MARKER ASSISTED SCREENING OF NEPALESE WHEAT GENOTYPES AND ADVANCED LINES FOR RESISTANCE TO DIFFERENT RACES OF WHEAT RUST SPECIES

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ABSTRACT

Primers tightly linked to genes of interest are valuable tools in rust resistant wheat breeding. A research was conducted with the objective to detect the source of rust resistant genes and further utilize as an important weapon in gene pyramiding. A total of forty gene-specific primer sets consisting of Lr, Yr and Sr associated gene specific markers were applied for screening thirty wheat genotypes. Based on the value of band size, the presence of concerned resistance gene was predicted and binary scoring was done for further analysis. Softwares Genealex and NTSYSpc2.1 were applied for detail analysis. Some genes like Lr29, Lr51, YrCH42, Sr36, etc. were detected in many of the tested genotypes but some genes like Lr47, Sr39, Sr24 were absent in tested lines. Among tested genotypes, Chyakhura-1 possesses highest number of rust resistant genes and Annapurna-4 bears lowest number of genes. Highest value of Jaccard coefficient was obtained between genotypes Danphe-1 and Danphe-2 (0.87) showing their greatest resemblance. UPGMA dendrogram grouped thirty genotypes into distinct four clusters. Percentage of Polymorphic loci was 88.57 and grand mean of observed heterozygosity (Ho) was found to be 0.193 (S.E 0.057).

Key words: Genotypes, Primers, Marker assisted screening, Polymorphism.

INTRODUCTION

Wheat is the third important cereal grain crop of Nepal after rice and maize. In Nepal, wheat crop occupies approximately 0.767 million hectares and produces 1.746 million metric tons of grains. National productivity of this crop is 2275 kg/ha (MoAC, 2012). There are numerous wheat diseases, caused by various pathogens. Among these, rust diseases have, for years, been a major concern and problem for breeders, farmers and commercial seed companies (Wiese, 1977; Marsalis and Goldberg, 2006). They are notable, historically, for their severe attacks on wheat crop.

Many rust resistance genes have been identified but a limited number of those genes are still effective to the pathogen population in our region. The released varieties are likely to be breakdown in terms of its resistance to biotic/abiotic stresses and also thereby need new cultivars having higher yield as well as better quality performance. The wheat rust fungi cause the potentially most devastating diseases of wheat and genetic control is a highly effective and cost-saving alternative to chemicals. The existing varieties are likely to breakdown in terms of its resistance to rust disease and also need further improvement in yield potential.

Nepalese wheat varieties are assumed to be the precious sources of resistant gene. But, the appropriate molecular genotyping of these cultivars is lacking. Hence, research on marker assisted rust resistant gene screening of wheat lines is new frontier in improving environment; increasing

production and productivity at low cost in the country. Developing, identifying, multiplying and disseminating high yielding varieties along with resistance to leaf, yellow and stem rusts is urgent need of country to safeguard the country from food shortages problem. Minimizing the loss caused by diseases is one of the solutions to increase the productivity. Any primers tightly linked to genes of interest are probably the most attractive markers since no further manipulations are needed for implementation. In this light; the current research is focused on marker assisted screening of wheat lines, using rust resistant gene specific markers.

MATERIALS AND METHODS

Selection of wheat genotypes

Thirty wheat genotypes were used for marker assisted screening using different races of rust resistant primers. The genotypes include Nepalese released and pre-released improved varieties of NARC, local cultivars, and other advanced breeding lines. This selection encompasses the broad range of genotypes so that more sources of various races of Lr, Yr and Sr genes can be identified. Nepalese genotypes used were mostly of mid-hill areas and accessions were collected from Agriculture-Botany Division, NARC; Khumaltar

Research laboratory

Detail molecular study was done in Molecular Laboratory, Biotechnology Division, National Agriculture Research Center (NARC), Khumaltar Nepal in 2012.

Genomic DNA extraction

Seeds of studied genotypes were kept for germination on Petridis, and 4-5 days seedlings were used as source of DNA. Leaves and roots were collected from seedlings was subjected to liquid nitrogen for DNA extraction using "Modified CTAB method "

DNA quantification and dilution

Dilution of DNA by adding 10 µl of DNA solution to 90 µl of distilled water in a micro-tube, were prepared and mixed well. Spectrophotometer was used for the quantification purpose. Based on the obtained values from DNA Quantification, concentration of DNA was calculated for working sample.

