GENETIC DIVERSITY IN A WHEAT CULTIVAR AND ITS VARIANTS UNDER SALINITY STRESS CONDITIONS USING MORPHOLOGICAL TRAITS AND RAPD MARKERS

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ABSTRACT

New variants, derived from the commercial wheat cultivar, Sids-1, using stepwise in vitro selection method by NaCl as selective agent, were evaluated, as well as their parent, using morphological traits and RAPD markers to determine the genetic diversity among them.

The standardized mean values of six morphological traits were used to determine a data matrix of pair-wise similarities among genotypes. The results showed that the average genetic similarity among the wheat cultivar (Sids-1) and its variants was 0.34 with values ranged from 0.0 to 1.0. The results, also, revealed that the Sids -1 cultivar and V_1 showed a very high degree of similarity (1.0), indicating that no genetic variations had been induced for V_1. On the other hand, Sids-1 cultivar showed the highest dissimilarity value with V_3 (100%).

The results of RAPD analysis for Sids -1 wheat cultivar and its nine variants showed that a total of 59 DNA fragments were produced by the five primers used, and 93 % of these bands were polymorphic. Pair-wise genetic distance estimates for the ten genotypes, based on RAPDs, ranged from 0.19 to 0.60. The closest variant to Sids-1 cultivar was V_4 (0.39), while the most distant variant to it was V_5 (0.19).

Comparison of matrices of RAPD and morphological data showed a low correlation between the two dendrograms ($r = 0.03$).

In conclusion, these results indicated that both morphological analysis and molecular markers showed a high degree of variation among the wheat variants analyzed. These variants might represent an important source of genetic diversity in wheat and could be used in future breeding programs. Although the correlation between morphological and RAPD data was low, both techniques could be complementary used in wheat characterization.

INTRODUCTION

Increasing wheat production in developing countries, under soil and water salinity, has become important in recent years. Salinization of soils in many parts of the world is reducing the available land for conventional agriculture. Egypt is one of the countries that suffers from sense salinity problems, 33 % of the cultivated land, which comprises only 3 % of the total land area in Egypt, is already salinized due to low precipitation (less than 25 mm annual rainfall) and irrigation with saline water (Ghassemi *et al.*, 1995). The development of salt-tolerant crop cultivars has been considered to be an efficient and economic mean of overcoming salinity problems (Epstein *et al*, 1980).

The conventional breeding systems have met with limited success in improving the response of many crops to salt stress (Epstein *et al*, 1980). The use of tissue culture techniques has the potential to increase the stress tolerance of plant cells, containing a complete species genome and, thus, are totipotance. Recently, *in vitro* selection schemes for the isolation of salt- tolerant cell lines have been successfully reported in various crop species (Barakat and Abdel-Latif, 1996 and Bajji *et al*, 2004).

Identification of genetic diversity is one of the important tools in plant breeding. Morphological and cytogenetic traits, used at present, are not stable, time-consuming and are affected by environmental conditions, but, molecular markers are stable (Boggini *et al*, 1990). Biochemical markers, such as isozymes and seed storage proteins, and DNA-based markers, such as restriction fragment length polymorphisms ($RFLP_s$) and random amplified polymorphic DNA (RAPD), have been used to evaluate the genetic diversity among wheat landraces (Autrique *et al.*, 1996 and Pujar *et al.*, 1999). Other molecular markers, such as microsatellites and amplified fragment length polymorphisms ($AFLP_s$), also, have been used to study genetic relationships among wheat species. Among all these molecular markers, $RAPD_S$ are simple, user-friendly, and cost-and time-effective. They have been successfully used for the evaluation of plant genetic resources in wheat genotypes (Joshi and Nguyen , 1993 and Vierling and Nugyen, 1992).Recently, Barakat and Milad (2006) reported that the use of RAPD technique to detect genetic variation at the level of DNA, among wheat cultivars and their somaclones, was sensitive and powerful. This would be of particular importance in the future, when dealing with *in vitro* selection to stress conditions.

The objective of the present study was to determine the genetic diversity among the wheat cultivar, Sids-1, and its selected variant lines, derived from step-wise *in vitro* selection to NaCl, using agronomical traits and RAPD markers. Assessment of genetic diversity in wheat variants would facilitate the development of variants for specific production constraints by providing an index of parental lines to be used in breeding programmes.

