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RESEARCH ARTICLE

Assessment of the combining ability and authentication of F_1 hybrids using SSR markers in wheat (*Triticum aestivum* L.)

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Abstract **Six wheat genotypes (three female and three male) were crossed for the study of some quantitative traits in wheat. Analysis of variance showed a highly significant difference for all the characters except flag leaf area, which was significant. Testers revealed that LU26S was the best general combiner only for plant height. Mehraj showed a good general combining ability effect on plant height, flag leaf area, peduncle length, and 1000-grain weight. Farid 2006 was the best male parent as general combiner for plant height, peduncle length, spike length, number of grains per spike, and grain yield per plant. The wheat parental lines revealed that 9381 was the best general combiner for plant height, flag leaf area, peduncle length, 1000-grain weight, and grain yield per plant. Whereas 9428 was the best general combiner for flag leaf area, spike length, and number of spikelets per spike. Among crosses, LU26S × 9272, LU26S × 9381, Mehraj × 9272, and Mehraj × 9381 showed a significant effect of specific combining ability (SCA) on grain yield per plant. Other crosses with significant and positive SCA effects were LU26S × 9272 on plant height and 1000-grain weight grain yield per plant, LU26S × 9428 on peduncle length, and Mehraj × 9381 on plant height and grain yield per plant. These crosses with significant effects of general combining ability (GCA) on grain yield per plant can be used in the development of new varieties. These crosses with nonadditive genes would give transgressive segregants. For yield improvement, vigilant selection of the potent transgressive segregants through family selection would be valuable for yield enhancement. A total of 15 SSR primers of Xgwm series and 5 of X series were used to find out the codominant loci in the hybrid and single dominant loci in parents. Out of 15 primers only, Xgwm-314 gave the polymorphic banding pattern. This primer showed the polymorphic dominant loci in the parents (LU26S, Mehraj, 9272 and 9381) and codominant loci midway between these parents. Therefore, this SSR primer was used to confirm the two best performing hybrids (LU26S × 9272 and Mehraj × 9381) on the bases of positively significant effects of GCA and SCA on plant height, 1000-grain weight and grain yield per plant, and other economically important traits. The two hybrids namely LU26S × 9272 and Mehraj × 9381 can be used in the further breeding program for the development of high yielding varieties.**

Keywords wheat, combining ability, hybrid authentication, SSR markers

Introduction

Bread wheat (*Triticum aestivum* L.) is a major food crop in the world and a staple food in Pakistan, where wheat occupies a pivotal position in its economy, contributing 13.8% to the value added in agriculture and 3.4% to the total GDP of the state. Manipulation of accessions for exploring genotypes with best combining ability and identification of transgressive segregates by using modern genetic tools is a prerequisite for high-yielding varieties. Combining ability analysis gives useful information regarding the selection of parents in terms of the performance of the hybrids. Such information is of great importance in forming and executing an efficient breeding program in order to achieve maximum genetic gain with the minimum resources and less time. Diallel

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mating design (Griffing, 1956) involving a large number of parents creates a problem in artificial crossing and is also unmanageable under field conditions, but line × tester analysis is efficient to evaluate a large number of parents for general and specific combining ability. Line × tester analysis (Kempthorne 1957) was used in the present study to determine the nature and relative contribution of general and specific combining ability estimates of the selected wheat cultivars as a means of selecting parents for the future hybridization program. Marker assisted selection (MAS) is a powerful tool for the indirect selection of difficult traits at early stages before production of next generation, thus it speeds up the process of conventional plant breeding and facilitates the progress of traits that cannot be improved easily by conventional methods. SSR markers are useful for a variety of applications in plant breeding and genetics because of their reproducibility, multiallelic nature, codominant inheritance, relative abundance, and good genome coverage (Morgante and Olivieri, 1993). These markers are an efficient tool for hybrid authentication.

Materials and methods

Assessment of combining ability

The investigations about morphological parameters were carried out in the experimental area of the Department of Plant Breeding and Genetics, University of Agriculture Faisalabad, Pakistan. The experimental material for estimation of combining ability comprised three lines and three testers of spring wheat, namely, LU26S, Mehraj, Farid-2006, 9272, 9428, and 9381, respectively. Three varieties, namely, LU26S, Mehraj, and Farid-2006 were crossed with university elite lines, namely, 9272, 9428, and 9381, by using line × tester mating design during the year 2008–2009, as described by Kempthorne (1957). Care was taken to avoid the contamination of genetic material during crossing.