Gene specific primers used for MAS

Wheat genotypes were mapped by using 40 gene-specific primer sets.

PCR Amplification

PCR process was accomplished by Thermocycler PCR machine (PTC-100, MJ Research, Inc., Watertown, Massachusetts, USA) with adjustment of appropriate amplification program for 35 cycles.

Gel electrophoresis and autoradiography

Amplification products was visualized with different sets of primers on 1.5% agarose gel with 1x TBE buffer and detected by staining with an ethidium bromide.

Statistical analysis

Based on the value of band size, the presence of associated trait in the genotypes was predicted. Different softwares such as, MS-EXCEL, NTSYS-PC, GeneAlex 6 (Peakall and Smouse 2006) were used for the data analysis in a binary matrix form. The amplified products were scored as bands on visualization on gel on UV illuminator. Only the reliable bands were included in analysis. These scored bands were computed into binary matrix. The presence of the corresponding resistant band was scored as "1" and absent bands was scored as "0". Genetic diversity measures such as the number of alleles per locus, size range, Shannon's Information Index, observed heterozygosity, expected heterozygosity, unbiased expected heterozygosity, fixation index and gene frequencies were estimated. The genetic similarities were calculated for each pair of inbred using the Jacard Similarity Coefficient matrix. The similarity matrix was used to construct UPGMA (un-weighted pair group methods using arithmetic averages algorithm) clustering using NTSYS-pc.V.2.11.

RESULTS AND DISCUSSION

Biotic stress resistance emerges as principal selection criteria in contemporary wheat selection objectives. Rust disease continues to cause huge losses worldwide in wheat production due to reliance on cultivars with race-specific resistance and the high level of virulence variation in rust pathogens. A new approach called "genomic selection" that is based on the widespread conventional selection with the use of information from the molecular markers will facilitate breeding program through better combination of cost, time, precision and durability. Marker assisted screening (MAS) is successful and innovative breeding tool in this context.

There is varying intensity of rust resistance gene present among the tested thirty genotypes. Genotypes also revealed diversity in terms of their relatedness with respect to different rust resistant genes. Out of forty gene specific markers used in study, five markers namely XABC465, RK2 (SCAR Lr 24), ARBI1RGA-2, Sr24#12 and Sr39#22r linked respectively with Lr47, Lr24, Lr24, Sr24 and Sr39 don't show any distinct bands. So, rest 35 markers were only used in Genetic diversity and analysis.

Banding patterns and screening of resistant genotypes

Out of thirty five markers that show distinct bands; four markers XGWM533, CsSr2, BE50070 and WMC 633 don't show any bands of resistant size but Primer XAGA7-1B showed resistant band in all of the tested genotypes.

Table 1. Marker assisted evaluation (Markerwise)

S.N.	Features/ Primers	Respective gene	Band size bp	No. of Genotypes with gene presence
1	XGWM319	Sr 36	170	27
2	Lr29F18	Lr29	900	27
3	XGDM87	Lr 50	110	7
4	XWMC44-1B	Lr46, Yr29	242	10
5	XGWM382	Lr50	139	5
6	XGWM413	Yr15	95	9
7	XGWM391	Sr 35	180	23
8	XCFA2076	Sr 35	210	23
9	XGWM498	YrCH42; Yr24; Yr26	159-161	29
10	XGWM533	Sr2	120	None
11	XABC465	Lr47	282	No Bands
12	XGWM259-1B	Lr46; Yr29	105	8
13	WMS639	Sr exp 2	200	10
14	XAGA7-1B	Lr 51	783	30
15	XBARC352-7D	Lr34	248	13
16	XGWM264	Yr 15	222	6
17	XGWM273	Yr 26	190	10
18	XWMC656	Yr 45	280	4
19	XBARC187-1B	Yr26, Yr CH42	220	20
20	XWMC631	Sr exp 1	220	26
21	XBARC6	Yr45	360	4
22.	STS Lr 19 (RK1)	Lr 19	130	7
23	CFD49	Sr Cad	275	18
24	XGWM192	Yr 46	230/130	29
25	XBARC80-1B	Lr46	95	9
26	XGWM130-7D	Lr34	132	20
27	XGWM18	Yr CH52	184	14
28	WMC313	Lr28	320	None
29	XGWM498-1Band	Yr CH42	160	11
30	PK54	Yr (hom to gama gladillin)	190	16
31	GWM508	Lr53	135	15
32	RK2 (SCAR Lr 24)	Lr 24	700	No bands
33	CFD1	Lr 53	225	10
34	ARBI1RGA-2	Lr24	161	No bands
35	Sr24# 12	Sr 24	500	No bands
36	Sr39#22r	Sr 39	487	No bands
37	E1(Exon 1)	Yr 10	754	18
38	WMC 633	Sr 22	117	None
39	BE500705	Sr 39	166	None
40	CsSr2	Sr 2	172	None

