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RESEARCH ARTICLE

Population structure and association analysis for downy mildew resistance in pearl millet from West Africa

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Abstract

Downy mildew (DM) caused by *S. graminicola* is the most important disease of pearl millet. It constitutes a serious threat to single cross F_1 hybrid. Marker-Traits Association analysis is a powerful method of detecting quantitative trait loci (QTL) underlying a trait. In this study, genetic variation for DM reaction was explored in 195 S_2 progenies of two pearl millet cytoplasmic male sterile (CMS) maintainer populations. Progenies were genotyped with 30 known SSR markers and phenotyped in greenhouse by screening with three different isolates of *S. graminicola* from Burkina Faso. Large variation (0-100% disease incidence) was observed for downy mildew reaction within the population. Heritability ranged from 93 to 96% for the three screening. The population was structured in three subpopulations based on genotypic data. Different statistical models were tested to account for population structure and control false positive. With general linear model (GLM), eight markers-trait associations were detected. Using GLM plus Q-matrix six significant marker-trait associations were found. And when mixed linear model (MLM) + Q-matrix + kinship was applied, four significant marker-trait associations were detected among which, three were validation of QTLs and one was a newly found QTLs. The associated markers reported in this study provide resources for marker-assisted selection for downy mildew resistance in Burkina Faso.

Key words: Pearl millet, genome wide association study, simple sequence repeat marker, B-line population, association analysis

Introduction

Pearl millet is a staple food crop for people living in the rural areas in many countries in the sub Saharan Africa. Cytoplasmic male-sterility (CMS) system has improved significantly yield of pearl millet by making possible commercial hybrid seed production (Bidinger *et al.*, 1994;

Kelley *et al*., 1996). However, DM caused by *Sclerospora graminicola* (Sacc.) Schroet, is a serious threat to single-cross hybrid varieties of pearl millet (Gowda *et al*., 2006; Sharma *et al*., 2008). High disease incidence (2% to 100%) was reported in India (Sharma *et al*. 2011). Up to 33% yield loss was reported in Nigeria (Gwary *et al*., 2009). High disease incidence was recorded in field screening in Burkina Faso (Wilson *et al*., 2008). The use of variety that is resistant to downy mildew offers a cost effective and sustainable control method against the pathogen.

Though reliable field and greenhouse screening procedures have been developed (Singh, 1995; Thakur *et al*., 2011) and successfully used to identify source of resistance to downy mildew (Singh and Singh, 1987; Thakur *et al*., 1992; Hash *et al*., 2006; Thakur *et al*., 2011), breeding for DM resistance will be facilitated by the use of marker-assisted selection. Molecular markers have been used in linkage mapping studies of bi-parental segregating population to identify quantitative trait loci (QTL) associated with DM resistance (Jones *et al*., 1995; Hash and Witcombe, 2001; Kebi, 2005; Gulia *et al*., 2007; Breese and Hash, 2008). A number of QTLs effective against one or more pathotypes of *S. graminicola* have been identified (Jones *et al*., 2002, 1995), and resistance alleles for some of these QTLs have been transferred in parental lines (Hash *et al*., 2006). However there is no report of QTL against *S. graminicola* isolates from Burkina Faso.

Association mapping has become a common approach to detect QTLs associated with complex traits in many crop species. The advantage of association mapping, compared to linkage mapping in bi-parental progeny are increased mapping resolution, reduced research time, and greater allele number in the germplasm set used for association mapping (Lüders *et al*., 2016). However, so far there are no reports on the use of association mapping to map downy mildew resistance traits in pearl millet. The objective of this study was to identify Simple Sequence Repeat (SSR) markers associated with downy mildew resistance in pearl millet using genome wide association study.

