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Analyses of genetic diversity among exotic- and indigenous- maize inbreds differing for responses to stored grain weevil (*Sitophilus oryzae* L) infestation

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Abstract

Sitophilus oryzae has emerged as one of the important storage grain pests of maize especially in Asia. It causes damage to the stored grains and affects significantly its quality and viability. A set of 48 diverse maize inbreds differing for degree of resistance against *S. oryzae* were analyzed for genetic diversity using 63 SSRs spread across the genome. The study generated a total of 177 alleles, with two to six alleles per locus. Seven unique and 13 rare alleles were detected among the inbreds. Polymorphism information content ranged from 0.04 to 0.67, and Jaccard's dissimilarity coefficient varied from 0.32 to 0.77 with a mean of 0.62. The cluster analyses grouped the genotypes into three major clusters, and the principle coordinate analysis revealed diverse nature of the inbreds across four quadrangles. Genetically distant resistant inbreds identified in the study can be used for generating heterotic cross combinations with resistance to *S. oryzae*. These diverse resistant inbreds can also be used for generating pools and segregating populations to derive new inbreds with improved resistance. Mapping populations developed from genetically diverse and phenotypically contrasting inbreds would help in identifying QTLs imparting resistance to infestation of *S. oryzae*. Genomic regions thus identified would help in improving the degree of resistance in susceptible maize through marker-assisted selection.

Keywords: maize, stored grain weevil, Sitophilus oryzae, resistance, SSR markers

Introduction

Grain weevil (Sitophilus sp.) is one of the important storage-pests of cereals worldwide and inflicts considerable damage to the stored grains (Tefera et al, 2013). *Sitophilus zeamais*, popularly phrased as «maize weevil» is mainly found in Latin America and Africa, while *S. granarius* orgranary weevil is prevalent in temperate regions. *S. oryzae*, also known as «rice weevil» is predominantly found in Asian countries, and is a major stored grain pest of rice and wheat. However, due to polyphagous nature it also infests maize grains particularly in the Asian countries (Hossain et al, 2007; Zunjare et al, 2014a). The storage loss of grains is normally 10 - 20%, but may extend upto 80% under the humidity and temperature favourable to growth and development of weevils (Masasa et al, 2013; Derera et al, 2014). In the developing countries, food grains are mostly stored in jute bags that contribute to increase in grain moisture during rainy season. This creates optimum conditions for the weevil infestation, resulting in loss of grain-weight and seed-viability, besides causing fungal growth on the seed surface (Hossain et al, 2007; Zunjare et al, 2014a). Hence, infestation of weevils poses a serious threat to household livelihoods as well as local/regional food security (Tefera, 2012). Insect control through pesticides is not considered a viable option due to serious health and environmental hazards, possibility of development of insect-pest resistance towards chemicals, besides incurring additional costs in cultivation (Dowd et al, 2005; Adarwah et al, 2012). Resistance breeding on the other hand, provides economical means to combat post-harvest losses in a sustainable and eco-friendly manner (Abebe et al, 2009; Mwololo et al, 2013; Garcia-Lara and Bergvinson, 2014; Castro-Alvarez et al, 2015).

Maize serves as an important crop in America, Africa and Asia, providing important source of energy to humans, besides serving as an important component of poultry and animal feeds (Shiferaw et al, 2011). Asia alone produces nearly 30% of the global maize production that amounts to 1,016.73 million metric tonnes (FAOSTAT, 2013). India produced 24.35 million metric tonnes of maize during 2013-14, and 23% of the produce is used for human food, while 63% is utilized for poultry- and animal-feed (Yadav et al, 2014; Gupta et al, 2015). The growing poultry industry is one of the key factors behind the growth in maize production especially in Asia, and the demand

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for maize in the developing world will be doubled by 2050 (Rosegrant et al, 2009).