Among tested genotypes, Chyakhura#1 have possess highest number of rust resistant genes (24) and Annapurna-4, bears lowest number of genes (12).

Table 2. Marker assisted evaluation (Genotypewise)

S.N.	Grown Parents	No. of Genes present based on MAS
1	WK 1182	13
2	WK 1204	18
3	WK 1481	16
4	WK 1627	21
5	WK 1661	20
6	WK 1803	17
7	WK 1804	13
8	WK 1905	20
9	WK 1906	16
10	WK 1907	18
11	WK 1909	19
12	WK 1912	22
13	Chyakhura # 1 (3 EBWYT 515)	24
14	Chewink # 1 (3 EBWYT 514)	22
15	Munal # 1	21
16	Danphe # 1	18
17	Danphe # 2	15
18	Annapurna - 4	12
19	Godwari Local	13
20	Hanse Seto	16
21	30 ESWYT 125	18
22	SW 2148	19
23	NOURIN 61 / MILAN// LONG MAI 19	17
24	KENYA NYANGUMI	16
25	BGUA/4/GOV/AZ//MUS/3/KEA	14
26	KENYA SWARA	15
27	CROC_1/ AE.SQUARROSA (205)// KAUZ/ 3/ SASIA	19
28	Bezostay 1	15
29	WK 1444	15
30	WK 1710	15

Genetic diversity measures

Among the set of gene specific markers used in study, 88.57% turned out to be polymorphic. Out of 35 markers that showed distinct bands; four markers namely XAGA7-1B, XGWM498,

XBARC187-1B and E1 (Exon 1) were found to be monomorphic.. Among Polymorphic markers, maximum number of different alleles (Na) was obtained in XGWM264 (6) followed by WMS639, XBARC80-1B and CsSr2F (5). Mean value of Na was found 3 (S.E. 0). Number of effective alleles (Ne) was maximum in Cssr2F primer (4.444) followed by XGWM 264 (4.073) and minimum in XGWM319 (1.220) among the polymorphic markers. Mean Value of Ne was obtained 2.377 (S.E. 0.164). Shannon's information index (I) was found maximum in XGWM264 (1.598) followed by CsSr2F (1.544). In contrast, I value was found minimum in XGWM 319 (0.325) and BE500705F (0.451) respectively. Mean Shannon's information index was observed to be 0.869 (S.E. 0.076). Observed heterozygosity (Ho) was found highest in XBARC6 and XGWM192 (1) among the polymorphic primers. Mean Ho value was observed to be 0.193 with S.E. 0.057. Expected heterozygosity (He) was found highest in CsSr2F (0.775) followed by XGWM264 (0.754) and mean expected heterozygosity was found 0.496 (S.E.0.039). Unbiased expected heterozygosity (UHe) was found maximum in CsSr2F (0.795) followed by XGWM264 (0.768) andWMS639 (0.762). Mean UHe value was observed to be 0.505 (S.E. 0.040). Fixation index value (F) was found maximum in 18 of the polymorphic markers (1). In contrast, minimum fixation index was found in primer XGWM192 (-1) and XGWM391 (-0.699). Mean value of F was observed to be 0.617 (S.E.0.104).

Jaccard coefficient similarity matrix

From the Jaccard coefficient similarity matrix (fig 1), highest value of Jaccard coefficient was obtained between genotypes Danphe-1 and Danphe -2 (0.87) showing their greatest resemblance with respect to presence of rust resistant genes. The value was followed by that between KENYA NYANGUMI and KENYA SWARA (0.78). Lowest coefficient was observed between genotypes WK 1906 and Godawari local as well as between Chewink# 1 and Annapurna- 4 (0.20) showing the least resemblance in terms of presence of rust resistant genes. The least value was followed in between the genotypes Godawari local and Danphe -1 (0.21).

