Electronic Journal of Plant Breeding, 7(3): 574-581 (September 2016)
ISSN 0975-928X
DOI: 10.5958/0975-928X.2016.00073.9

Research Article

Genetic characterization of *Opaque-2* Maize plants derived from the First Backcross Generation

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(Received:14 Jun 2015; Accepted:20 July 2016)

Abstract
Maize has poor nutritional value due to deficiency of two essential amino acids – tryptophan and lysine. Marker assisted selection in combination with conventional breeding can greatly accelerate the introgression of opaque2 gene into normal maize. Parents of a perspective hybrid HKI287 and HKI1126 were undertaken for conversion to develop a QPM hybrid suitable for Central and other parts of India. Plants were selected for the presence of *opaque2* using two markers (phi057 and umc1066) as indicated by the amplified products of 140-160 bp. The phi057 marker identified 36 out of 60 BC1F1 HKI1126 plants (60%) and umc1066 marker identified 24 out of 48 BC_1F_1 HKI287 plants (50%). Microsatellite markers located on different chromosome were used to characterize the effect of first-generation backcrosses by monitoring the level of homozygosity and the parental genomic recovery. The maximum genome recovery for BC1F1 HKI1126 was 75.2% and for BC_1F_1 HKI287 77.3%.

Key words
Backcross, Maize, microsatellite markers, genome recovery, genetic variability

Introduction

Maize (*Zea mays* L.) also known as corn, is the world's third most important cereal crop after rice and wheat, lacking two essential amino acids tryptophan and lysine content. Like other cereals, the maize proteins (zeins) have poor nutritional value for monogastric animals and human beings because of reduced content of amino acids - lysine and tryptophan (Bjarnason and Vasal, 1992) leading to harmful consequences such as growth retardation, protein energy malnutrition, anaemia, pellagra, free radical damage etc. Malnutrition and protein deficiency specifically are major challenges affecting developing countries. Since the discovery of the *opaque2* maize mutant various experiments have continued to improve the protein quality in normal lines as the endosperm of this mutant contains lysine at higher levels (69%) unlike that of normal maize endosperm (Yang *et al*., 2004). In this context, genetic improvement mediated by backcrossing allows the expansion of genetic diversity in subsequent generations, and also making targeted and predictable improvements. Introgression of *o2* gene into normal elite maize lines through normal convention breeding is still underway. To accelerate the time taken to obtain a true QPM, marker assisted breeding is gaining recognition as it can reduce time, costs, and also ensure quality of QPM (Frisch *et al.*, 1999a; Frisch *et al*., 1999b). In our study two non QPM lines i.e. HKI1126 and HKI287 were selected as recipient for conversion to QPM lines using HKI161 and HKI193-1 as donors. Both recipient parents were selected on the basis of their agronomic responses. HKI1126 is a high yielding, bold seeded inbred line with resistance against maize late blight (MLB) disease and HKI287 is multiple disease resistant, early maturity and high yielding inbred line (Sain Dass *et al*., 2008; Sain Dass *et al*., 2009). Donor parents (HKI161 and HKI193-1) were selected because of presence of *o2* gene with expression of higher level of lysine and tryptophan.

Among the different classes of markers available for molecular analysis, microsatellites (SSR) have been widely used for marker-assisted selection in backcross programs (Benchimol *et al.*, 2005; Xi *et al*., 2008). During the past years, SSR has become one of the most popular molecular markers due to the massive amount of sequences available in databases, reflecting the progress of genome research (Leal *et al*., 2010). During genetic analysis in plant breeding, SSR markers exhibit a variety of applications due to its multi-allelic nature, reproducibility, high information content, codominant inheritance, high abundance, and extensive coverage of the genome (Gupta and Varshney, 2000) but distributed in a non-random way (Wang *et al.*, 2008).

During present investigation, microsatellite markers were used to characterize the effect of the first-generation backcross in two maize populations to monitor the level of homozygosity, the genomic proportion of parental genotypes in the offspring. In addition, favourable segregating genotypes were also identified for generation advance.

Materials and method

Two non QPM lines *i.e*. HKI1126 and HKI287 were selected for conversion to QPM lines using HKI161 and HKI193-1 as donors. Two hundred plants were selected for each cross in which HKI287, HKI1126 used as female parents and HKI193 and HKI161 used as male parents. HKI287crossed with HKI193-1 and HKI1126 crossed with HKI161.