Information on germplasm diversity and genotype relationships are fundamental to any crop improvement programme (Sserumaga et al, 2014). Characterization of maize inbreds differing for their responses to *S. oryzae* infestation therefore, assumes great significance in the resistance breeding programme. Though limited studies on molecular diversity of maize inbreds differing for various insect-pests are available, no study to best of our knowledge has been reported with reference to responses of maize inbreds to infestationof *S. oryzae*. In the present study, genetic diversity analyses were undertaken among a set of maize inbreds differing for their responses to *S. oryzae*, to (i) investigate patterns of genetic relationships among the maize inbreds, (ii) identify potential heterotic cross combinations with resistance to *S. oryzae* infestation, and (iii) to identify suitable parents for generating mapping population(s) for localizing QTLs underlying the resistance to *S. oryzae* infestation.

Materials and Methods

Plant material

A set of 48 diverse maize inbreds differing for their responses to weevil infestation, were selected for molecular diversity analyses. Among the inbreds, 37 were of indigenous origin, while 11 inbreds were of exotic nature developed at CIMMYT, Mexico (CML-line), CIMMYT-HarvestPlus Programme (HP-line) and Kasetsart University, Thailand (KUI-line). These inbreds earlier evaluated against *S. oryzae* infestation, possessed varying responses with cumulative resistance index (CRI) ranging from 0.25 - 2.93 (Zunjare et al, 2014b). Higher value of CRI is indicative of resistance, while lower value depicts increased susceptibility (Zunjare et al, 2014b). CML394 and CML442 were used as resistant checks in the inbred panel (Dhliwayo and Pixley, 2003). Responses of these inbreds to weevil infestation have been mentioned in Table 1.

Genomic DNA isolation and PCR amplification

Leaf samples were collected from young seedlings and DNA isolation was carried out using CTAB method (Saghai-Maroof et al, 1984). The quality of DNA was checked using 0.8% agarose gel electrophoresis, followed by dilution with Tris-EDTA buffer to the concentration 10 ng μl^{-1}, the final concentration for PCR reaction. Primer sequence information for the maize SSR loci at different genomic bin locations is available in public domain (MaizeGDB; http://www.maizegdb.org). Among 63 SSRs (distributed throughout the genome) used in the analyses, 13 loci were having di-repeat motifs, 45 loci possessed tri-repeat motifs and two loci possessed tetra-repeats, while motif number could not be assigned to three loci. PCR amplification was carried using a procedure optimised at the laboratory, and PCR-amplified products were resolved in 3.5% agarose gel (Choudhary et al, 2015).

Statistical analyses

Total number of alleles, major allele frequency, gene diversity, heterozygosity and polymorphic information content (PIC) were estimated using PowerMarker V3.0 (Liu and Muse, 2005). Any allele appearing in only one genotype was considered as unique allele, while allele with a frequency of <0.05 was considered as rare allele. Genetic dissimilarity was calculated for pairwise comparison of genotypes using Jaccard's coefficient with 1000 bootstraps. Neighbour-Joining method implemented in DARwin-5.0 was used for constructing the dendrogram, and principal coordinate analysis (PCoA) was calculated to supplement the clustering pattern (Perrier et al, 2003).

Table 1 - Details of inbred lines used in the study.

