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QTL mapping of pearl millet rust resistance using an integrated DArT- and SSR-based linkage map

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Abstract Rust, caused by the fungus *Puccinia substriata* var. *indica*, is one of the most important production constraints of pearl millet worldwide, leading to grain yield losses of up to 76 % as well as major losses in fodder yield and quality. Here, we report the development of a linkage map integrating Diversity Arrays Technology (DArT) markers and simple sequence repeat (SSR) markers, using this to identify quantitative trait loci (QTLs) for pearl millet rust resistance. Genotyping data from 256 DArT and 70 SSR markers on 168 F_7 recombinant inbred lines from cross 81B-P6 × ICMP 451-P8 were used to construct a linkage map comprised of 286 loci (229 DArT and 57 SSR markers) spanning a total length of 740.3 cM (Haldane) with an average adjacent marker distance of 2.7 cM. Linkage group 7 (LG7) (153.5 cM) was the longest and LG6 the shortest (45.0 cM). The map was used to identify a major QTL for rust resistance with an LOD score of 27 on LG1, which explained 58 % of the observed phenotypic variation. In addition, two putative modifiers of small effect were detected, one each on LG4 and LG7. The novel rust resistance QTL identified on LG1 is thought to confer a durable slow-rusting phenotype, which is still effective in India more than 20 years after it was first deployed in the previously popular single-cross hybrid MH 179 (ICMH 451). The flanking markers reported here provide a framework for marker-assisted selection and possible future map-based cloning of this resistance gene.

Keywords *Pennisetum glaucum* · Molecular markers · Diversity Arrays Technology · Linkage map · Rust resistance · QTL mapping

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Introduction

Pearl millet, *Pennisetum glaucum* (L.) R. Br. ($2n = 2x = 14$), also known as cattail millet, bulrush millet, candle millet, *cumbu* and *bajra*, is the sixth most important cereal following rice, wheat, maize, barley and sorghum. It is a C4 grass with the highest levels of tolerance to heat and drought among tropical cereals and is grown on more than 29 million ha in arid, semi-arid, subtropical and tropical regions of Asia, Africa and Latin America where it produces staple food grain and fodder. It is still sometimes regarded as an 'orphan' crop and has received relatively little attention from researchers outside of India compared to its potential contribution to humanity. There is a need to better understand the genetic basis of economically important traits in this crop and develop more efficient genomic tools for use in its cultivar development. Compared to better studied cereals such as rice, wheat, maize, barley and sorghum, there has been relatively little research on the development and application of molecular genetic tools for pearl millet (Liu et al. 1994, 1996, 1997; Jones et al. 1995, 2002; Busso et al. 1995, 2000; Burton and Wilson 1995; Morgan et al. 1998; Devos et al. 2000; Poncet et al. 2000, 2002; Allouis et al. 2001; Gale et al. 2001; Qi et al. 2001, 2004; Bhattacharjee et al. 2002; Breese et al. 2002; Yadav et al. 2002, 2003, 2004; Azhaguvel et al. 2003; Budak et al. 2003; vom Brocke et al. 2003; Bidinger et al. 2005, 2007; Hash and Witcombe 2001; Hash et al. 2003, 2006; Bertin et al. 2005; Mariac et al. 2006a, b, 2011; Gulia et al. 2007; Senthilvel et al. (2008); Saïdou et al. 2009, 2014a, b; Stich et al. 2010; Supriya et al. 2011; Kholová et al. 2012; Nepolean et al. 2012; Sehgal et al. 2012, 2015; Rajaram et al. 2013; Vengadessan et al. 2013; Kannan et al. 2014; Ramana Kumari et al. (2014); Aparna et al., 2015; Gemenet et al. 2015; Moumouni et al. 2015). The RFLP- and SSR-based genetic linkage maps developed so far for pearl millet provide less than optimal genome coverage and marker density (Liu et al. 1994; Qi et al. 2004; Gulia et al. 2007; Rajaram et al. 2013). A consensus map of 353 RFLP and 65 SSR markers was developed (Qi et al. 2004) by integrating genetic maps produced in four different crosses of pearl millet where 85 % of the markers are clustered and occupy less than one third of the total map length. Extreme localization of recombination is toward the chromosome ends, resulting in gaps on the genetic map of 30 cM or more in the distal regions (Devos et al. 2000; Qi et al. 2004). The unequal distribution of recombination has consequences for the transfer of genes controlling important agronomic traits from donor to elite pearl millet germplasm (Qi et al. 2004). To date, only approximately 200 PCR-compatible markers have been mapped in pearl millet (Morgan et al. 1998; Gale et al. 2001; Gulia et al. 2007; Rajaram et al. 2013). The length of published linkage maps so far ranges from 280 cM (Jones et al. 2002) to 675 cM (Senthilvel et al. 2008). Hence, there is a continuing need to fill the gaps in these maps, further saturate them and extend the portion of the mapped genome further into subtelomeric regions to facilitate further application of genomic tools for improvement of this species. DArT has the potential to generate hundreds of high-quality genomic dominant markers with a cost- and time-competitive trade-off (Kilian et al. 2005) and can be used for construction of high-density genetic linkage maps with even distribution of markers over the genome, which offer real advantages for a range of molecular breeding and genomic applications. Supriya et al. (2011) developed a DArT platform for pearl millet and used this for diversity analysis and high-density linkage map construction. Other new genotyping technologies capable of highly parallel analysis would represent a major step forward in this crop. Recently, another high-throughput and low-cost genotyping method named genotyping-by-sequencing (GBS) has been developed and has proven its efficiency in other crops such as maize and barley (Elshire et al. 2011), sorghum (Nelson et al. 2011; Morris et al. 2013a, b; Lasky et al. 2015) and pearl millet (Moumouni et al. 2015).