Materials and methods

Plant Material

A total of 195 selfed S_2 progenies derived from two cytoplasmic male sterile B populations MS-SOSAT-B (99 progenies) and MS-CIVAREX-B (96 progenies) were used in this study. MS-SOSAT-B population was developed by crossing an open pollinated variety (OPV) SOSAT-C88 with a CMS inbred line 38A (A_4 CMS system) from the International Research Institute for Semi-Arid Tropics (ICRISAT), Niger. Selected S_1 progenies from SOSAT-C88 that maintained sterility were recombined to form MS-SOSAT-B population. Similarly, MS-CIVAREX-B was developed by crossing CIVAREX 06-05 population with CIVAREX-A inbred lines both from "l'Institut de l'Economie Rural" (IER) Mali. Seed of the two B-line populations were sown in 30-rows plot of 6 m length in 2013 rainy season. Inter-row and intra-row plant spacing was maintained at 80 cm and 60 cm, respectively. Individual plants were self-pollinated in each population to develop the S_1 progenies. Each S_1 was sown in ear-to-row design of 6 m length at Sadore, ICRISAT Niger during the 2014 off season. S_2 progenies of each population were produced by selfing every panicle on each row. Bulked seed from each S_1 family plants formed the S_2 progeny seed.

DNA extraction and genotyping using SSR marker

Bulk DNA was extracted from meristem tissue samples collected on 10 – 15 plants from each S_2 progeny family using Qiagen DNeasy mini kit. DNA quality was checked by agarose gel electrophoresis (1%); the quantification was

done using a NanoDrop 2000 spectrophotometer. The DNA was further diluted to 5ng/µl and sent for SSR genotyping at the Genomics Service Laboratory of the M.S. Swaminathan Center of Excellence in Genomics, at ICRISAT, India. The 195 S_2 progeny families were screened using 30 SSR markers selected from pearl millet consensus linkage map (Rajaran *et al.,* 2013). Multiplex PCR was carried out to amplify SSRs. PCR reactions were carried out in 5µl of reaction containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.4 pm primers, 0.2 mM dNTPs, and 0.2U tag polymerase. The following program was used: 94^o C for 1 minute and 72^o C for 1 minute followed by 40 cycles of 94^o C for 1 minute, 51^o C for 1 minute, 72^o C for 1 minute and final extension at 72^o C for 20 minute. The PCR product was then analyzed using the ABI 3730 DNA analyzer. Two µl of PCR product of each marker of the multiplex set (markers labeled with different dyes) were pooled together for simultaneous detection of the amplified alleles. Seven µl of formamide and 0.2 µl of fragment-size standard GenScan™ 500 LIZ were added to the pooled PCR product and run on an ABI 3730 DNA genetic analyzer. Data were collected automatically by the detection of the different fluorescence and analyzed using GenMapper 4.0 software.

Screening of S_2 progenies

The 195 S_2 progenies families along with the two original populations, one susceptible check (7042S) and one resistant check (ICMVIS90311) were screened at seedlings stage in greenhouse against three different *S. graminicola* pathotypes from Burkina Faso at the "Centre de Recherches Environnementales et Agricoles" (CREAF) Kamboinse in Burkina Faso in 2015. The screening was done following the greenhouse screening technique of downy mildew of pearl millet (Thakur *et al.,* 2011). The sporangia inoculum concentration used was $2.4x10^5 ml^{-1}$. The experiment was conducted in a randomized complete bloc design (RCBD) with three replications, 30 seedlings per replication.

Analysis of phenotypic data

Disease was scored by counting the number of seedlings presenting typical symptom of downy mildew 14 days after inoculation. DM incidence was computed as percent of infected seedlings. Disease scores made on pot-replicate less than eight seedlings were removed and considered as missing data to avoid inaccurate estimation of percent disease incidence. Data set was subjected to analysis of variance (ANOVA) in Breeding View version 1.5 (BMS 3.9) using the following mixed linear model:

$$Y_{ij} = \mu + Ri + \text{Bj} + eij$$

Where Y_{ij} is the pot observation corresponding to genotype j of replicate i, μ is the population means, Ri is the effect of replicate i, Bj is the effect of genotype j, and eij is the residual.
Each screen was first analyzed separately using General Linear Model (GLM). Then the combined analysis was done using Restricted Maximum Likelihood (ReML) mixed models. Replications were treated as fixed whereas DM pathogen isolates, genotypes and all interactions were treated as random. The phenotypic mean BLUEs (Best Linear Unbiased Estimates) were estimated and were used for association studies. Broad sense heritability (H^2) was computed considering the percentage of genotypic variance over the phenotypic variance including genotype x pathotype interaction.