S. No.	Inbred	Institution	CRI*	S. No.	Inbred	Institution	CRI
1	CML394	CIMMYT, Mexico	2.93	25	BAJIM-06-14	CSK-HPKV, Bajaura, India	2.05
2	CML207	CIMMYT, Mexico	2.83	26	BAJIM-08-11	CSK-HPKV, Bajaura, India	2.31
3	CML288	CIMMYT, Mexico	2.54	27	BAJIM-10-17	CSK-HPKV, Bajaura, India	2.27
4	CML290	CIMMYT, Mexico	0.25	28	Pant102	GBPUAT, Pantnagar, India	1.90
5	CML442	CIMMYT, Mexico	2.85	29	Pant104	GBPUAT, Pantnagar, India	1.94
6	CML480	CIMMYT, Mexico	1.10	30	Pant109	GBPUAT, Pantnagar, India	2.89
7	CML487	CIMMYT, Mexico	0.27	31	DMRQPM60	ICAR-IIMR, New Delhi, India	1.53
8	CML505	CIMMYT, Mexico	2.57	32	CM150	AICMIP, New Delhi, India	0.49
9	CML61	CIMMYT, Mexico	2.69	33	CM502	AICMIP, New Delhi, India	2.72
10	CI4	CIMMYT, Mexico	1.09	34	BML15	ANGRAU, Hyderabad, India	1.82
11	HPKP1	HarvestPlus Program	2.23	35	V334	ICAR-VPKAS, Almora, India	2.47
12	KUI3	Kasertsart University, Thailand	1.01	36	V364	ICAR-VPKAS, Almora, India	0.90
13	MGB1	ICAR-IARI, New Delhi, India	2.83	37	V372	ICAR-VPKAS, Almora, India	2.65
14	MGB2	ICAR-IARI, New Delhi, India	1.73	38	VQL1	ICAR-VPKAS, Almora, India	1.13
15	MGHC1	ICAR-IARI, New Delhi, India	2.75	39	VQL2	ICAR-VPKAS, Almora, India	0.45
16	MGHC2	ICAR-IARI, New Delhi, India	2.43	40	LM11	PAU, Ludhiana, India	2.50
17	BLSB-RIL107	ICAR-IARI, New Delhi, India	2.35	41	LM13	PAU, Ludhiana, India	2.78
18	HKI1105	CCS-HAU, Uchani, India	0.60	42	LM15	PAU, Ludhiana, India	1.05
19	HKI1344	CCS-HAU, Uchani, India	1.32	43	LM16	PAU, Ludhiana, India	2.63
20	HKI170	CCS-HAU, Uchani, India	1.74	44	EI116	MPUAT, Udaipur, India	1.84
21	HKI193-1	CCS-HAU, Uchani, India	0.72	45	SKV18	UAS, Nagenahelli, India	2.35
22	HKI193-2	CCS-HAU, Uchani, India	0.55	46	SKV21	UAS, Nagenahelli, India	2.89
23	HKI209	CCS-HAU, Uchani, India	2.79	47	NAI147	UAS, Nagenahelli, India	2.49
24	HKI323	CCS-HAU, Uchani, India	1.98	48	KDMI4	UAS, Arabhavi, India	1.44

*CRI: Cumulative Resistance Index (Zunjare et al, 2014b)

Table 2 - Primer details and summary statistics of genotyping assay of 48 inbred lines.