The F_1 seeds along with their parents were planted in the field during the cropping season of 2009–2010 by randomized complete block design with three replications using interplant and interrow distance of 15 cm and 30 cm, respectively. Two seeds per hole were sown with the help of a dibbler and later thinned to single seedling per hole after germination. For the entire experiment, other cultural and agronomic practices were kept constant. At maturity, ten guarded plants from each row were taken randomly from each plot, and data were recorded for nine morphological traits, like plant height, flag leaf area, number of tillers per plant, peduncle length, spike length, number of spikelets per spike, number of grains per spike, 1000-grain weight, and grain yield per plant. Thus, the data collected were subjected to analysis of variance technique (Steel et al., 1997). The traits showing significant genotypic difference will be further analyzed for combining ability studies using line × tester analysis, as outlined by Kempthorne (1957).

Authentication of hybrids by using SSR markers

This part of research was carried at the Center of Agriculture Biochemistry and Biotechnology (CABB), Faisalabad, Pakistan, for the authentication of hybrids derived from three varieties and three lines by using microsatellite or simple sequence repeat (SSR) markers.

DNA isolation

Genomic DNA was isolated from 7-day-old seedlings of all the parents and hybrids by following the method of Doyle and Doyle (1990) with little modification. Two gram of fresh leaves were crushed with motor and pestle in the presence of 10 mL preheated (65ºC) 2×CTAB solution to make the homogeneous mixture, which was taken into 50 mL falcon tubes. The homogenized samples were incubated in a water bath at 65ºC for 30 min and shacked gently after every 5 min. Tubes were placed at room temperature for 5 min and gently shaken. Equal volume of chloroform-isoamylalchol (24 : 1) was added into the tubes, which then were centrifuged at 3500 r/min for 15 min. Supernatant was taken in fresh falcon tubes, and 2/3 volume of ice-chilled isopropanol was added to precipitate the DNA. After tubes were spun and supernatant was discarded, DNA pallet was taken out, washed with 70% ethanol, air-dried, and resuspended in 400 µL of 0.1 d_3H_2O. To remove any RNA from the preparation, 2 µL RNase was added to the pellet and incubated at 37ºC overnight. Extracted DNA was stored at −20ºC.

PCR amplification and electrophoresis

The PCR conditions were optimized according to the procedure as described by Dograr and Akkaya (2001) for the amplification of DNA. PCR conditions for SSR analysis were optimized in eppendorf DNA mastercycler/mastercycler gradient, Germany. A total of 15 codominant SSR primer pairs were used in PCR reaction for all genotypes and their crosses. For SSR analysis concentrations of genomic DNA, 10 × PCR buffer, $MgCl_2$, dNTPs, primer, and *Taq* DNA polymerase were optimized. The concentrations of PCR reagents were used to make the final reaction mixture of 20 µL (1×) as follows.

Reagent concentration volume consisted of template DNA (10 ng) 2.0 µL, dNTP's (2.5 mM) 4.0 µL, buffer (10×) 2.0 µL, $MgCl_2$ (25 mM) 1.6 µL, Primer-F (30 ng/µL) 1.5 µL, Primer-R (30 ng/µL) 1.5 µL, *Taq* DNA polymerase (5 U/µL) 0.25 µL, and double distilled H_2O ultra pure 7.15 µL (total volume 20 µL).

PCR was carried out in eppendorf mastercycler/mastercycler gradient (Germany). The PCR profile used to amplify the genomic DNA is given as follows:

Steps	Temperature	Time
1	T = 94ºC	3 min (Denaturation)
2	T = 94ºC	30 s (Denaturation)
3	T = 48ºC	30 s (Annealing)
4	T = 72ºC	1 min (Extension)
5 followed by 35 cycles		
6	T = 72ºC	10 min (Final extension)
7	T = 20ºC	until the tubes were removed

Gel electrophoresis

First, the PCR products were electrophoresed on 3.0% agarose gels using 0.5 × Tris Borate EDTA (TBE) buffer and visualized by ethidium bromide staining under UV light and photographed using gel documentation system (GDS).