$H^2 = [Vg/(Vp)] *100$, (Hallauer *et al.* 2010).

Where, Vg is the genotypic variance, Vp is the phenotypic variance.

Analysis of population structure

The population structure was analyzed using STRUCTURE 2.2 software program (Vinod, 2011). Five independent interactions of running were performed with the number of subpopulation (*k*) ranging from 1 to 10. The optimal k was determined by joining the log probability of data [(LnP (D)] from the STRUCTURE output with the different *k* value. Based on the optimal (k = 3), each S_2 progeny was assigned to a subpopulation for which its membership value (Q value) was > 0.5. The population structure matrix (Q) and the genetic relatedness among S_2 progenies, kinship matrix (K), were generated for further analyses. Negative kinship values were set as zero.

Association analysis

To account for the population structure and avoid false positive association, population structure (Q-matrix) and the relatedness between individuals (Kinship) were estimated and two statistical models were tested; General Linear Model with Q-matrix (GLM+Q) and Mixed Linear Model with Q-matrix and K matrix (MLM Q+K), in addition to the general linear model (GLM) considered as a naïve method in association analysis. Both the Q and K matrix were set as covariates and they remained constant for testing all the markers. Variance components of polygene effects and the residuals were estimated using the likelihood equations (Kang *et al*., 2008) computed with the sofware TASSEL 5. Markers were considered being significantly associated with DM resistance trait on the basis of their significant association threshold $P \leq 0.05$.

Results and discussion

Variation among progenies for reaction to downy mildew

Analysis of variance showed significant difference among progenies and among DM isolates. Isolate-progeny interaction was also significant. The variation in replication is also significant (Table 1). The analysis of genetic variation (Table 2) and the frequency distribution (Fig. 1, 2, 3) indicated a large variation of DM incidence in the S_2 progeny families for each pathotype. High value of heritability (>90%) was also found for each screening (Table 2).

Table 1: Restricted maximum likelihood analysis of S_2 progenies for disease incidence to three downy mildew

Source	DF	MS
Replication	2	4890.242**
S_2 Progenies	197	1518.3544**
Isolates	2	7211.083**
Isolates x S_2 Progenies	400	1840.0327**
Residuals	200	601.8631
Total	602	948.4685

**** Significant at P ≤ 0.01, DF= degree of freedom, MS= mean squares**

Table 2: Means downy mildew incidence, ranges and heritability of S_2 the progenies

***Sclerospora graminicola* Pathotypes**	**Range (%)**	**DMI Mean (%)**	**Heritability (%)**
DM14	0 - 97	69	93
DM15	0 - 98	63	94
DM5	1 - 98	57	96

Table 3: Markers associated with downy mildew resistance in pearl millet S_2 progeny populations using GLM, GLM (Q) and MLM (Q+K)

Isolates	Marker	LG	GLM		GLM+Q		MLM Q+K	
			P value	% variance	P value	% variance	P value	% variance
DM14	XPSMP2248	6	**0.000**	9.6	**0.000**	9.4	0.993	0.0
DM14	XPSMP2084	4	**0.004**	5.9	0.2094	1.5	0.6312	0.5
DM14	XPSMP0066	4	**0.015**	4.2	0.7053	0.3	0.9354	0.1
DM14	XPSMP2059	2	**0.018**	2.8	**0.028**	2.2	**0.015**	2.9
DM14	IPES0003	2	**0.018**	2.8	**0.029**	2.2	**0.015**	2.9
DM15	XPSMP2085	4	**0.040**	3.4	**0.050**	3.1	**0.044**	3.4
DM15	XPSMP0066	4	**0.040**	3.2	**0.021**	3.8	**0.011**	4.7
DM5	XPSMP2248	6	**0.000**	12.4	**0.000**	12.3	0.540	0.7