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MATERIALS AND METHODS

Plant materials:

Variants, derived from the commercial wheat cultivar, Sids-1, using a stepwise *in vitro* selection method by NaCl as selection agent (Barakat and Abdel-Latif,1996). In such stepwise, the calli derived from immature embryos were transferred to salt-free medium and was, then, subcultured monthly on a fresh medium with gradually increasing concentration of NaCl by 3g until it reached 12g/L. Then, the calli were transferred to the regeneration medium free of NaCl.

Regenerated plants were grown during 2004/2005 winter to obtain R_1 seeds. R_1 plants were selfed to obtain R_2 seeds during the season of 2005/2006. The R_2 seeds of nine variants for salt tolerance were successfully obtained.

Growing under salinity conditions:

R_2 seeds derived from the variants, as well as the parent cultivar, were grown under greenhouse conditions. They were sown in pots, 20 cm in diameter and 15 cm length. Three seeds from each genotype were sown per pot, three pots were used for each genotype. The pots were filled with sand washed with water three times. Salinity solutions were prepared, using NaCl at the levels of 1.2 and 7.0 dsm^{-1}. All pots were irrigated, using water at the level of 1.2 dsm^{-1} salinity, till complete emergency of seeds. This process took one month. From this stage to maturity, pots were irrigated with 7.0 dsm^{-1} saline water.

Morphological characters assessment and data analysis:

The variants, as well as the parental cultivar, (Sids-1), were harvested on May, 2007. The grain yield per pot, 100-kernel weight, number of spikes per pot, grain weight per spike, number of kernels per spike and number of days to heading were recorded.

The standardized traits mean values (mean of each trait was subtracted from the data values and the results were divided by the standard deviation) were used to determine a data matrix of pairwise similarities among genotypes, according to Jaccard coefficient (Jaccard, 1908).

The relationships between the Jaccard distance matrix, based on morphological characters and genetic distance matrix obtained with RAPD analysis, were analyzed, according to Mantel (1967), using NTSYS-pc.

RAPD analysis:

PCR analyses were carried out, using genomic DNA from the wheat cultivar, Sids-1, and its selected variant lines. The selected variant lines were designated as V_1 to V_9.

DNA extraction:

Genomic DNA was extracted from fresh leaves of the wheat cultivar and its variant lines, using CTAB (Saghai -Maroof *et al.*, 1984). RNA was removed from the DNA preparation by adding 10µl of RNAase (10mg /ml) and, then, incubating for 30 min. at 37°C. DNA sample concentration was quantified by using a spectrophotometer (Beckman Du-65).

PCR amplification:

Five primers (Table 1) were used in the experiment to amplify the templated DNA. Each amplification reaction was performed in a 25-µl vol., containing 50 ng of genomic DNA, 1x PCR buffer Mg Cl_2 (60 mM KCl, 10mM Tris- HCl (pH 9.0), 2mM $MgCl_2$ and 1% Triton x-100), 200 mM each of dATP, dCTP, dGTP and dTTP (promega), 50 pM primer, 50ng template DNA and 1.5 µl of Taq DNA polymerase. Amplifications were carried out in an MJ Research PTC-100 thermal cycler with amplification conditions, adopted from Williams *et al.* (1990), DNA denaturation at 94°C for three minutes and 45 cycles of melting at 94°C for one min., annealing at 36°C for one min. and extending at 72°C for two min. This was followed by a seven min. final extension step at 72°C, then, the reactions were kept at 10°C. RAPD fragments were size- fractionated in a 2% agarose gel in TBE 0.5 x TBE buffer, with a 1-kb ladder molecular weight marker. Gels were stained in ethidium bromide solution and, then, photographed.

Data analysis:

The RAPD bands were scored as one for the presence or zero for absence of a particular DNA fragment of a similar length. Only reproducible clear amplification bands were scored for the construction of the data matrix .Pair-wise comparisons of genotypes, based on the presence or absence of unique and shared polymorphic products, were used to regenerate similarity coefficients, according to Jaccard (1908). The similarity coefficients were used to construct a dendrogram by UPGMA (Unweighted Pair- Group Method with Arithmetical Averages), using NTSYS-pc (Rohlf, 1993).