Results and discussion

Table 1 reveals that there was a significant difference in combining ability among the replications only for flag leaf area but nonsignificant combining ability for the rest of the traits studied. Highly significant differences of combining ability were recorded among genotypes for plant height, peduncle length, spike length, number of spikelets per spike, number of grains per spike, and grain yield, while a significant different combining ability for flag leaf area, number of tillers per plant, and 1000-grain weight indicated the existence of adequate genetic variability for genetic analysis. The perusal of the results in Table 1 showed that female parents (lines) accounted a nonsignificant combining ability for peduncle length, while a highly significant one was shown for plant height, flag leaf area, number of tillers per plant, spike length, spikelets per spike, number of grains per spike, and 1000-grain weight except a significant one for grain yield per plant. Male parents (testers) showed highly significant differences in combining ability effects on plant height, peduncle length, and grain yield per plant, while a significant difference affected the number of grains per spike. Line × tester interaction in the present study was significant for plant height, peduncle length, number of spikelets per spike, and grain yield per plant. Crosses had a significant combining ability effect on all the tested traits. However, the parents × crosses combination also had a significant combining ability effect on peduncle length, spike length, and number of grains per spike. These results indicated that for genotypes, parents, crosses, and parents × crosses, all lines and testers markedly differ in the combining ability effects on grain yield and most of the other traits under study.

Table 2 exhibits that among the male parents, LU26S showed the best performance in plant height, number of tillers per plant, 1000-grain weight, and grain yield per plant. Farid 2006 showed the better performance in the number of spikelets per spike and number of grains per spike, while Mehraj exhibited higher values in spike length and 1000-grain weight. Among female parents, 9428 showed the best performance in plant height, number of grains per spike, and 1000-grain weight, while 9272 did in leaf area and peduncle length and 9381 in spike length and grain yield per plant.

Among the crosses, LU26S × 9428 and Mehraj × 9428 expressed the best performance in plant height, and the following crosses, Mehraj × 9272, Farid × 9381, LU26S × 9428, Farid × 9381, Farid × 9428, and Farid × 9381, were the most promising in flag leaf area, number of tillers per plant, peduncle length, spike length, number of grains per spike, and number of spikelets per spike, respectively. LU26S × 9381 and Farid × 9381 were the best crosses in 1000-grain weight and grain yield per plant, respectively.

General combining ability

In case of plant height, negative general combining ability effects are important since more emphasis is placed upon selection for short stature segregates in segregating population because the short stature line is more responsive to fertilizer and tolerant to lodging will be bred ultimately. From this point of view, 9272 among female parents showed a negative but significant value (Table 3). LU26S and Farid 2006 among male parents illustrate the most significant GCA

Table 1 Analysis of variance for various quantitatively inherited traits of wheat genotypes derived from line x tester analysis three lines and three testers

SOV	*df*	Plant height (cm)	Flag leaf area (cm^2)	Tillers per plant	Peduncle length (cm)	Spike length (cm)	Spikelets/ spike	Grains/ spike	1000-grain weight (g)	Grain yield per plant (g)
Reps	2	28.65NS	355.71**	1.28 NS	3.88 NS	0.41 NS	0.780 NS	0.25 NS	1.42 NS	1.22 NS
Genotype	14	86.65**	13.578	2.37*	15.50**	1.28**	1.81**	45.53**	48.36**	6.65**
Parents	5	58.67**	6.28 NS	2.87*	16.18**	0.82*	1.23**	31.09**	45.55 NS	7.51**
P × C	8	2.80 NS	31.36 NS	2.35 NS	24.90**	2.44**	0.05 NS	221.58**	6.53 NS	4.17 NS
Crosses	8	114.61**	15.89*	2.06*	13.90**	1.42**	2.38**	32.54**	55.33*	6.43**
Lines	2	69.80**	47.87**	3.74*	4.19 NS	5.06**	6.12**	89.03**	145.33**	5.91*
Testers	2	168.51**	5.04 NS	0.50 NS	12.81**	0.43 NS	0.26 NS	7.97*	12.44 NS	10.07**
L × T	1	110.07**	5.33 NS	2.00 NS	19.30**	0.09 NS	1.58**	16.59**	31.77 NS	4.87**
Error	28	12.51	5.61	1.10	1.84	0.28	2.02	0.42	0.87	4.56
Total		147.01	10.78	1.124	4.19	1.97	1.26	9.84	10.67	8.53

* and ** represent significant differences at 5% and 1% levels of probability respectively. NS means nonsignificant, P × C means parents versus crosses, and L × T means line × tester.