UPGMA dendrogram and principal coordinates analysis

Based on Jaccard coefficient similarity matrix, UPGMA dendrogram was constructed. UPGMA dendrogram grouped thirty genotypes into distinct four clusters (Figure 1). This result was similar and same clusters are also obtained from Principal coordinate analysis (1 vs 2) (Figure 2). Distribution of different genotypes in different cluster is shown in Table 3.

Table 3. Grouping of genotypes into clusters as revealed by UPGMA dendrogram and PCA analysis

Number	Genotypes
Cluster I (7 genotypes)	WK 1182; WK 1481; WK 1803; WK 1804; WK 1204; WK 1627 and WK 1661.
Cluster II (8 genotypes)	NOURIN 61 / MILAN// LONG MAI 19; KENYA NYANGUMI; KENYA SWARA; BGUA/4/GOV/AZ//MUS/3/KEA; CROC_1/ AE.SQUARROSA (205)// KAUZ/ 3/ SASIA; Bezostay 1; WK 1444 and WK 1710
Cluster III (10 genotypes)	WK 1905; WK 1906; WK 1907; WK 1909; Chyakhura # 1; Chewink # 1; Munal # 1; Danphe # 1; Danphe # 2 and WK 1912
Cluster IV (5 genotypes)	Annapurna – 4; 30 ESWYT 125; ; Godwari Local; Hanse Seto; SW 2148

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	1.00																													
2	0.41	1.00																												
3	0.56	0.36	1.00																											
4	0.44	0.60	0.46	1.00																										
5	0.45	0.52	0.53	0.67	1.00																									
6	0.45	0.39	0.50	0.46	0.42	1.00																								
7	0.42	0.33	0.47	0.43	0.47	0.53	1.00																							
8	0.37	0.31	0.34	0.40	0.49	0.39	0.55	1.00																						
9	0.26	0.33	0.30	0.36	0.44	0.39	0.49	0.60	1.00																					
10	0.40	0.30	0.37	0.36	0.37	0.42	0.53	0.49	0.55	1.00																				
11	0.41	0.31	0.39	0.29	0.37	0.34	0.38	0.36	0.40	0.58	1.00																			
12	0.25	0.34	0.33	0.37	0.33	0.38	0.30	0.34	0.39	0.43	0.42	1.00																		
13	0.25	0.36	0.30	0.39	0.40	0.30	0.29	0.41	0.46	0.44	0.55	0.57	1.00																	
14	0.28	0.27	0.29	0.42	0.34	0.33	0.35	0.45	0.37	0.51	0.45	0.44	0.67	1.00																
15	0.31	0.46	0.30	0.36	0.37	0.26	0.29	0.39	0.36	0.37	0.44	0.33	0.50	0.60	1.00															
16	0.39	0.36	0.34	0.38	0.35	0.32	0.30	0.38	0.36	0.41	0.41	0.33	0.44	0.58	0.71	1.00														
17	0.42	0.38	0.39	0.41	0.39	0.30	0.32	0.38	0.34	0.39	0.38	0.33	0.41	0.50	0.64	0.87	1.00													
18	0.39	0.31	0.42	0.29	0.39	0.27	0.26	0.33	0.31	0.25	0.25	0.19	0.25	0.20	0.29	0.34	0.39	1.00												
19	0.39	0.40	0.33	0.30	0.25	0.36	0.28	0.24	0.20	0.26	0.28	0.27	0.22	0.19	0.24	0.21	0.25	0.41	1.00											
20	0.45	0.41	0.42	0.41	0.40	0.34	0.42	0.41	0.32	0.37	0.39	0.38	0.34	0.31	0.34	0.32	0.34	0.42	0.57	1.00										
21	0.40	0.32	0.40	0.44	0.45	0.31	0.40	0.42	0.32	0.33	0.34	0.29	0.31	0.32	0.31	0.35	0.40	0.42	0.31	0.45	1.00									
22	0.38	0.34	0.42	0.34	0.40	0.25	0.34	0.36	0.29	0.32	0.36	0.33	0.28	0.29	0.35	0.34	0.39	0.34	0.33	0.48	0.40	1.00								
23	0.27	0.33	0.41	0.43	0.39	0.41	0.40	0.38	0.43	0.41	0.38	0.32	0.43	0.34	0.29	0.25	0.27	0.26	0.27	0.36	0.39	0.38	1.00							
24	0.33	0.32	0.44	0.36	0.39	0.41	0.44	0.38	0.46	0.41	0.41	0.27	0.37	0.35	0.32	0.32	0.34	0.41	0.29	0.37	0.42	0.34	0.60	1.00						
25	0.37	0.30	0.41	0.41	0.47	0.37	0.41	0.38	0.51	0.46	0.36	0.33	0.39	0.38	0.30	0.34	0.39	0.34	0.21	0.32	0.35	0.32	0.54	0.56	1.00					
26	0.32	0.29	0.41	0.31	0.34	0.36	0.33	0.33	0.40	0.41	0.40	0.30	0.36	0.35	0.30	0.32	0.34	0.36	0.24	0.32	0.39	0.27	0.50	0.78	0.64	1.00				
27	0.36	0.35	0.36	0.45	0.43	0.27	0.35	0.40	0.35	0.45	0.42	0.41	0.45	0.46	0.36	0.35	0.38	0.35	0.28	0.40	0.34	0.31	0.39	0.50	0.58	0.52	1.00			
28	0.39	0.41	0.49	0.51	0.49	0.44	0.39	0.36	0.46	0.44	0.36	0.31	0.41	0.42	0.37	0.41	0.43	0.41	0.27	0.34	0.35	0.30	0.51	0.65	0.65	0.64	0.50	1.00		
29	0.37	0.36	0.50	0.44	0.45	0.32	0.34	0.39	0.39	0.42	0.34	0.35	0.39	0.38	0.32	0.37	0.41	0.47	0.31	0.34	0.35	0.30	0.38	0.58	0.52	0.60	0.60	0.71	1.00	
30	0.39	0.38	0.38	0.54	0.52	0.31	0.28	0.33	0.33	0.36	0.26	0.27	0.31	0.34	0.27	0.33	0.38	0.43	0.23	0.27	0.36	0.29	0.32	0.45	0.51	0.42	0.56	0.63	0.65	1.00