For DNA extraction, green young and healthy leaves from 200 plants of each cross were collected one month after sowing (before flowering) in the morning hours from the field during *Kharif* 2010 for extraction of genomic DNA. The collected samples were placed in cooling pads to transfer and then stored at –20°C and DNA was isolated (Saghai-Maroof *et al*., 1984).

Two SSR markers were used for foreground selection *umc1066* F 5' ATGGAGCACGTCATCTCAATGG-3', R 5'-AGCAGCAGCAACGTCTATGAC ACT-3' (Babu *et al*., 2005) and *phi057* F 5'-CTCATCAGTGCCGTCGTCCAT-3', R 5'-CAGTCGCAAGAAACCGTTGCC-3' (Vivek *et al*., 2008). The amplification in 10µl reaction volume containing PCR buffer 10x, $MgCl_2$ 1.5 mM, dNTPs 100µM, primer 10pmol, *Taq* Polymerase 0.5 unit was carried out using Thermo Hybaid (Px2) PCR Machine. The basic PCR profile was 5 min at 94°C, 35 cycles of 30s at 94°C, 30s at 50-65°C, 45s at 72°C and 5min at 72°C for final extension. Amplified products were resolved on 4% Agarose and denaturing PAGE as per requirement for the generation of micro-satellite fingerprints. In PAGE, bands were visualized by silver staining and photo-documented using the Syngene© bio-imaging system.

The DNA of the parents of both populations was initially used to perform the optimization of the reaction and the screening of 110 synthesized primers. The markers covering all chromosomes were selected for the study. The information about primers is available on maize genome database (http://www.maizegdb.org). After the optimization of the reaction conditions, 30 primers for each population were selected, because they had a greater complementarity, reproducibility and presence of polymorphism among the parents (Table 1 and 2).

For the data scoring P_1 (male parent) was considered as B allele and P_2 (female parent) was considered as A allele on the basis of samples having both the alleles were considered as heterozygous and other apart from this considered as specific allele. Homozygosity and genome recovery of recurrent parent in BC_1F_1 generation was calculated and a dendogram was generated by polymorphic SSR markers using the software 'Power Marker' (Liu and Muse, 2005). The analysis of the level of homozygosity of the genotypes evaluated proceeded from the calculation of the ratio between the number of homozygous loci and the total number of loci analyzed per genotype.

Result and discussion

Of the 110 microsatellite loci analyzed in this study, only 30 for each population showed polymorphism between the parental genotypes and were therefore selected for the amplification reactions in the populations similarly Benchimol *et al*. (2005) screened more than 250 SSR markers, used to genotype BC_1 generation and found only 53 polymorphic markers and thus used for backcrossing in maize. The present study was conducted to analyse genome recovery of recurrent parent in BC_1F_1 using SSR markers. Two non QPM lines i.e. HKI1126 and HKI287 were selected for conversion to QPM lines using HKI161 and HKI193-1 as donors respectively. For the foreground selection two specific primers namely phi057 and umc1066 were used. Thirty six heterozygous plants in BC_1F_1 HKI1126 and 24 heterozygous plants in BC_1F_1 HKI287 were used for background selection using polymorphic SSR markers for each population. In both the populations selected polymorphic SSR markers covered all the chromosomes (n=10) of maize. For HKI1126 population highest numbers of markers (five) were found polymorphic from chromosome5 followed by four markers from chromosome10 and three each markers from chromosome1, 2, 3, 6 and 7. From chromosome4 and 9 only two markers were found to be polymorphic. Single marker (umc1817) from chromosome8 amplified polymorphic alleles between both the parents (Table 1). In case of HKI287 population, six polymorphic SSR markers were from chromosome3 followed by four markers each from chromosome5 and 10, three each markers from chromosome2, 7 and 9 while, two each from chromosome1, 6 and 8 (Table 2). Only one marker (umc1758) amplified polymorphic alleles from chromosome4 between both the parental DNA.