Table 2 Mean values for various quantitatively inherited traits of crosses derived from line × tester analysis

Genotypes	Plant height (cm)	Flag leaf area (cm^2)	Tillers per plant	Peduncle length (cm)	Spike length (cm)	Spikelets /spike	Grains /spike	1000-grain weight (g)	Grain yield per plant (g)
9272	103.1	28.89	9.20	33.40	13.17	23.20	64.200	32.67	28.33
9428	107.9	28.59	9.27	31.67	13.36	21.47	66.667	38.00	30.00
9381	104	27.45	9.00	31.10	13.57	22.07	67.067	37.33	28.22
LU26S	113	23.17	10.87	30.37	11.57	19.93	57.733	34.00	27.00
Mehraj	108.5	26.66	9.87	35.80	14.53	20.80	61.133	33.33	21.11
Farid-2006	92.3	23.02	10.60	28.60	12.30	21.07	60.200	26.67	23.44
LU26S × 9272	108.73	28.97	8.53	34.27	12.73	21.40	64.40	38.67	24.89
LU26S × 9428	112.03	27.73	10.53	31.73	12.73	21.53	59.40	39.33	23.11
LS26S × 9381	108.06	28.03	10.33	32.80	12.93	21.67	64.33	40.00	23.33
Mehraj × 9272	109.6	30.15	10.20	37.47	13.53	21.40	66.53	39.33	20.22
Mehraj × 9428	110.23	28.40	9.93	34.40	13.03	21.20	66.40	37.33	23.56
Mehraj × 9381	108.53	29.98	10.20	34.77	12.97	20.47	65.13	34.67	29.11
Farid × 9272	102.66	26.21	8.27	31.97	13.13	21.40	66.40	28.67	24.00
Farid × 9428	105.33	28.13	8.00	31.07	12.37	22.47	65.67	32.00	27.00
Farid × 9381	99	29.03	9.40	32.60	13.90	21.40	73.80	36.67	30.00

effect on plant height. These potential parents can be used in the further breeding programs. For flag leaf area, positive general combining ability effects are more important because the flag leaf area has much contribution in the photosynthetic activity and, ultimately, in the grain yield, which is our main objective. Female parents 9428 and 9381 showed a positively significant GCA effect on flag leaf area followed by the two male parents LU26S and Mehraj. Number of tillers per plant also plays an important role in the grain yield as more number of tillers are expected to result in better yielding ability. General combining ability effects calculated for this trait were of moderate magnitude. Among female parents, 9272 and 9381 exhibited significant and positive general combining ability effects. Like plant height, shorter peduncle length is a required trait because an increase in peduncle length ultimately increases in the plant height, and preference is always given to plant with short stature. One male parent Farid-2006 depicted significant GCA effects on peduncle length and can be exploited for breeding dwarf genotypes. Mehraj 9381 and 9272 showed a significant GCA effect on 1000-grain weight, while 9381 also demonstrated highly significant GCA effect on grain yield per plant. It can be depicted from our study that Mehraj 9381 and 9272 should be exploited in further breeding endeavors due to their better GCA estimates.

Specific combining ability

Crosses between the parents with positive specific combining ability effects on desirable traits are likely to give transgressive segregants. Specific combining ability effects of the crosses depicted that there were some crosses showing significant SCA effects on grain yield per plant (LU26S × 9272, LU26S × 9381, Mehraj × 9272, and Mehraj × 9381;

Table 3 Estimation of general combining ability for various quantitatively inherited traits of three lines and three testers of wheat

Parent	Plant height (cm)	Flag leaf area (cm^2)	Tillers/plant	Peduncle length (cm)	Spike length (cm)	Spikelets/spike	Grains/spike	1000-grain weight (g)	Grain yield per plant (g)
Line									
9272	−1.8037	−2.663	0.644	−0.604	−0.803	−0.859	−3.104	−4.222	−1.25
9428	−1.4037	1.363	−0.644	−0.137	0.683	0.785	3.185	0.444	−0.73
9381	3.2074	1.299	0.000	0.741	0.120	0.074	−0.081	3.778	1.98
Tester									
LU26S	−1.874	0.151	−0.267	0.485	−0.114	0.052	−0.681	−0.444	−1.99
Mehraj	3.074	0.544	0.089	0.874	−0.139	−0.193	−0.393	1.333	−0.47
Farid-2006	−4.948	−0.695	0.178	−1.359	0.253	0.141	1.074	−0.889	2.46
S.E of GCA for lines	1.65	1.09	0.33	0.68	0.23	0.24	1.05	1.09	0.97
S.E of GCA for testers	1.65	1.09	0.33	0.68	0.23	0.24	1.05	1.09	0.97