DM14, DM15 and DM5 = *S. graminicola* isolates (different pathotypes); Bold indicate significant probability of marker-downy mildew resistance association

Fig. 1: Frequency distribution of downy mildew incidence (%) among S_2 progenies (Pathotype DM5)

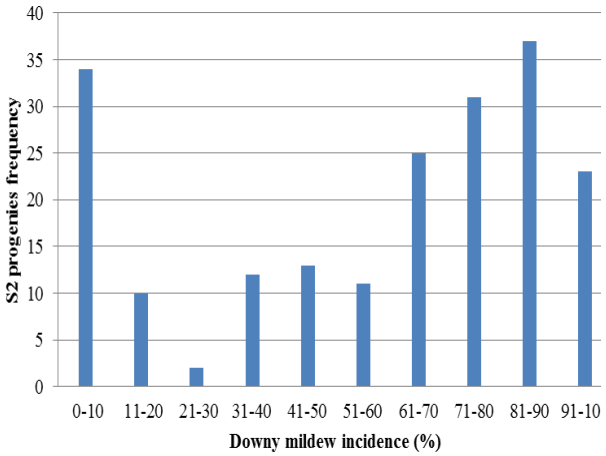


Fig. 2: Frequency distribution of downy mildew incidence (%) among S_2 progenies (Pathotype DM14)

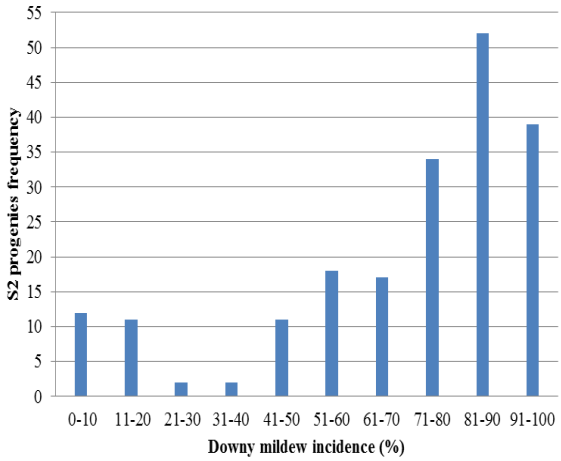


Fig. 3: Frequency distribution of downy mildew incidence (%) among S_2 progenies (Pathotype DM15)