RESULTS AND DISCUSSION

Morphological diversity analysis:

The UPGMA dendrogram obtained, using morphological characters (Fig.1) clearly separated the Sids-1 cultivar and its nine variants into three clusters. The first cluster consisted of four variants; i.e., V_3, V_8, V_4 and V_9. The second cluster includes V_5, V_6, V_1, V_7, as well as the Sids-1 cultivar. The latter cluster included only V_2.

The first and the second clusters diverged at a similarity index of 0.28. However, the third cluster diverged at a similarity index of 0.11.

The average genetic similarity among the wheat cultivar (Sids-1) and its variants was 0.34 , with values ranging from 0.0 to 1.00 (Table 2). The Sids-1 cultivar and V_1 showed a very high degree of similarity (1.0), indicating that this pair closely related genotypes, as well as no genetic variation, had been induced for V_1. On the other hand, the V_2 with either V_3 , V_4 , V_5 , V_8 or V_9 had the highest dissimilarity values (100%) . Also, it was observed from Table 1 that the V_3, with either Sids-1 or V_1 and the V_5 with V_9, had the highest dissimilarity values (100 %).

RAPD analysis:

Five primers were screened for their ability to amplify the genomic DNA of the wheat cultivar (Sids-1) and its variants. The number of DNA fragments amplified ranged from 9 to 18, depending on the primer and the DNA sample with a mean value of 11.8 bands per primer (Table 2). These results were almost similar to those reported in other wheat cultivars and their somaclones (Barakat *et al*,2005; Barakat and Milad,2006). On the other side, these results are considered rather high for RAPD amplification, compared to the average number of amplified bands recorded in other cereal crops; namely, three fragments in *Triticum turgidum* L. (Joshi and Nguyen, 1993) and 6.7 fragments in *Zea mays* L. (Heun and Helentjaris,1993).

The size of fragments ranged from 150 to 850 bp. A total of 59 fragments were produced by the five primers. From these 59 fragments, 7% were not polymorphic, whereas, the remaining bands (93%) were polymorphic in one or another of the ten genotypes (one cultivar and variants).However, Figure 2 shows the amplification profiles, generated by primer 1 (5' TTCCCCCGCT 3') across the Sids-1 cultivar and its variants. There were eleven scorable bands of this primer, and ten of them were polymorphic across the genotypes, with a percent of 91 polymorphism. In the present investigation, results of RAPD analysis had proved to be a very useful and rapid method to detect variation among different wheat genotypes, even when a very high number of markers had not been scored. Wolff (1996) reported that the choice of the primers might be an important factor in obtaining a rapid discrimination among samples.

Pair-wise genetic distance estimates for the ten genotypes, based on $RAPD_S$, are shown in Table 3. The genetic distances ranged from 0.19 to 0.60. The closest variant to the Sids-1 cultivar was V_4 (0.39), while the most distant variant to such cultivar was V_5 (0.19).

The UPGMA cluster of the Sids-1 cultivar and its variants further revealed interesting associations, based on $RAPD_s$ (Fig.3). The results of characterization analysis revealed a high diversity between the cultivar and its variants. Three clusters could be observed. The first cluster included five variants (V_1, V_2, V_3, V_4 and V_5). The second cluster included the other four variants (V_6 toV_9) .The two clusters diverged at similarity index of 0.39. The last cluster included only the Sids-1 cultivar. Moreover, the last cluster and the two other clusters diverged at a similarity index of 0.28.It could be observed from Figure 3 that the shortest genetic distance (the highest similarity value) was found between V_8 and V_9 , whereas, the highest distance (the low similarity value) was observed between the parent (Sids-1) and V_5.

The RAPD data, obtained in the present study, showed a good fitness among the wheat cultivar (Sids -1) and its variants under salt-stress conditions .These results, also, indicated that the RAPD technique was effective to detect the genetic variation at the level of DNA among the wheat cultivar and its variants, as well as the stepwise *in vitro* selection, was a useful method to induce variation at cell level.

The estimates of genetic relationship could be helpful for organizing germplasm for conservation of genetic resources for the identification of genotypes for selection of parents for hyberdization for predicting favorable heterotic combinations. This, also, helps to reduce the number of samples required for sampling of genetically variable broad range of genotypes in breeding programs .Genotypes, with the most distinct DNA profiles, are likely to contain the greatest number of novel alleles, which are likely to undercover the largest number of unique and potentially agronomically useful alleles.