Table 4). They also exhibited significant SCA effects on some of the yield contributing traits. Other crosses with significant and positive SCA effects were LU26S × 9272 on plant height and 1000-grain weight grain yield per plant, LU26S × 9428 on peduncle length, and Mehraj × 9381 on plant height and grain yield per plant. These crosses with significant GCA effects on grain yield per plant can be used in the development of new varieties. These crosses with nonadditive genes would give transgressive segregants. For yield improvement, watchful selection of the potent transgressive segregants through family selection would be valuable for yield enhancement. Nonadditive gene action was important for grain yield and other yield-related components. Prevalence of nonadditive gene effects on most of the traits would propose the selection of desired genotypes that must be practiced in the later generations.

Hybrid authentication with SSR markers

The full potential of any hybrid can be realized only by using good quality seeds and hence determination of genetic purity is an essential requirement for its commercial success (Naresh et al., 2009). Conventionally, authentication of hybrids is done through grow-out test (GOT) based on morphological and floral characteristics of plant on maturity. Due to time consuming and environmental influences, GOT is not an effective method for hybrid purity assessment and is currently replaced with effortless, rapid, unbiased, and lucrative DNA-based assay. Randomly Amplified Polymorphic DNA (PAPD) and Simple Sequence Repeat (SSR) markers are properly used for the assessment of the hybrid purity in crop plants (Ilbi, 2003; Liu et al., 2007; Sundaram et al., 2008). A total of 15 SSR primers of Xgwm series and 5 of X series were used to find out the codominant loci in the hybrid and single dominant loci in parents (Table 5). Three primers from X series, namely, X66-5b, X-135-1a, and X-129-2b, gave the polymorphic band in hybrids but not single banding pattern in the parent, so it was concluded that these primers can be used to confirm the hybrids under study. Out of 15 primers, Xgwm-314 and Xgwm-311 gave the polymorphic banding pattern. The primer Xgwm-314 gave ambiguous polymorphic banding pattern that was not used to confirm the hybrids, while the primer Xgwm-311 showed the polymorphic dominant loci in the parents (LU26S, Mehraj 9272 and 9381) and codominant loci midway between these parents. Therefore, this SSR primer was used to confirm the two best performing hybrids (LU26S × 9272 and Mehraj × 9381) on the bases of positively significant general and specific combining ability effects on plant height, 1000-grain weight and grain yield per plant, and other economically important traits (Fig. 1).

Similar results for hybrid authentication with SSR markers were also reported by Chabane et al. (2007) in wheat land races, L: et al. (2008) in melon, Asif et al. (2009) in cotton, Naresh et al. (2009) in safflower, and Hashemi et al. (2009) in Iranian rice.

Conclusion

Combining ability analysis showed that the genotype 9381 was the best general combiner. This parent can be used in transgressive breeding. While the specific combining ability analysis indicated that LU26S × 9272 and Mehraj × 9381 were the two best hybrids. Their hybridity was confirmed by the primer Xgwm-314, which showed the polymorphic dominant loci in the parents (LU26S, Mehraj 9272 and 9381) and codominant loci midway between these parents. High-yielding segregants can be selected from these two combinations in succeeding generations.

Table 4 Estimation of specific combining ability for various quantitatively inherited traits for line × tester analysis of wheat

Cross	Plant height (cm)	Flag leaf area (cm^2)	Tillers/plant	Peduncle length (cm)	Spike length (cm)	Spikelets/spike	Grains/spike	1000-grain weight (g)	Grain yield/ plant (g)
LU26S ×9272	6.5295	−1.176	0.689	−1.707	0.0359	−0.719	−1.274	3.111	9.296
LU26S × 9428	0.8259	1.663	−0.667	3.337	−0.205	0.393	1.837	0.667	−0.593
LU26S × 9381	−7.3519	−0.487	−0.022	−1.630	0.1693	0.326	−0.563	−3.778	−8.704
Mehraj × 9272	−3.7741	0.513	0.311	0.859	−0.083	0.904	−1.096	−2.889	−6.259
Mehraj × 9428	−0.1741	−0.433	0.022	−1.263	0.1326	−0.585	1.081	0.667	−0.815
Mehraj × 9381	3.9481	−0.080	−0.333	0.404	−0.049	−0.319	0.015	2.222	7.074
Farid ×9272	−2.7519	0.663	−1.000	0.848	0.0470	−0.185	2.370	−0.222	−3.03
Farid ×9428	−0.6519	−1.230	0.644	−2.074	0.0726	0.193	−2.919	−1.333	1.407
Farid ×9381	3.4037	0.567	0.356	1.226	−0.119	−0.007	0.548	1.556	1.630
S.E (SCA effects)	2.86	1.90	0.58	1.18	0.41	0.42	1.81	1.89	1.68
S.E (gi-gj) for lines	2.34	1.55	0.47	0.96	0.33	0.34	1.48	1.54	1.37
S.E (gi-gj) for testers	2.34	1.55	0.47	0.96	0.33	0.34	1.48	1.54	1.37
S.E (Sji-Ski)	4.05	2.68	0.82	1.67	0.57	0.59	2.56	2.67	2.36