S. No.	Primers	Bin	Repeats	Major Allele Frequency	No. of Allele	Gene Diversity	Heterozygosity	PIC
1	bnlg1014	1.01	(AG)14	0.77	2	0.35	0.00	0.29
2	bnlg439	1.03	-	0.54	2	0.50	0.08	0.37
3	umc1558	1.04-1.05	(AG)7	0.96	2	0.08	0.00	0.08
4	umc1812	1.06	(ACC)6	0.89	2	0.20	0.10	0.18
5	umc1122	1.06-1.07	(CGT)7	0.68	3	0.49	0.08	0.44
6	umc1446	1.08	(TAA)7	0.88	2	0.22	0.00	0.19
7	umc2240	1.08-1.09	(AC)6	0.56	2	0.49	0.00	0.37
8	umc2100	1.12	(ATT)4	0.67	2	0.44	0.00	0.35
9	bnlg1092	2.00-2.01	(AG)30	0.49	6	0.70	0.11	0.67
10	umc1552	2.01-2.02	(GGA)7	0.78	2	0.34	0.00	0.28
11	bnlg125	2.02-2.03	-	0.45	3	0.64	0.10	0.57
12	umc2129	2.07	(CGC)5	0.53	3	0.6	0.10	0.52
13	umc2380	2.07-2.08	(GCT)5	0.6	2	0.48	0.09	0.37
14	umc2077	2.09	(AGC)4	0.71	2	0.41	0.00	0.33
15	umc2214	2.1	(CTT)4	0.39	3	0.66	0.02	0.58
16	umc2101	3.00-3.01	(AG)7	0.58	3	0.50	0.00	0.40
17	umc2377	3.01	(GAC)4	0.71	4	0.46	0.13	0.43
18	phi374118	3.02	ACC	0.44	3	0.65	0.00	0.57
19	umc2259	3.03	(CCG)6	0.64	3	0.51	0.02	0.44
20	umc1717	3.04	(GAAA)4	0.43	4	0.70	0.02	0.65
21	umc2127	3.05	(GGC)6	0.67	3	0.48	0.02	0.41
22	umc1052	3.09	(AAC)5	0.43	3	0.65	0.10	0.57
23	umc1136	3.09-3.10	(GCA)5	0.73	4	0.42	0.00	0.37
24	umc1758	4.01-4.02	(CTT)5	0.75	2	0.38	0.00	0.30
25	bnlg1937	4.05-4.06	(AG)21	0.9	4	0.19	0.04	0.19
26	bnlg1023	4.06	(AG)19	0.47	3	0.62	0.09	0.54
27	umc1775	4.07-4.08	(CGC)5	0.94	3	0.12	0.06	0.12
28	umc2139	4.09	(GCC)4	0.88	2	0.22	0.08	0.19
29	umc1532	4.1	(AAAT)4	0.67	3	0.48	0.02	0.41
30	bnlg1890	4.11	(AG)26	0.54	5	0.63	0.06	0.58
31	umc1761	5.02	(GCA)5	0.69	2	0.43	0.00	0.34
32	umc2296	5.03	(AGT)4	0.46	3	0.57	0.15	0.48
33	umc2298	5.03-5.04	(GCG)4	0.7	2	0.42	0.10	0.33
34	umc2201	5.06-5.07	(GCG)5	0.75	4	0.41	0.08	0.39
35	umc2143	5.08	(TTC)4	0.85	2	0.25	0.00	0.22
36	umc1153	5.09	(TCA)4	0.69	2	0.43	0.00	0.34
37	umc1186	6.01-6.02	(GCT)5	0.54	2	0.50	0.00	0.37
38	umc1178	6.02	(GGC)6	0.89	2	0.20	0.15	0.18
39	umc1257	6.02-6.03	(CAC)4	0.87	4	0.23	0.06	0.22
40	umc1857	6.04	(TAA)6	0.54	2	0.50	0.04	0.37
41	umc2141	6.05	(CT)8	0.58	2	0.49	0.00	0.37
42	umc2375	6.06	(GCG)4	0.54	3	0.60	0.00	0.53
43	umc2165	6.07	(TTC)12	0.52	4	0.58	0.12	0.49
44	umc2324	6.08	(CAC)4	0.92	2	0.15	0.00	0.14
45	umc2325	7.01	(TGG)7	0.49	4	0.63	0.04	0.57
46	umc1831	7.02	(AG)8	0.53	3	0.61	0.02	0.54
47	umc2332	7.04	(CTC)5	0.52	3	0.59	0.21	0.52
48	umc2334	7.05	(GGA)4	0.51	3	0.57	0.15	0.48
49	umc1359	8	(TC)12	0.73	4	0.44	0.08	0.4
50	umc1872	8.02	(GCA)6	0.35	4	0.72	0.24	0.66
51	phi119	8.02	AG	0.69	3	0.44	0.00	0.36
52	bnlg240	8.06	-	0.6	3	0.55	0.02	0.48
53	phi028	9.01	GAA	0.55	2	0.49	0.00	0.37
54	umc2393	9.00-9.01	(ACG)7	0.86	2	0.24	0.07	0.21
55	umc2336	9.02-9.03	(TGT)4	0.56	3	0.53	0.10	0.42
56	bnlg1209	9.04	(AG)12	0.54	3	0.59	0.02	0.52
57	umc2134	9.05-9.06	(TTC)6	0.69	3	0.44	0.00	0.36
58	umc1714	9.07-9.08	(AGG)8	0.81	3	0.32	0.10	0.29
59	umc1381	10.03	(AAC)4	0.63	2	0.47	0.00	0.36
60	umc1179	10.03	(AAG)4	0.98	2	0.04	0.00	0.04
61	umc1827	10.04-10.05	(GAC)6	0.79	2	0.33	0.00	0.28
62	umc2156	10.04-10.05	(TCG)5	0.83	2	0.28	0.00	0.24
63	umc2043	10.05	(TCC)4	0.49	3	0.61	0.13	0.53
Mean				0.66	2.80	0.45	0.05	0.38