Apart from grain, pearl millet is also important as a forage and stover crop (Anand Kumar 1989; Andrews and Kumar 1992). Its pre-flowering vegetative parts provide excellent forage because of their low hydrocyanic (HCN) acid content and high levels of protein, calcium, phosphorous and other minerals (Athwal and Gupta 1966). Sorghum is the main C4 forage, having potential for toxic levels of HCN, which are hazardous to livestock when fed green. In contrast, pearl millet forage (and grain) has low levels of cyanogenic glucosides, but juvenile plants can accumulate nitrates at levels that are dangerous for livestock and when stressed are known to accumulate oxalates to levels that make the forage unpalatable (Anand Kumar 1989;

Andrews and Kumar, 1992). Lack of adequate foliar disease resistance can dramatically reduce the livestock feed value of pearl millet green forage, hay, silage or crop residues remaining after harvest of a pearl millet grain crop (Monson et al. 1986; Wilson et al. 1991). Among the various foliar diseases of economic importance in pearl millet such as downy mildew (*Sclerospora graminicola* (Sacc.) J. Schroet.), rust (*Puccinia substriata* var. *penicillariae* (Speg.) Ramachar & Cumm.) and blast (*Pyricularia grisea* (Cke.) Sacc.), rust, caused by the fungus *P. substriata* var. *penicillariae* (de Carvalho et al. 2006), is the most important forage production constraint for this crop worldwide, leading to losses of up to 76 % in grain production and major losses in fodder yield and quality (Wilson et al. 1996) as well as substantial reductions in biomass yield and quality when pearl millet is grown as the mulch component of minimum tillage systems in Brazil (de Carvalho et al. 2006). Visual effects of this rust are severe, ranging from death of young plants from early infection to premature desiccation and/or death of leaves with later infection. Green yield, dry-matter yield and forage quality as measured by in vitro digestibility are negatively correlated with rust severity (Monson et al. 1986; Wilson et al. 1991, 1996). Therefore, improving pearl millet rust resistance to reduce yield and quality losses has become a high priority for breeders in regions where this disease is prevalent. Although resistance to rust has been reported in some pearl millet germplasm accessions and breeding lines (Rao and Rao 1983; Wilson 1993a; Singh et al. 1997), identification of new physiological races of the pathogen (Wilson 1991, 1993b; Tapsoba and Wilson 1996) suggests that continuous evaluation of new sources of resistance is required. Rust resistance has been reported to be conferred by a single dominant gene and susceptibility by its recessive allele (Andrews et al. 1985; Hanna et al. 1985; Wilson 1993a), with several different sources of major gene and quantitative resistance having been identified and exploited (Rao and Rao 1983; Singh et al. 1987, 1990; Singh 1990; Wilson 1993a, 2006; Wilson et al. 1994, 2001). Quantitative trait locus (QTL) mapping is a highly effective approach for studying genetically complex forms of plant disease resistance. Morgan et al. (1998) used a combination of RAPD and RFLP markers to map the *Rr1* gene from wild pearl millet (*P. glaucum* spp. *monodii*) to linkage group 3 (LG3); however, this major gene resistance was overcome by the pathogen population in the southeastern USA soon after its deployment in forage and grain hybrids following its backcross transfer to elite hybrid seed parent maintainer background Tift 85D2A1/85D2B1 (Hanna et al. 1987; Wilson 1993b; Wilson et al. 1994, 1996). Hash et al. (2003) suggested quantitative trait loci (QTL) mapping and marker-assisted selection (MAS) for stover yield, foliar disease resistance and in vitro estimates of the nutritive value of various stover fractions for ruminants in pearl millet and sorghum as ways to improve the economic value of residues of these crops that are available following grain harvest. In addition to this, QTL mapping of downy mildew resistance (Jones et al. 1995, 2002; Hash and Witcombe 2001; Breese et al. 2002; Gulia et al. 2007), rust and blast resistance (Morgan et al. 1998), drought tolerance (Yadav et al. 2002, 2004; Bidinger et al. 2007; Kholová et al. 2012; Sehgal et al. 2012, 2015; Aparna et al. 2015) and the association of flowering time with the genotype × environment interaction of grain and stover yield (Yadav et al. 2003) has been done. However, so far there are no reports on the identification and mapping of rust resistance QTLs in pearl millet that are effective in Asia or Africa. This article reports the development of an integrated high-density genetic linkage map based on DArT and SSR markers that has been used for mapping QTLs for pearl millet rust resistance that is effective in India.