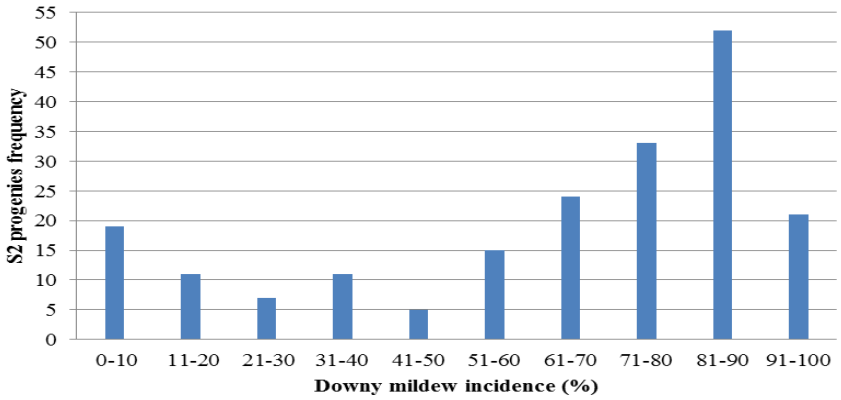
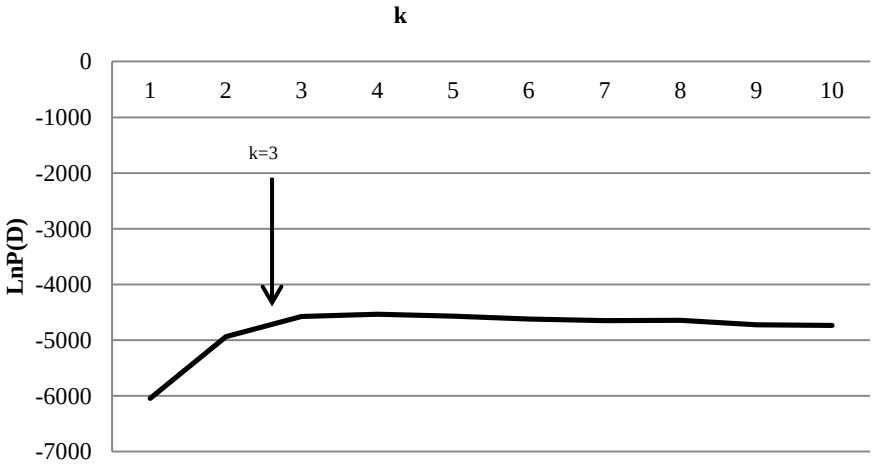
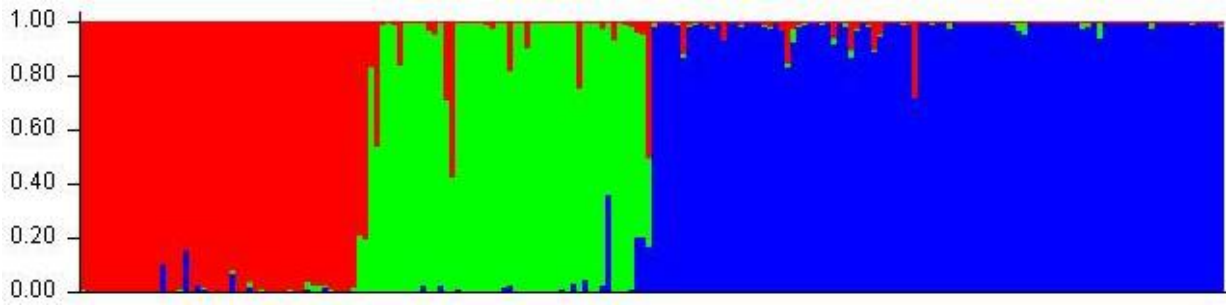


Fig. 4: Log probability data [Ln P (*D*)] as function of *k* (number of groups) from the STRUCTURE run showing the optimum k value



The plateau of the graph at *k* = 3 indicates the minimum number of groups (subpopulations) possible in the panel

Fig. 5: Barplot showing genetic diversity structure for 195 S_2 pearl millet progenies at *k* = 3 using the program STRUCTURE 2.3.2



Population structure of the S_2 progeny lines

In association analysis variability in the population is required for accurate identification of marker-trait association. From the 30 SSR markers used, 23 were able to amplify and therefore were considered for this study. A total of 137 alleles were detected and the number of alleles per locus varied from 2 to18, with an average of 5.95 alleles per locus. The population structure was analyzed with the 23 SSR markers. The STRUCTURE software was run for k = 1 to 10 based on the distribution of the 137 alleles at the 23 SSR loci among the S_2 progenies. LnP (D) appeared as an increasing function of k. But it reached a plateau at k = 3 (Fig. 4). Hence a k value of three subpopulations was selected to describe the population structure of the 195 S_2 progenies. The three subpopulations (G1, G2 and G3) included 52, 47 and 99 progenies (Fig. 5), which had an F_{ST} equal to 0.1636, 0.4648, and 0.4390, respectively. Hence, it can be assumed that in this study, the genetic diversity structure analysis indicated that the S_2 progenies developed from the two West African OPVs were structured in three different subpopulations. A similar population structure was also found in inbred lines developed from West African pearl millet germplasm (Gemenet *et al.*, 2015). The parental lines used in this study were developed using different genotypes from West Africa. SOSAT_C88 is and OPV developed from "Suna" originated from Senegal

and "Sanio" from Mali and it is cultivated across several countries in West Africa. CIVAREX population is also a landrace from Mali. As pearl millet has a high cross pollinating rate, these genotypes may have accumulated alleles from several genotypes over the time. This could explain why the population structure of inbred lines drawn from these populations is comparable to other population structure of West African germplasm. It was reported that the most probable reason for the differentiation between West African germplasm could be the flowering time which is shown to be a major adaptation trait in pearl millet (Mariac *et al.,* 2011). The strong population structure observed in this study may result in Type I error if it is not take into account during the association analysis (Zhu *et al.,* 2002). Several statistical models have been proposed to control spurious LD caused by population structure (Pasam *et al.,* 2012). Two of these models were tested in this study in comparison to the simple t-test.