In case of BC_1F_1 HKI1126 population homozygosity was highest ranging from 0.333 to 0.724 with an average of 0.51 while genome recovery was 0.666 to 0.862 with an average of 0.75. Out of 36 plants of BC_1F_1 HKI1126 analysed only two were observed with more than 85% genome recovery. While, in BC_1F_1 HKI287, homozygosity ranged from 0.30 to 0.767 with an average of 0.55 and the genome recovery from 0.65 to 0.883 with an average of 0.77. Out of 24 plants of BC_1F_1 HKI287 two plants exhibited more than 85% genome recovery. Similar results with approximately 80% genome recovery have also been observed by Prigge *et al.*, 2009 during their studies on three initial backcross generations

of marker assisted backcrossing in maize. An average of 74.3% recovery of the recurrent parent for the first backcross generation did not differ from the expected value (75%). These results are similar to the results obtained in the present study. Similar result found in wheat using marker assisted backcross selection approach to transfer Yr 15 stripe rust resistance gene from Avocet S*6/ Yr 15' to a susceptible soft white spring wheat cultivar 'Zak' using 55 SSR markers (Randhawa *et al.*, 2009) . When equally spaced (10cM) background markers were used in BC_1 generation for recurrent parent genome recovery then 79.9% recurrent parent genome were recovered which shows the similar result found in present investigation (Herzog and Frisch, 2011). In a BC_1 population from a crossing between two contrasting genotypes, it is expected that an average of 75% of the progeny genome is similar to the recurrent genitor (Collard *et al*., 2005).

A dendrogram was generated using the software 'Power Marker' by polymorphic SSR markers. In case of BC_1F_1 1126, five plants viz., 5, 9, 10, 11 and 35 (Fig. 1) are close to recurrent parent HKI1126 and also came under the range of high homozygosity i.e. > 60% and also have > 80% genome recovery (Table 3). In case of BC_1F_1 287, five plants *viz*., 6, 12, 14, 20, and 24 (Fig. 2) showed closeness with recurrent parent HKI287 and came under the range of high homozygosity i.e. >65% and genome recovery i.e. >83% (Table 4). From the findings, plants which possess high homozygosity and genome recovery are found suitable for growing next generation.

From obtained result, it can be inferred that the number of SSR loci analysed were enough to effectively determine the degree of homozygosity of the progeny evaluated. Furthermore, we observed that in the population with higher number of the fixed loci, creating an expectation of obtaining of self-fertilizing plants with a high degree of stability and a level of homozygosity greater than or equal to 95%, which will be a satisfactory level in this breeding program for QPM development.

The backcross procedure has gained extensive applications in recent years due to the increasing efforts to transfer transgenes to commercial elite inbred lines (Lewis and Kernodle, 2009). Although it is recommended to be a well-known method for introgression or substitution of a target gene, higher number of generations are required to recover the recurrent parent genome and the presence of targeted portions of the donor parent genome linked to the favourable allele transferred there are few problems inherent to the backcross (Benchimol *et al.*, 2005). The well-established alternative in plant breeding is the procedure of molecular marker-assisted backcross (Schmierer *et al*., 2004), which helps in classical procedures, accelerating the recovery of recurrent parent genome and reducing the number of generations required for introgression of the gene of interest (Hospital and Charcosset, 1997). This strategy can also increase genetic gain and economic efficiency in relation to classical procedures (Kuchel *et al*., 2005).

Acknowledgement
The authors acknowledge the funding from Department of Biotechnology, Government of India.

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Table 1. Markers used for BC_1F_1 1126