Materials and methods

Plant material

A mapping population of 168 F_7 RILs derived from cross 81B-P6 × ICMP 451-P8 was used to construct an integrated DArT + SSR based linkage map and was screened for rust resistance.

DNA extraction and quantification

Pot-grown pearl millet seedlings grown under greenhouse conditions at ICRISAT- Patancheru were used. The youngest 3–5 leaves were taken, and DNA was extracted using the SDS-potassium acetate method (Dellaporta et al. 1983). DNA quantification was done by agarose gel electrophoresis (0.8 %), and it was

further diluted to 50 ng/µl for DArT and 5 ng/µl for SSR genotyping.

Genotyping of individual DNA samples using DArT array

One hundred sixty-eight F_7 RILs of the mapping population were used individually to prepare the genomic representations using the same complexity reduction method used for library construction (*Pst*I/*Ban*II), and genotyping was done as described in Supriya et al. (2011).

Genotyping with Simple Sequence Repeats (SSRs)

PCR using SSR markers

A set of 30 genic and genomic SSRs (27 fluorescently labeled SSRs and 3 M13-labeled SSRs) (Table 1) was screened using the two parental lines and 168 F_7 RILs. Multiplex PCR was carried out to amplify SSRs. For M13-labeled primers a three-primer strategy was used with a 1:15:15 ratio for the forward primer with an M13 tail, regular reverse primer and universal fluorescent-labeled M13 primer, respectively. PCR reactions were carried out in 5 µl reactions containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.4 pm primers, 0.2 mM dNTPs and 0.2 U *Taq* polymerase (NEB, UK). Touchdown PCR was performed using the following program: 94 °C for 3 min and five cycles of touchdown at 94 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min followed by 40 cycles of 94 °C for 1 min, 51 °C for 1 min, 72 °C for 1 min and final extension at 72 °C for 20 min.

Analysis of PCR products on the ABI 3730 DNA analyzer

Two µl of PCR product was taken from each marker of the multiplex set (markers labeled with different dyes) and pooled together for simultaneous detection of the amplified alleles. Seven µl of formamide and 0.2 µl of fragment-size standard GeneScan^{TM} 500 LIZ were added to the pooled PCR product and run on an ABI 3730 DNA genetic analyzer (Applied Biosystems). The data were collected automatically by the detection of the different fluorescences and analyzed using GeneMapper v4.0 software (Applied Biosystems).