Results and Discussion

SSR polymorphism

A total of 177 alleles from 63 SSR loci were generated across genotypes (Table 2). The number of alleles per SSR locus ranged from two to six, with an average of nearly three. Among the loci, bnlg1092 and bnlg1890 generated six and five alleles, respectively, thereby confirming the presence of wide genetic diversity among the inbreds. The average major allele frequency was 0.66, with a range of 0.35 (umc1872) to 0.98 (umc1179; Table 2). The highest gene diversity observed was 0.72 (umc1872), while the lowest was 0.04 (umc1179) with an average of 0.45. The PIC ranged from 0.04 (umc1179) to 0.67 (bnlg1092) with a mean of 0.38 (Table 2). Sixteen loci were found to be having PIC value more than 0.50, which is indicative of the higher discriminating power of SSR loci used in the study (Table 2). Rakshit et al (2010) while

working with a set of maize inbred lines differing for resistance to pink borer resistance reported an average PIC of 0.57 with a range of 0.27 to 0.84. The study also detected seven unique and 13 rare alleles that provided a prospect for unambiguous separation of the respective inbred lines from others (Sivaranjani et al, 2014; Choudhary et al, 2015; Pandey et al, 2015). The mean heterozygosity across locus was 0.05, indicating the inbreds attained high degree of homozygosity upon inbreeding. However, some loci such viz., umc1872 (0.24), umc2332 (0.21), umc2334 (0.15), umc1178 (0.15) and umc2296 (0.15) showed high heterozygosity (Table 2). Some loci regardless of repeated cycles of selfing over many generations tend to segregate due to residual heterozygosity. Other possible reasons include mutation at specific allele or amplification of similar sequences from different genomic regions due to duplication (Semagn et al, 2006). Inbreds bred conventionally often show some degree of heterozygosity as compared to doubled haploid based inbreds (Sivaranjani et al, 2014; Muthusamy et al, 2015).

Genetic relationships among inbreds

The genetic dissimilarity of the parental pairs was found to range from 0.32 (HKI193-1 and HKI193-2) to 0.77 (HKI193-1 and VQL1). The average dissimilarity value across all genotypes was 0.62, indicating presence of genetically diverse inbreds in the panel. The low genetic dissimilarity between HKI193-1 and HKI193-2 is due to derivation of both the inbreds from a common ancestor, CML193. Cluster diagram grouped the selected set of 48 genotypes into three distinct clusters (Figure 1). The CIMMYT inbreds were found to be distributed in all three clusters. Cluster A1 was comprised of 16 inbred lines, of which three were from CIMMYT (CML207, CML288, and CML480), one inbred developed by Kasetsart University, Thailand and 12 inbreds from India (HKI193-1, HKI193-2, HKI1105, BML15, LM15, BLSB-RIL107, BAJIM-10-17, BAJIM-08-11, BAJIM-06-14, SKV21, KDMI4, and EI116). As expected, genetically similar sister lines, HKI193-1 and HKI193-2 were grouped together. Cluster A2 comprised of MGHC1, MGHC2, MGB1, VQL1, V364, V372, V334, HKI209, HKI1344, LM11 of Indian origin and CML505, CML61, CML394, CML442, and HPKP1 of CIMMYT origin. Cluster B had two sub-clusters (Cluster B1 and B2), and consisted of 13 inbreds, of which only one inbred (CML290) is from CIMMYT, while the rest of the inbreds were developed by the various breeding centres of India. The Indian inbreds in Cluster B included DMRQPM-60, CM502, CM150, LM13, LM16, HKI170, VQL2, SKV18, NAI47, CI4, Pant 102, and MGB2. The third cluster (Cluster C) was relatively a small cluster which comprised of one CIMMYT (CML487) and three Indian lines (Pant109, Pant104, and HKI323). Rakshit et al (2010) carried out molecular diversity analysis among 23 maize inbred lines for pink borer infestation, and the genotypes were grouped into two main clusters, with five and six sub-clusters each. The genetic relationship were further elucidated and reconfirmed by Principal Coordinate Analysis (PCoA; Figure 2). The analyses showed that the inbreds were distributed in all the four quadrangles, signifying their genetic variability. The CIMMYT inbreds were present in all the four quadrangles along with the Indian inbred lines.