Association analysis of DM resistance

In our study several significant putative marker-trait associations were identified for DM resistance. A total of eight markers were found to be significantly associated with the resistance to downy mildew using through the naïve method; five were associated with the resistance to the pathotype DM 14 with phenotypic variance of 2.8 – 9.6%, two were associated with the resistance to the pathotype DM 15 with 3.2-3.4% phenotypic variation, and one was associated with the resistance to the pathotype DM5 explaining up to 12.4% phenotypic variation. Using the GLM plus Q-matrix six markers showed significant association with the resistance to downy mildew (Table 3); three markers including XPSMP2248 on LG 6, XPSMP2059, and IPES0003 on LG 2 were associated with the resistance to the pathotypes DM14 explaining 2.2% to 9.4% of the phenotypic variation, XPSMP2248 was also associated with the resistance to the pathotype DM5 explaining 12% of the phenotypic variation, and XPSMP2085 and XPSMP0066 on LG 4 were associated with resistance to DM15 explaining 3.1 and 3.8% of the phenotypic variation, respectively. With the mixed linear model using Q and K matrix as covariate with fixed effect, four markers were found to be significantly associated with resistance to two downy mildew pathotypes (Table 3). Of these markers, two were associated with the resistance to DM14 with 2.9% phenotypic variation, and the two others were associated with resistance to DM15 with 3-4.7% phenotypic variation. Several significant putative marker-trait associations were identified for DM resistance. However, the number of significant associations varied according to the model. With the t-test (GLM), eight putative marker-trait associations were identified. Using the Q-model which account for the population structure, the number of significant putative marker-trait association decreased to six and the magnitude of most of the significance decrease also. Using the mixed linear model with population structure and kinship (MLM Q+K model) to account for population structure and the relatedness between individuals, the number of marker-trait association identified decreased to four. There is evidence from this study that population structure has affected the detection of significant marker-trait association for DM resistance. The Q-model was also useful in controlling inflation in the P-values. In general, the phenotypic variation accounted for the QTLs were low. This could be explained by the limited number of SSR markers used in this study.

Four SSR markers have been constantly significantly associated with DM resistance across all models tested. As they were revealed by the mixed model considered as the most efficient model in Genome-Wide association (GWAS) analysis in controlling false positive

association (Liu *et al.,* 2016), those markers could be considered as the most reliable markers associated with downy mildew resistance. Marker-trait association for downy mildew resistance was found on different chromosomes for different pathotype indicating that different QTLs are involved in the resistance against each pathotype. Therefore, a stable resistance to DM might involve different QTLs. The involvement of different QTLs in the resistance against different isolate of *S. graminicola* was reported in several studies (Jones *et al.,* 1995, 2002; Breese, *et al.,* 2002; Kebi, 2005; Gulia *et al.,* 2007; Breese and Hash, 2008).

Among the associated markers, IPES0003 is located in the same genomic region with a QTL detecting resistance against *S. graminicola* isolates of Sadore (Niger) and Bamako (Mali) (Azhaguvel, 2001), XPSMP2085 is located in the same genomic region with a QTL detecting resistance against *S. graminicola* isolates of Maiduguri (Nigeria) and Bengou (Niger) (Jones et *al.,* 1995) and Xipes0066 is located in the same genomic region with a QTL detecting resistance against *S. graminicola* isolates of Sadore, Bamako, Benin Kebi (Nigeria) (Breese *et al*., 2002). These three SSR markers could be considered as validated markers from previous study. However, XPSMP2059 identified on LG 2 was not found in any genomic region previously associated with DM resistance. Therefore, this SSR marker is a newly identified QTL for DM resistance. This marker needs to be validated before it becomes a useful molecular marker in selection for DM resistance in pearl millet.

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