Linkage map construction

The scores of all polymorphic DArT and SSR markers were converted into genotype codes ('A', 'B') according to the scores of the parents. Data for 40 polymorphic ICRISAT pearl millet EST stress (IPES) EST-SSR markers (Rajaram et al. 2013) were also added prior to linkage map construction, and linkage groups were obtained using JoinMap (Stam 1993) at logarithm of odds (LOD) threshold values ranging from 2 to 10. The order of markers in each linkage group was finalized by RECORD software (van Os et al. 2005) and the Haldane mapping function. The graphical representation of the map was drawn using MapChart software (Voorrips 2002). DArT markers were named with the prefix "PgPb" where 'Pg' stands for *P. glaucum*, 'P' for *Pst*I (primary restriction enzyme used) and 'b' for *Ban*II (secondary restriction enzyme used) followed by numbers corresponding to unique clone ID following Supriya et al. (2011).

Phenotyping and QTL mapping for rust resistance

Greenhouse screening for rust resistance

Seed of susceptible check entries (ICMB 89111 and ICMB 06222) and resistant (ICML 11 and ICMP 451) and 167 F_7 RILs segregating for rust resistance from the cross 81B-P6 (susceptible) × ICMP 451-P8 (resistant) were sown in pots (15 seeds/pot) filled with a sterilized soil-sand-farmyard manure (FYM) mix (2:1:1 by volume) and placed in a completely randomized design in a greenhouse maintained at 35 °C. The experiment was conducted with four replications, and there were two pots per replicate for each entry. Pots were watered daily, and seedlings were thinned to ten plants/pot. Twelve days after germination, when the seedlings were at the third leaf stage, they were spray-inoculated with an aqueous urediniospores suspension ($\approx 1.0 \times 10^5$ urediniospores ml^{-1}) of *P. substriata* (spores were collected from the Pathology Section, ICRISAT-Patancheru) and exposed to high humidity (>90 % RH) under misting. Rust severity was recorded 10 days after inoculation using the modified Cobb's rating scale for the percentage of infected leaf area within each pot (Thakur et al. 2011).

Table 1 Pearl millet SSR markers used for genotyping of 168 F_7 RILs derived from cross (81B-P6 × ICMP 451-P8)