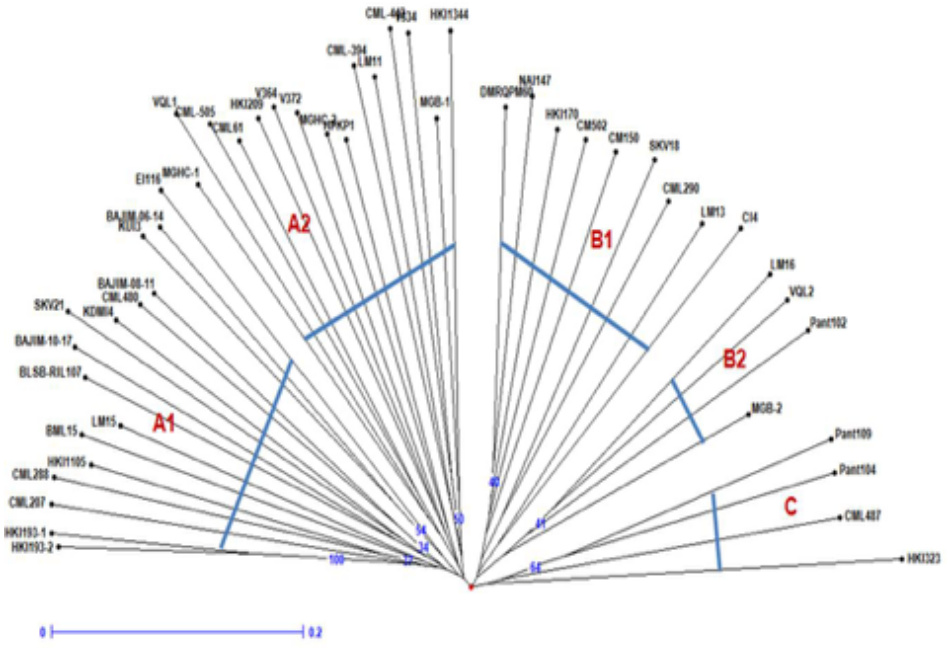

Figure 1 - Cluster analyses depicting genetic relationship among inbreds. Bootstrap value ≥ 30 is presented.

The two sister lines, HKI193-1 and HKI193-2 were placed at upper right quadrangle. It is important to note that HKI1105 and HKI323, the parental inbreds of HM4 hybrid were present in two different quadrangle (upper right and lower left, respectively), depicting their genetic distance. Similar observation was also noticed in case of VQL1 and VQL2 (parental inbreds of Vivek QPM-9), LM15 and LM16 (parental inbreds of PMH-2; Figure 2).

Potential utilization of inbreds in resistance breeding programme

The wide genetic diversity as observed in the present study signifies that the genes governing weevil resistance are possibly from diverse pedigree, and different sets of genes could be responsible for imparting resistance in diverse genetic background. Based on the genetic relationships and higher degree of resistance as depicted by higher values of CRI, CML394, SKV21, Pant109, CML442, CML207, MGB1, HKI209, LM13, MGHC1 and CM502, can be potentially utilized in the resistance breeding programme. Firstly, crosses viz. SKV21 × Pant109, SKV21 × LM13, SKV21 × CM502, MGB1 × LM13, MGB1 × CM502, MGB1 × Pant109, HKI209 × LM13, HKI209 × CM502, HKI209 × Pant109, MGHC1 × LM13, MGHC1 × CM502, MGHC1 × Pant109, LM13 × CM502, LM13 × Pant 109, CM502 × Pant109 may be attempted to exploit heterosis for grain yield with weevil resistance. Rakshit et al (2010) reported considerable diversity among 23 inbred lines differing for resistance against pink borer; and provided ample scope for selection of parents for utilization in heterosis breeding. Secondly, resistant white inbreds viz. CML394, CML442, and CML207 can be used as potential donors for the resistance genes. Thirdly, genetically diverse resistant inbreds can be crossed to derive new resistant inbreds from the F_2 segregants. Preponderance of additive gene action in imparting resistance against *S. zeamais* in maize has been reported by several researchers (Dhliwayo and Pixley, 2003; Kanyamasoro et al, 2012; Castro-Alvarez et al, 2015). Our research findings have also observed the importance of additive gene actions for imparting resistance in maize against *S. oryzae* (Zunjare et al, 2014b). This suggests that it is also possible to develop promising inbreds with higher degree of resistance through transgressive segregants generated from two diverse resistant inbreds (Castro-Alvarez et al, 2015). Fourthly, pool(s) can be constituted from the resistant inbreds, and intra-population recurrent selection can be employed to increase the frequency of the desirable alleles contributing to resistance. The improved pools thus developed can be potentially used to derive new inbreds with better degree of resistance to weevil infestation. Garcia-Lara and Bergvinson (2014) employed intra-population recurrent selection in P84 population, and observed 2-3 fold increase in level resistance against *S. zeamais*. Further, these resistant inbreds can be crossed with highly susceptible inbreds *viz*. CML290, CML487,

