1 AY951944.1
Sfa-3/ SDN2: El-Nielain	SCoT-3	500	1- *T. aestivum* chromosome 3B, genomic scaffold, cultivar Chinese Spring. 2- *T. aestivum* chromosome arm 3DS-specific BAC library, contig ctg447	562 96.9	16351 96.9	95% 75%	3e-156 3e-16	85% 66%	HG670306.1 HE774676.1
Sfa-8/ EGY3: Sachah	SCoT-8	150	1- *T. aestivum* clone wlsu2.pk0001.h3:fis, full insert mRNA sequence 2- *T. aestivum* MADS-box transcription factor TaAGL29 (AGL29) mRNA, complete cds	104 89.7	104 89.7	100% 87%	2e-21 4e-17	90% 90%	BT009458.1 DQ512346.1
Sfa-11/ MAR 2: Arrehane	SCoT-11	650	1- *T. aestivum* chromosome 3B, genomic scaffold, cultivar Chinese Spring.	102	949	67%	6e-18	71%	HG670306.1
Sfa-47/ EGY3: Sachah	SCoT-47	500	No hits found for this sequence in *T. asetivum* or any other plant.						
Sfa-48/ EGY3: Sachah	SCoT-48	200	1- *T. aestivum* chromosome 3B, genomic scaffold, cultivar Chinese Spring. 2- *T. aestivum* ammonium transporter Amt1;1 mRNA, complete cds. 3- *T. aestivum* wPR4f-b gene for wheatwin6-b defense protein, exons 1-2 (defense protein to bacteria).	192 69.8 48.2	16733 69.8 48.2	81% 45% 20%	1e-47 8e-11 3e-04	100% 90% 100%	HG670306.1 AY525637.2 AM116976.1

and their rational comparison may emerge novel insights and give a better understanding of the domestication of *T. aestivum* L. genetic diversity. In addition, this study provided for the first time how high resolution SEM and SCoT polymorphism analysis were positioned and integrated together in the characterization and discrimination of some Egyptian *T. aestivum* cultivars.

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Author Affiliations

Top

[1]*Botany Department, Faculty of Science, Ain Shams University, Cairo, Egypt*
[2]*Botany Department, Faculty of Science, Zagazig University, Sharqia, Egypt*