Table 5 Description of wheat microsatellite primer sets and loci used for hybrid authentication

Locus	Left primer	Right primer	Repeat	An. Temp.	Opata (bp)	Synth. (bp)
Xgwm190-5D	GTG CTT GCT GAG CTA TGA GTC	GTG CCA CGT GGT ACC TTT G	(CT)22	60°C	201	253
Xgwm448-2A	AAA CCA TAT TGG GAG GAA AGG	CAC ATG GCA TCA CAT TTG TG	(GA)29	60°C	203	243
Xgwm484-2D	ACA TCG CTC TTC ACA AAC CC	AGT TCC GGT CAT GGC TAG G	(CT)29	55°C	153	143
Xgwm46-7B	GCA CGT GAA TGG ATT GGA C	TGA CCC AAT AGT GGT GGT CA	(GA)2GC(GA)33	60°C	186	179
Xgwm311-2D	TCA CGT GGA AGA CGC TCC	CTA CGT GCA CCA CCA TTT TG	(GA)29	60°C	157	143
Xgwm314-3D	AGG AGC TCC TCT GTG CCA C	TTC GGG ACT CTC TTC CCT G	(CT)25imp	55°C	182	171
X-132-6D	CCAAAGACTGCCCATCTTTCATC TCT C	CATGACTAGCATGGGTGTGACAT TA	(CA)30, (TA) 21	50°C	–	–
X-107-4B	ATTAATACCTAGAGGGAGGTGC	GGTCTCAGGAGCAAGAACAC	(CT)21	50°C	–	–
X-131-1B	AATCCCCACAGATTCTTCT	AGTTCGTGGGTCTCTGATGG	(CT)22	50°C	–	–
X-157-2D	GTCGTCGCGGTAAGCTTG	GAGTGAACACACGAGGCTTG	(CT)14	50°C	–	–
X-165-4A	TGCAGTGGTCAGATGTTTCC	CTTTCTTTCAGATTGCGCC	(GA)20	50°C	–	–

An. temp. means annealing temperature, Synth. means synthetic wheat, and imp means imperfect repeat.

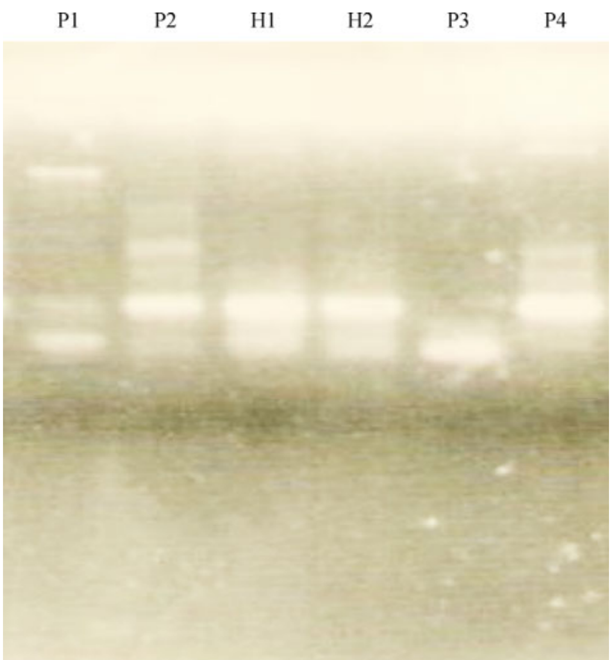

Figure 1 Authentication of two best performing hybrids on the bases of high SCA and mean values for yield and other economic traits. Here P1 is male parent (LU26S), P2 is female parent (9272), H1 is hybrid between P1 and P2, P3 is male parent (Mehraj), P4 is female parent (9381), and H2 is Hybrid between P3 and P4.

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