S. No.	Primers	Forward Sequences (5'-3')	Reverse Sequences (5'-3')	Ch. No.
1	umc1605	TGCTGTGCAGTTCTTGCTTCTTAC	AGCTTCACGCTCTTCTAGACCAAA	1
2	umc1725	CTACGGCCAGAGTATCGGTCAC	TATGGTGGGAGAGACGACAGGTAT	1
3	umc2189	CCAGGAGAGAAATCAACAAAGCAT	GGAGAAGCACGCCTTCGTATAG	1
4	umc1256	CCCGGCTAGAGCTATAAAGCAAGT	CTAGCTAGTTTGGTGCGTGGTGAT	2
5	umc1552	ACGTGGTCATCACTCACCGC	AAGGAGGAGCGTTCTCGTGG	2
6	phi127	TTTTCTGCAGGGATAACATTTGTG	ATAGGAGGTGAGGTGAGGAGGAAG	2
7	umc2276	TCTCGCTGTCCTTCGATTAGTACGG	AATGCAGGCGATGGTTCTCCGGCCT	3
8	umc1273	GTTCGCTGCTGCTTCTTATATGCT	AATTGGCGCAGGCTATAGACATTT	3
9	umc2101	AGCTGCTGAAGATGAAGGACAGG	TCACCGTCGAGAACGACGAC	3
10	umc2061	CATCCTCCAAAAGCACTACGT	CAGCTGTCCGACACTTATTCTGTA	4
11	umc1869	CGAGCGCTCTAGACACGATTTT	GAACTGGAGGAGCGAGCATGTAT	4
12	umc1429	GGGCCCTGTTAATCCTCATCTG	TCCTCCTTTCTCTCATGTTTCTCG	5
13	umc2373	ACCCAAGTGAGGTGAAGTGAAGC	TATGGTACAGGCACAGCAGCAGTA	5
14	umc1153	CTTTTGTGATGTCTGCAATATGCC	TTAGTAGGTGCATTGGATGCTCAA	5
15	umc1060	TTCAATCCGTAGGTCTGGTGCTAT	GCGGCTGCGTTTTTATTCAAACTTGTT	5
16	bnlg1118	CAGAGTTGATGAACTGAAAAAGG	CTCTTGCTTCCCCCCTAATC	5
17	umc1110	GAAAACTAATCAAACGCAACCAGG	GATGGAGTGAGGATTAGCAGCCTA	5
18	umc2165	CATACACCAAGAGTGCAGCAAGAG	GGAGGTCTGGAATTCTCCTCTGTT	6
19	umc1105	CTTCCTCCTCACCTCACCTCCTAT	GGTAGCCAATCCTTCCTTCCTATG	6
20	umc1296	CTCTCCCGGCTCTGACCTAGC	GCTGGAGATAGGCATCCAGACAC	6
21	umc2190	CCACATTTGGCTGAATTTGTTGTA	CTTGTTGGCTAGAAATTTGCCTTG	7
22	umc2332	GTCGGAGAAGGAGCTACTGAGCTA	CACAGGTACGTCTGGATGCTGT	7
23	umc2334	ATGGCCTCCGTGCTGAAGAT	CATCTGATGGTGTTGTAGCAGCAG	7
24	umc1817	CTACGCAGGCTTCAACCACC	GTACTGGTGATGATGGTACCCCTG	8
25	umc1743	AACCTCAAGATCACCAACATCCTC	CACCCTGCTGTCAGATGGATACTT	9
26	umc2089	CCTTCAAACCAAATGTACAGCAGC	CTCCTCAACGACAGCGTGTACC	9
27	umc1569	CGTAAGTACAGTACACCAATGGGC	ACACCGACTACAAGCCTCTCAACT	10
28	umc2021	AAACTCAAGCTCGGAATGTACTGC	CGATACTGATCTACTTCACGCTGG	10
29	umc1827	GCAAGTCAGGGAGTCCAAGAGAG	CCACCTCACAGGTGTTCTACGAC	10
30	umc2017	TCCCTCTTGAGTGTTTATCACAAA	GTTTCCATGGGCAGGTGTAT	10