Sample no.	Marker locus	Size (bp)	Repeat motif	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)	References	Linkage group
1	*Xctm10*	180–200	(CT)22	GAGGCAAAAGTGGAAGACAG	TTGATTCCCGGTTCTATCGA	Budak et al. (2003)	LG3
2	*Xctm12*	310–340	(CT)12	GTTGCAAGCAGGAGTAGATCGA	CGCTCTGTAGGTTGAACTCCTT	Budak et al. (2003)	LG1
3	*Xctm25*	255–280	(CT)34	GCGAAGTAGAACACCGCGCT	GCACTTCCTCCTCGCCGTCA	Budak et al. (2003)	
4	*Xpsmp2031*	180–200	(CCA)3(TCC)3	CACATCCGCAAGAGACACCAAAT	TTTGGGGGTGTAGGTTTTGTTG	Qi et al. (2004)	
5	*Xpsmp2069*	210–230	(CA)26	CCCATCTGAAATCTGGCTGAGAA	CCGTGTTCGTACATGGTTTTGC	Qi et al. (2001)	LG1
6	*Xpsmp2080*	155–190	(AC)14	CAGAATCCCCACATCTGCAT	TGCAACTGAGCGAAGATCAA	Qi et al. (2004)	LG1
7	*Xpsmp2089*	110–130	(AC)15	TTCGCCGCTGCTACATACTT	TGTGCATGTTGCTGGTCATT	Qi et al. (2004)	
8	*Xpsmp2208*	230–300	(GT)10	GAAAGAGCAAACTGAACAATCCC	ACTTTGCCCTGGATGATCCTC	Qi et al. (2001)	LG5
9	*Xpsmp2219*	210–280	(GT)7	ACTGATGGAATCTGCTGTGGAA	GCCCGAAGAAAAGAGAACATAGAA	Qi et al. (2001)	LG5
10	*Xpsmp2225*	220–240	(GT)12	CCGTACTGATGATACTGATGGTT	TGGGAGGTAAGCTCAGTAGTGT	Qi et al. (2001)	LG2
11	*Xpsmp2227*	175–190	(GT)7	ACACCAAACACCAACCATAAAG	TCGTCAGCAATCACTAATGACC	Allouis et al. (2001)	LG3
12	*Xpsmp2229*	220–280	(GT)5	CCACTACCTTCGTCTTCCTCCATTC	GTCCGTTCCGTTAGTTGTTGCC	Allouis et al. (2001)	LG3, LG5 and LG7
13	*Xpsmp2231*	210–235	(TG)12GG(TA)4	TTGCCTGAAGACGTGCAATCGTCC	CTTAATGCGTCTAGAGAGTTAAGTTG	Qi et al. (2001)	LG2
14	*Xpsmp2232*	220–240	(TG)8	TGTTGTTGGGAGAGGGTATGAG	CTCTCGCCATTCTTCAAGTTCA	Allouis et al. (2001)	LG2
15	*Xpsmp2236*	210–235	(TG)4(GT)4	ATAAGTGGGACCCACATGCAGCAC	CGAAAGACTAGCAAAATTGCGCCTTC	Allouis et al. (2001)	LG7
16	*Xpsmp2237*	245–265	(GT)8	TGGCCTTGGCCTTTCCACGCTT	CAATCAGTCCGTAGTCCACACCCCA	Allouis et al. (2001)	LG2
17	*Xpsmp2248*	145–165	(TG)10	TCTGTTTGTTTGGGTCAGGTCCTTC	CGAATACGTATGGAGAACTGCGCATC		
18	*Xpsmp2249*	115–160	(GT)7imperfect	CAGTCTCTAACAAACAAACACGGC	GACAGCAACCAACTCCAAACTCCA	Allouis et al. (2001)	LG3
19	*Xpsmp2251*	140–200	(TG)6	TCAAACATAGATATGCCGTGCCTCC	CAGCAAGTCGTGAGGTTCGGATA	Allouis et al. (2001)	
20	*Xpsmp2255*	255–300	(TG)34	CATCTAAACACAACCAATCTTGAAC	TGGCACTCTTAAATTGACGCAT	Allouis et al. (2001)	LG6
21	*Xpsmp2261*	165–190	(GA)16	AATGAAAATCCATCCCATTTCGCC	CGAGGACGAGGAGGGCGATT	Allouis et al. (2001)	

Table 1 continued

Sample no.	Marker locus	Size (bp)	Repeat motif	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)	References	Linkage group
22	*Xpsmp2266*	180–200	(GA)17	CAAGGATGGCTGAAGGGCTATG	TTTCCAGCCCACACCAGTAATC	Allouis et al. (2001)	
23	*Xpsmp2270*	130–155	(GA)26imperfect	AACCAGAGAAGTACATGGCCCG	CGACGAACAAATTAAGGCTCTC		
24	*Xpsmp2273*	140–160	(GA)12	AACCCCACCAGTAAGTTGTGCTGC	GATGACGACAAGACCTTCTCTCC	Allouis et al. (2001)	LG1
25	*Xpsmp2275*	260–290	(GTT)10	CCAGTGCCTGCATTCTTGGC3	GCATCGAATACTTCATCTCA	KM Devos (pers. comm)	LG6
26	*Xpsmp2085*	155–170	(AC)11	GCACATCATCTCTATAGTATGCAG	GCATCCGTCATCAGGAAATAA	Qi et al. (2004)	LG4
27	*Xicmp3027*	185–210	(GAT)6	ACACCATCACCGACAACAAA	AGTGACCTGGGGTACAGACG	Senthilvel et al. (2008)	LG5
28	*Xicmp3032*	180–200	(GCT)8(ACAT)3	AGGTAGCCGAGGAAGGTGAG	CAACAGCATCAAGCAGGAGA	Senthilvel et al. (2008)	LG1
29	*Xicmp3050*	195–215	(TA)8	ATGTCCAGTGTTGACGGTGA	CGGGGAAGAGACAGGCTACT	Senthilvel et al. (2008)	LG6
30	*Xicmp3088*	150–175	(TCC)8	TCAGGTGGAGATCGATGTTG	TTACGGGAGGATGAGGATG	Senthilvel et al. (2008)	LG1

Statistical analysis of disease severity data

Statistical analysis was performed using Genstat 12th edition from Rothamsted, UK. Analysis of variance (ANOVA) was performed using a completely randomized design, and the mean of rust severity %, S.E., C.V. and heritability were calculated.