Comparison between RAPD and morphology:

In order to compare the extent of agreement among dendrograms, derived from morphology and RAPD markers, a distance matrix was constructed for each assay and compared, using the Mantel matrix correspondence test. Comparison of matrices of RAPD and morphological data showed a low correlation among dendrograms ($r=0.03, p=0.974$). Despite this low correlation between morphological and molecular analysis, there were similar genotypes formed in the respective dendrograms .The formation of three clusters was consistently found in both analysis, however, some discrepancies between the two dendrograms could be found. For example, the variant 2 (V_2) was clearly separated in the morphological analysis, while, in the RAPD analysis, the same variant was clustered in the first cluster. Another discrepancy was concerned with the wheat cultivar (Sids -1), which did fall into the second cluster in the morphological dendrogram, but, in the molecular analysis, it was separated in the last cluster. Several reasons might be responsible for the discrepancy among results, based on morphology and RAPDs. Although some RAPD markers might be associated with functionally important loci (Penner, 1996), most RAPDs might be amplified from noncoding regions of the genome (Williams *et al*,1990). Morphological traits are, generally, believed to be under the pressure of natural selection, and their expression is partially

under the influence of environmental factors .In contrast to morphological traits, RAPD variation is directly based on DNA sequence variation. A single nucleotide change can result in a change of the RAPD phenotype. Beside, these differences of morphological and RAPD markers, different combinations of alleles might result in morphological similarities or differences that are not proportional to the underlying genetic differences.

The low correlation between RAPD and morphological traits had been reported in other studies in European barley varieties (Schut *et al*, 1997), in rye grass varieties (Roldan-Ruiz *et al*, 2001) and synthetic hexaploid wheat and their parents (Lage *et al*, 2003). Schut *et al*. (1997) reported that no significant correlation between molecular markers and 25 morphological traits in barley. Working with sixteen ryegrass varieties. Rolden-Ruiz *et al*. (2001) reported a correlation value of r = -0.06 between molecular markers and fifteen morphological characters. Similar results were found in wheat by Lage *et al* (2003), who detected differences between dendrograms generated from morphological traits and molecular data, and they suggested that morphological and molecular differences were apparently independent, due to different selection and evolutionary factors. Although AFLP markers can cover a high proportion of the genome because of the high number of bands scored in each analysis, due to its neutral origin, there is no guarantee that such bands fall in coding regions of the genome involved in morphological and agronomic traits.

In the present investigation, the characterized variants were mainly classified, according to agronomic traits under salinity stress conditions, which were complex and multigenic characters. Such characters were environmentally affected and, therefore, liable to subjective evaluation. In this sense, the molecular characterization was more efficient in the generation of an unbiased picture of diversity than an agronomic approach. However, the agronomic characterization was still important in wheat germplasm management, and determination of molecular diversity should not be seen, as replacing traditional characterization, but, rather as a complement to it.

In conclusion, these results indicated that both morphological analysis and molecular markers showed a high degree of variation among the wheat variants analyzed. These variants should represent an important source of genetic diversity in wheat and could be used in future breeding programs. Although the correlation between morphological and RAPD data was low, both techniques could be complementary used in wheat characterization.

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Table 1: Morphological similarity matrix, based on Jaccard coefficient for Sids-1 wheat cultivar and its variant lines determined for analysis, using the mean values of six morphological characters with three replications.

	Sids -1 (P)	V1	V2	V3	V4	V5	V6	V7	V8	V9
Sids -1 (P)	1.00									
V_1	1.00	1.00								
V_2	0.33	0.33	1.00							
V_3	0.00	0.00	0.00	1.00						
V_4	0.5	0.5	0.00	0.25	1.00					
V_5	0.25	0.25	0.00	0.33	0.25	1.00				
V_6	0.75	0.75	0.25	0.2	0.4	0.5	1.00			
V_7	0.5	0.5	0.17	0.33	0.5	0.33	0.67	1.00		
V_8	0.2	0.2	0.00	0.67	0.5	0.25	0.4	0.5	1.00	
V_9	0.25	0.25	0.00	0.33	0.67	0.00	0.2	0.3	0.67	1.00