Table 2. Markers used for BC_1F_1 287

S. No.	Primers	Forward Sequences	Reverse Sequences	Ch. No.
1	bnlg176	TTACACCAAGGTCCGAAACAAGAT	TCTTGGAAGGCAAGACTCTACCTG	1
2	bnlg 1811	CTCTCGTCTCATCACCTTTCCCT	CTGCATACAGACATCCAACCAAAG	1
3	umc2125	CAAGGGTAAGGGCAAGATGGTAGT	CTGAGGTCTACCTCGGCCATC	2
4	umc1256	CCCGGCTAGAGCTATAAAGCAAGT	CTAGCTAGTTTGGTGCGTGGTGAT	2
5	umc2214	ACCCCCTGATTCTCTCTTACGTTT	CTGGATGAGGAGGAAGAATACGAG	2
6	umc1639	CTAGCCAGCCCCCATTCTTC	GCAAGGAGTAGGGAGGACGTG	3
7	umc1361	GATGCTCAAGGAGCAGCGAC	CAGGTGGTACGCCATGAACC	3
8	umc1273	GTTCGCTGCTGCTTCTTATATGCT	AATTGGCGCAGGCTATAGACATTT	3
9	umc1136	TTTCGACTGCTAGTGTACTTGGGG	CTCTACATCTTCAGCGTCTCCACA	3
10	umc1501	TTCAGGTGTGCACTGACTCTGACT	ATGCTCAAGCTCAACAGCACTTC	3
11	umc1644	CCATAAACTGTTCCTTTGGCACAC	CTTTCACGTGTTAAGGGAGACACC	3
12	umc1758	ACCTTAGTTACACAGGCACACGGT	GGTGATGGGATTTTCGCATTATTA	4
13	umc2298	CTGTACATGGATATGGCATTGGTG	GCATATACACCACCTTGGACAACA	5
14	umc1429	GGGCCCTGTTAATCCTCATCTG	TCCTCCTTTCTCTCATGTTTCTCG	5
15	umc1110	GAAAACTAATCAAACGCAACCAGG	GATGGAGTGAGGATTAGCAGCCTA	5
16	umc1153	CTTTTGTGATGTCTGCAATATGCC	TTAGTAGGTGCATTGGATGCTCAA	5
17	umc1105	CTTCCTCCTCACCTCACCTCCTAT	GGTAGCCAATCCTTCCTTCCTATG	6
18	umc1186	TCAAGAACATAATAGGAGGCCCAC	AGCCAGCTTGATCTTTAGCATTTG	6
19	umc2332	GTCGGAGAAGGAGCTACTGAGCTA	CACAGGTACGTCTGGATGCTGT	7
20	umc1251	GGTAGTGCTTGGTATTGACATCAGA	CTCTTGAAGATGGTCCTAGCATTG	7
21	umc2190	CCACATTTGGCTGAATTTGTTGTA	CTTGTTGGCTAGAAATTTGCCTTG	7
22	umc1817	CTACGCAGGCTTCAACCACC	GTACTGGTGATGATGGTACCCCTG	8
23	umc1872	GAATAAGACCAGACAGCACCGAAC	AAGATTGTATAAATGGCAGCCACG	8
24	umc2130	CGAGTTACCTTTGGCACTAGCACT	ATCATGACGTATCTTTCCGAGAGC	9
25	umc2393	CAACTCGATCCAGACCACACATAG	CTCTTGGTTGTTTGTTTCCTTGCT	9
26	umc2358	GCACGAGGTTTCCCTTGCTC	GACTCGCGAATAAGGTCTGGGTT	9
27	umc1381	CTCTAGCTACGAGCCTACGAGCA	CCGTCGAGTCAACTAGAGAAAGGA	10
28	umc2017	TCCCTCTTGAGTGTTTATCACAAA	GTTTCCATGGGCAGGTGTAT	10
29	umc1678	GGCTCGACTTCGAGGACACC	GAGGAGGAGAGGGACAGGGAAG	10
30	umc2021	AAACTCAAGCTCGGAATGTACTGC	CGATACTGATCTACTTCACGCTGG	10

Table 3. Homozygosity (H) and Genome recovery (R) of BC_1F_1 plants

F_1 X HKI1126									F_1 X HKI287					
Plant	**H**	**R**	**Plant**	**H**	**R**	**Plant**	**H**	**R**	**Plant**	**H**	**R**	**Plant**	**H**	**R**
1	0.50	0.75	13	0.40	0.70	25	0.333	0.666	1	0.50	0.75	13	0.634	0.816
2	0.50	0.75	14	0.666	0.833	26	0.40	0.70	2	0.40	0.70	14	0.70	0.85
3	0.379	0.689	15	0.517	0.758	27	0.50	0.75	3	0.60	0.80	15	0.467	0.734
4	0.50	0.75	16	0.517	0.758	28	0.50	0.75	4	0.466	0.734	16	0.344	0.672
5	0.724	0.862	17	0.551	0.776	29	0.566	0.783	5	0.517	0.758	17	0.30	0.65
6	0.50	0.75	18	0.344	0.672	30	0.50	0.75	6	0.767	0.883	18	0.60	0.80
7	0.344	0.672	19	0.433	0.717	31	0.366	0.683	7	0.634	0.816	19	0.30	0.65
8	0.466	0.733	20	0.40	0.70	32	0.50	0.75	8	0.533	0.767	20	0.70	0.85
9	0.666	0.833	21	0.566	0.783	33	0.60	0.80	9	0.533	0.767	21	0.467	0.734
10	0.60	0.80	22	0.433	0.716	34	0.413	0.706	10	0.634	0.816	22	0.634	0.816
11	0.70	0.85	23	0.566	0.783	35	0.620	0.810	11	0.466	0.734	23	0.60	0.80
12	0.566	0.783	24	0.533	0.766	36	0.466	0.733	12	0.667	0.833	24	0.667	0.833
			Average				**0.504**	**0.752**					**0.547**	**0.773**