Figure 1. Jaccard's similarity table matrix between tested genotypes

1. WK 1182 2. WK 1204 3. WK 1481 4. WK 1627 5. WK 1661 6. WK 1803 7. WK 1804 8. WK 1905 9. WK 1906 10. WK 1907 11. WK 1909 12. WK 1912 13. Chyakhura # 1 (3 EBWYT 515) 14. Chewink # 1 (3 EBWYT 514) 15. Munal # 1 16. Danphe # 1 17. Danphe # 2 18. Annapurna – 4 19. Godwari Local 20. Hanse Seto 21. 30 ESWYT 125 22. SW 2148 23. NOURIN 61 / MILAN// LONG MAI 19 24. KENYA NYANGUMI 25. BGUA/4/GOV/AZ//MUS/3/KEA 26. KENYA SWARA 27. CROC_1/ AE.SQUARROSA (205)// KAUZ/ 3/ SASIA 28. Bezostay 1 29. WK 1444 30. WK 1710

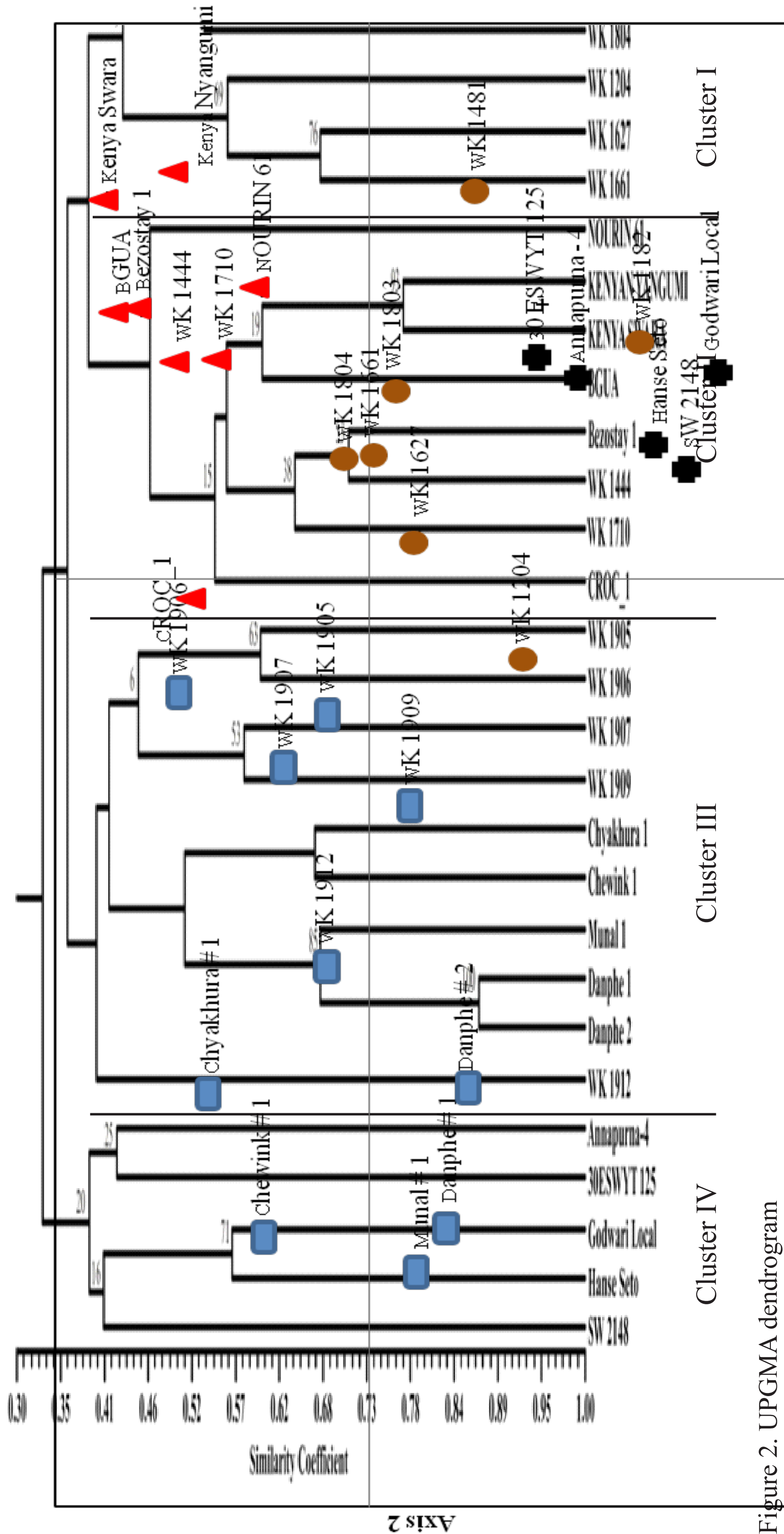
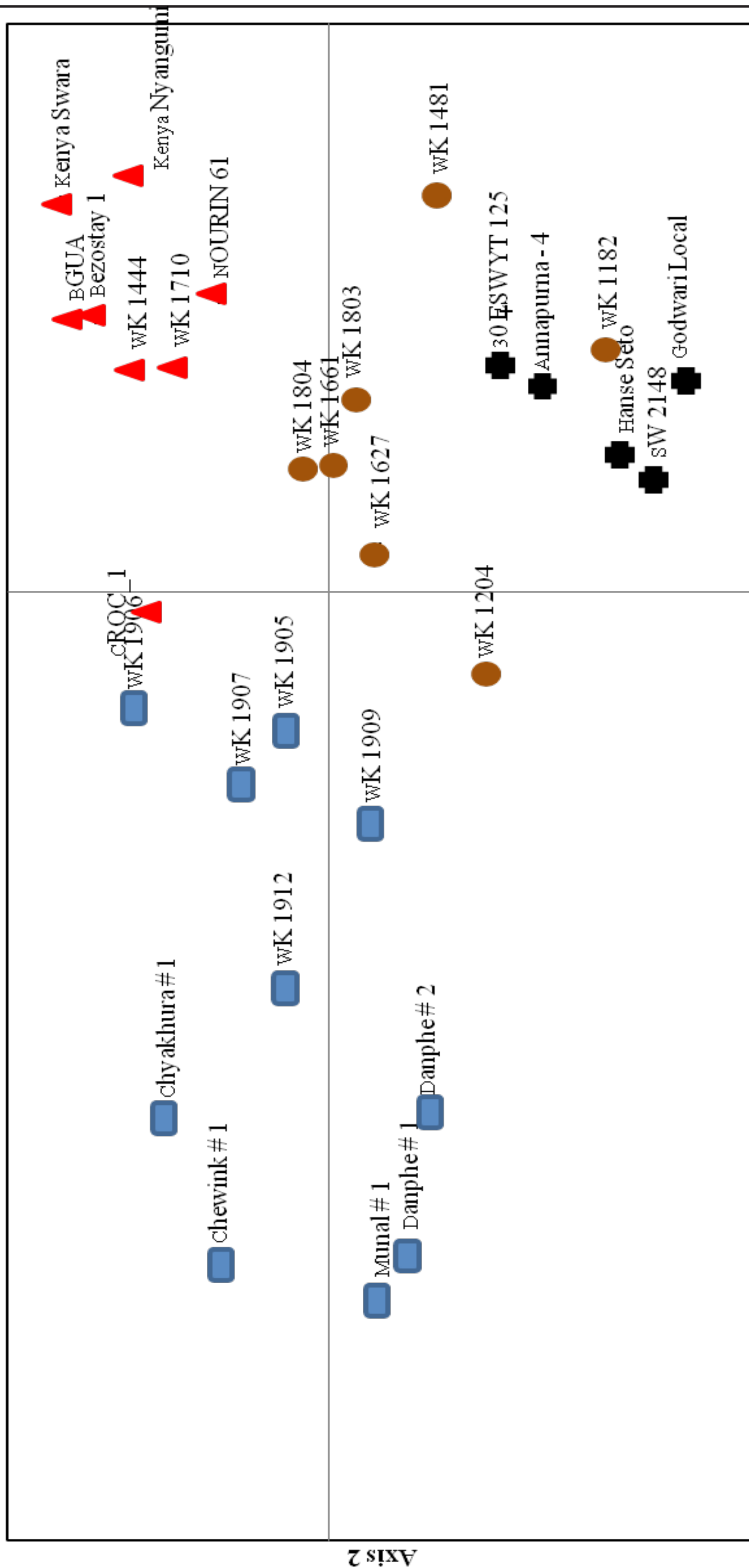