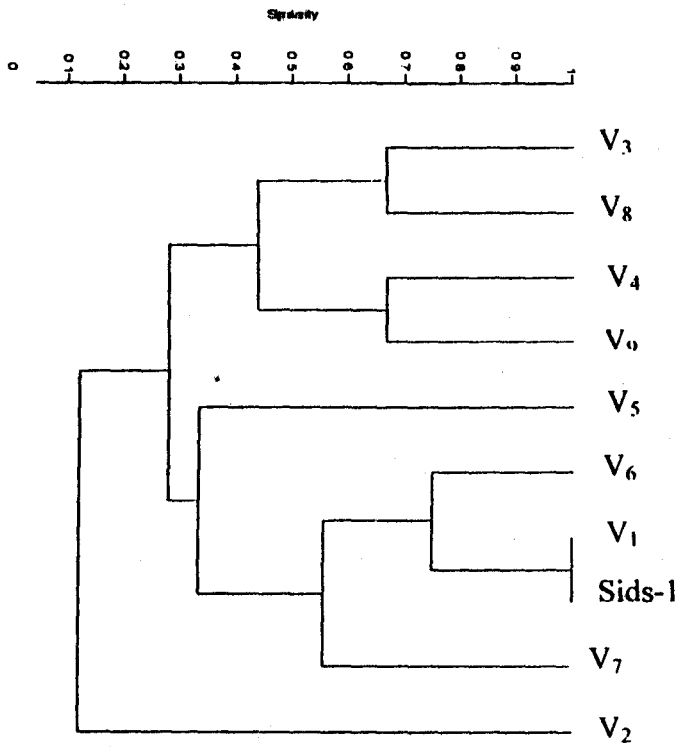

Fig. 1: Dendrogram for Sids-1 wheat cultivar and its variant lines, based on a cluster analysis (UPGMA) of phenotypic similarities (Jaccard coefficient) for six morphological characters with three replications.

Table 2: Number of amplified and polymorphic bands, using five primers, in Sids-1 wheat cultivar and its variant lines.

Primer number	Nucleotide sequence 5` to 3`	No. of amplified bands (a)	No. of polymorphic bands (b)	Degree of polymorphism b/a (%)
1	TTCCCCCGCT	11	10	91
2	GGGTAACGCC	10	8	80
3	CAATCGCCGTTCG	11	10	91
4	GCGATAG	9	9	100
5	AGCCAGCGAA	18	18	100

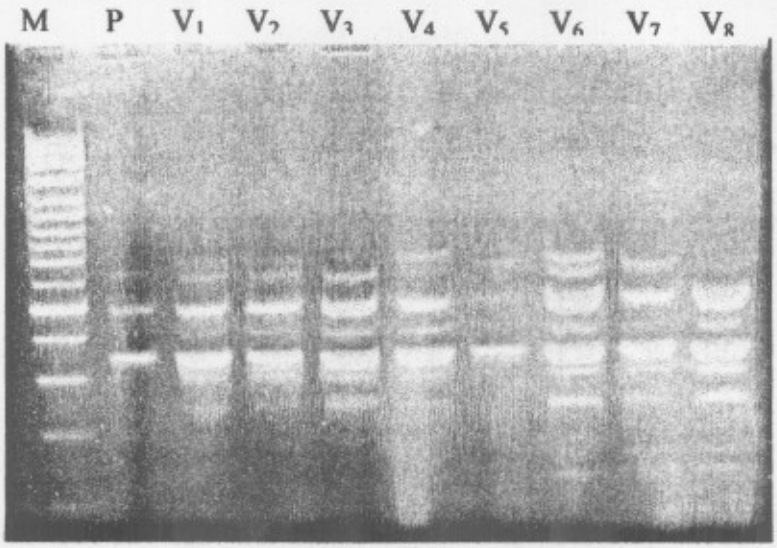

Figure 2 : RAPD polymorphic in Sids -1 wheat cultivar and its variants. Primer (5'TTCCCCCGCT 3')

Table 3: RAPD similarity matrix, based on Jaccard's coefficient for Sids-1 wheat cultivar and its variant lines determined from analysis, using five primers that amplified 59 DNA fragments.