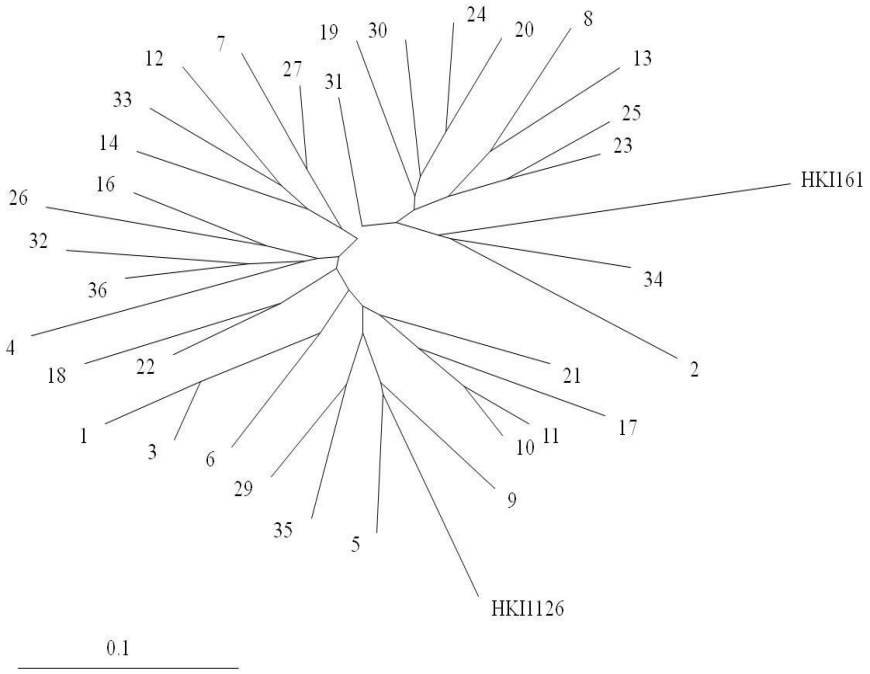

Fig. 1. Dendrogram showing relationship among BC_1F_1 plants and recurrent parent HKI1126

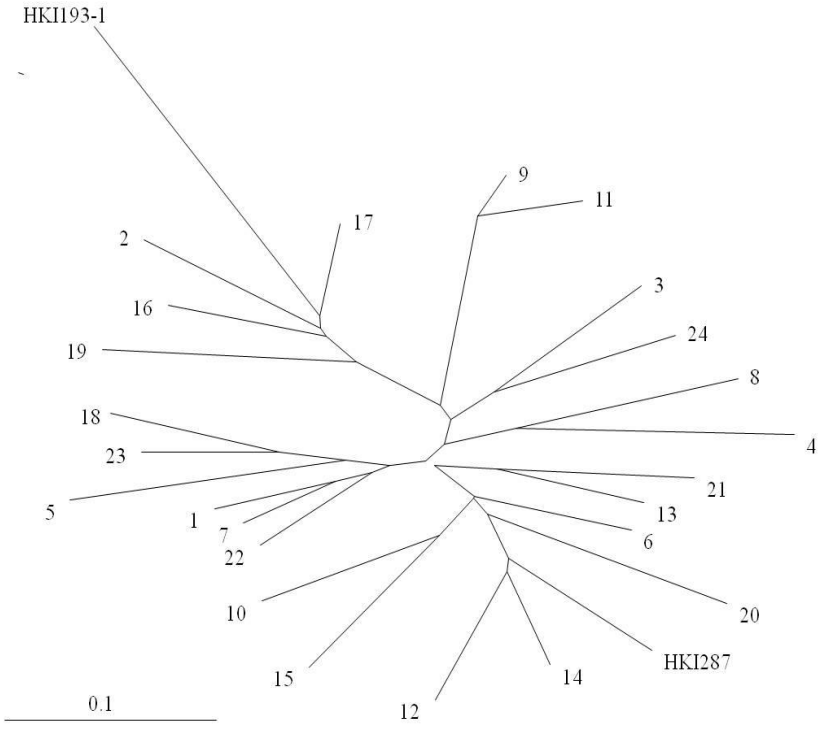

Fig. 2. Dendrogram showing relationship among BC_1F_1 plants and recurrent parent HKI287