Figure 2. UPGMA dendrogram

●: Cluster I, ▲: Cluster II, ■: Cluster III, and ✚: Cluster IV

Figure 3. Principal coordinate analysis (1 vs. 2)



: Cluster I, : Cluster II, : Cluster III, and : Cluster IV

Figure 3. Principal coordinate analysis (1 vs. 2)

Tests for Hardy- Weinberg equilibrium

Chi-square test was conducted in case of each polymorphic marker to test for Hardy-weinberg equilibrium. Out of 31 polymorphic markers; all except for XCFA2076 were found highly significant. This indicates that the bands present in most of the polymorphic markers are significantly different among each other. Highest chi-square value was obtained in XBARC 80-1 B (120 at df 10) followed by WMS639 (116 at df 10). In contrast, minimum chi-square value was found in primer XCFA2076 (1.767 at df 3).

CONCLUSION

There is varying intensity of rust resistance gene present among the tested thirty genotypes. Five gene specific markers didn't show any distinct bands and out of thirty five markers that show distinct bands; four markers XGWM533, CsSr2, BE50070 and WMC 633 don't show any bands of resistant size. In contrast, Primer XAGA7-1B showed resistant band in all of the tested genotypes. Genotype Chyakhura#1 possess highest number of rust resistant genes and Annapurna-4, bears lowest number of genes. Among the set of gene specific markers used in study, 88.57% primers revealed polymorphism. Thus, from this study, the presence of rust resistant genes in some Nepalese wheat genotypes and advanced lines were detected with the help of gene specific molecular markers. Genetic relationship among the genotypes with respective to presence of rust resistance gene was also assessed. The current study is worthy as an important tool in gene pyramiding of rust resistant genes in wheat. The results would be equally beneficial as vital means of genetic improvement for wheat rust resistance.

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