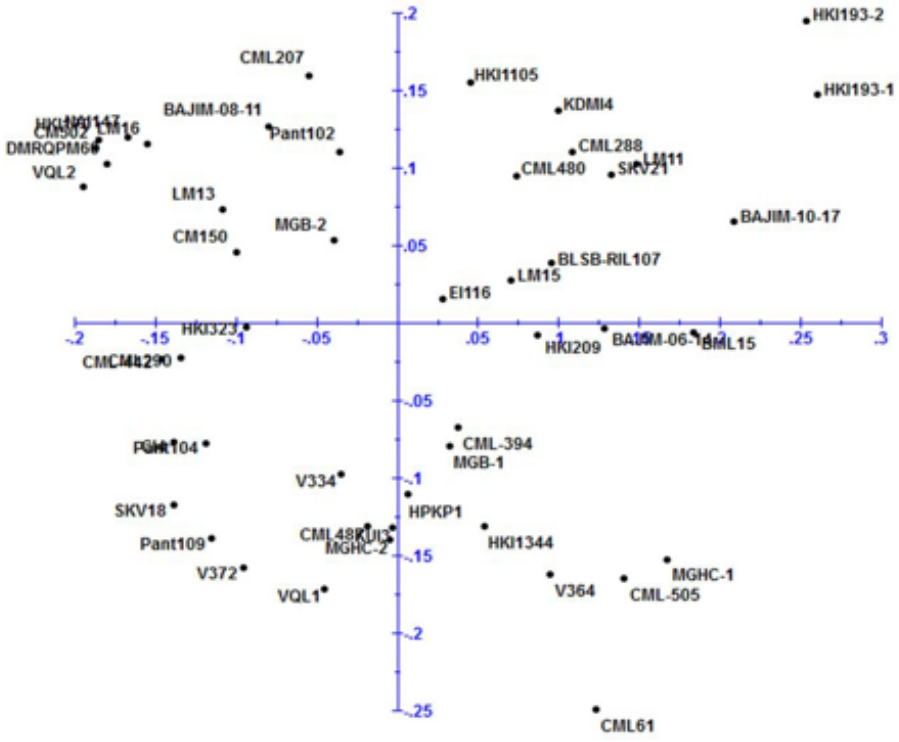

Figure 2 - Principal Coordinate Analysis of inbreds used in the study.

VQL2, and CM150 to generate mapping populations for identifying genetic loci underlying resistance to weevil infestation. QTLs conferring resistance to *S. zeamais* have been identified in maize (Garcia-Lara et al, 2009; Castro-Alvarez et al, 2015). Using recombinant inbred line population of tropical maize three major QTLs for resistance to *S. zeamais* were detected (Castro-Alvarez et al, 2015). So far, there is no report of QTL-mapping in maize against infestation of *S. oryzae*. Mapping population(s) developed from the contrasting inbreds identified in the study can potentially help in identifying key genomic regions that can be transferred to an otherwise agronomically superior but susceptible genotypes using marker-assisted selection (Garcia-Lara et al, 2009; Castro-Alvarez et al, 2015).

Conclusions

The present study depicted wide genetic variation among diverse inbreds differing for resistance to *S. oryzae*. Pattern of genetic relationships among the inbreds depicted through cluster analyses and PCoA, can be exploited effectively in the breeding programme. Genetically diverse resistant inbreds can be used for generating possible heterotic crosses, besides serving as important germplasm for creating transgressive segregants and pools with enhanced degree of resistance. Suitable mapping population(s) can be developed for identification of QTLs imparting resistance against S. oryzae.

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