	Sids -1 (P)	V1	V2	V3	V4	V5	V6	V7	V8	V9
Sids -1 (P)	1.00									
1	0.23	1.00								
2	0.20	0.61	1.00							
3	0.34	0.58	0.58	1.00						
4	0.39	0.53	0.50	0.54	1.00					
5	0.19	0.40	0.52	0.32	0.45	1.00				
6	0.32	0.32	0.35	0.37	0.33	0.30	1.00			
7	0.24	0.29	0.40	0.31	0.34	0.45	0.43	1.00		
8	0.26	0.36	0.50	0.40	0.34	0.46	0.48	0.58	1.00	
9	0.26	0.44	0.49	0.38	0.41	0.48	0.50	0.49	0.60	1.00

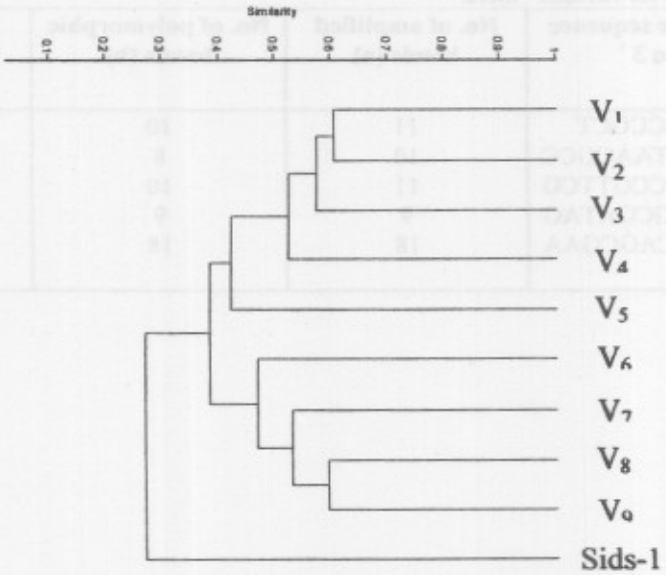

Fig. 3: Dendrogram for Sids-1 wheat cultivar and its variant lines, based on cluster analysis (UPGMA) of genetic similarities (Jaccard coefficient) for RAPD data.

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الملخص العربي

البعد الوراثي لصنف قمح و ال variants الناتجة منه تحت ظروف الاجهاد الملحي باستخدام الصفات المورفولوجية وRAPD marker.

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تم تقويم تسعة variants ناتجة من صنف القمح المحلي" سدس-١" بالإضافة إلى الأب باستخدام طريقة stepwise للانتخاب المعملي في وجود كلوريد الصوديوم كمادة للانتخاب وذلك باستخدام الصفات المورفولوجية و RAPD markers لتقدير البعد الوراثي بينهم . استخدمت نتائج ست صفات مورفولوجية لتقدير درجة القرابة الوراثية بين صنف القمح "سدس-١" و ال variants الناتجة منه. و قد أظهرت النتائج أن متوسط القرابة الوراثية بينهم كان ٠,٣٤ و تراوحت القيم ما بين ٠,٠-١,٠. كما أظهرت النتائج انه لا توجد أي اختلافات وراثية بين الأب" سدس-١" و V_1 حيث كان معامل التشابه الوراثي (١,٠) . و في الجانب الأخر فان V_5 أظهر اختلافات وراثية عالية جدا (١٠٠%) بالمقارنة بالأب" سدس- ١" .

أظهرت نتائج تحليل الRAPD للصنف " سدس-١" و التسعة Variants الناتجة منه أظهرت أن V_5 كانت أكثر ال variants اختلافا عن الأب "سدس-١" حيث كان معامل التشابه الوراثي بينهما ٠,١٩ .

أظهرت المقارنة بين نتائج التحليل المورفولوجي و تحليل ال RAPD تلازما منخفضا بين الطريقتين (ر=٠,٠٣) .

وبناء عليه فان النتائج أثبتت أن كل من التحليل المورفولوجي و Molecular markers اظهر درجة كبيرة من الاختلافات بين التراكيب الوراثية المختبرة ، هذه الاختلافات قد تعتبر مصدرا مهما للاختلافات الوراثية في القمح يمكن استخدامه في برامج التربية في المستقبل. و على الرغم من أنخفاض درجة التلازم بين الطريقتين فانه يمكن استخدام كل منهما كطريقتين مكملتين لبعضهما البعض لتوصيف القمح.