QTL analysis

For QTL mapping, the linkage map constructed with marker data from 146 F_7 RILs derived from the cross (81B-P6 × ICMP 451-P8) was used. The entry means of raw data scored for resistance percentage were used for QTL analysis, which was performed by composite interval mapping (CIM) with PlabQTL (Utz and Melchinger 1996) using a LOD of 2.5 as the threshold value for QTL significance.

Results

Genotyping using DArT array and SSRs

After screening a mapping population of 168 F_7 RILs from the cross 81B-P6 × ICMP 451-P8, 256 polymorphic clones (DArT markers) were identified in a total of 7680 clones (3.3 % of polymorphic clones) on the array. The call rate ranged from 80.3 to 98.4 % with an average of 89.5 %, and the scoring reproducibility was 100 %. The DArT markers used displayed high polymorphism information content (PIC) values, ranging from 0.27 to 0.50 with an average of 0.46. The *P* and *Q* values, which are measurements of variation, were calculated as described by Storey and Tibshirani (2003), and across individuals they ranged from 65.1 to 92.9 % (average 81.3 %) and 64.8 to 92.5 % (average 80.9 %), respectively. Out of 30 SSR primer pairs (Table 1) used for capillary electrophoretic separation of fluorescent-labeled PCR products, 25 SSRs (83.3 %) detected reliably scorable polymorphism.

Genetic linkage mapping

To assemble the linkage map, 326 polymorphic markers (256 DArT and 70 SSRs) were used. Out of these, 286 loci (229 DArT markers and 57 SSRs) were distributed across the expected 7 linkage groups using LOD thresholds ranging from 2 to 10 and a

recombination frequency (r) threshold <0.4 using JoinMap, and 40 markers (27 DArTs and 13 SSRs) remained unlinked, probably because of the extremely high recombination rates observed in subtelomeric regions of pearl millet chromosomes (Devos et al. 2000). The order of markers in each linkage group was finalized using RECORD software. The map built with JoinMap was inflated by 47 % when compared with that built using RECORD. Markers violating map stability were removed, and linkage groups were reanalyzed to construct a stabilized map, which spanned a total length of 740.3 cM (Haldane) (Fig. 1a, b) with an average adjacent-marker distance of 2.7 cM, and an average density of 0.39 markers/cM. The total number of mapped loci per linkage group ranged from 23 on LG6 to 59 on LG2, and the average was 40.9 loci/LG. The longest individual linkage group map was for LG7 (153.5 cM), the shortest was for LG6 (45.0 cM), and the average LG length was 105.8 cM. The density of markers on the individual linkage groups ranged from 0.29 markers/cM on LG5 to 0.51 markers/cM on LG6. Map distances between two consecutive markers varied from 0 to 21 cM, and 263 of the 279 intervals (94.3 %) were <10 cM. There were only 16 intervals (5.7 %) larger than 10 cM, and the largest gap between markers was observed on LG7 (21.0 cM). Many DArT markers were present as clusters in subtelomeric regions (e.g., the top of LG1) (Fig. 1a). Of the 286 markers placed on the genetic map, 54 were distributed on LG1, 59 on LG2, 35 on LG3, 42 on LG4, 27 on LG5, 23 on LG6 and 46 on LG7 (Table 2).

Significant segregation distortion from the expected 1:1 Mendelian ratios was found for 124 (38.0 %) out of 326 markers genotyped across these 146 RILs. Sixty markers (18.4 %) showed distortion in favor of the 81B-P6 allele, whereas 64 (19.6 %) showed distortion in favor of the ICMP 451-P8 allele. Of the 286 markers mapped, 118 (41.2 %) showed distorted segregation with 57 markers (19.9 %) showing distortion in favor of the 81B-P6 allele and 61 (21.3 %) in favor of the ICMP 451-P8 allele. Distorted markers (Fig. 1a, b) favoring the 81B-P6 alleles were found primarily on LG2 (49 out of 59 markers mapping to this group), LG3 and LG6, while those favoring the ICMP 451-P8 alleles were mapped on LG1, LG3, LG4 (41 out of 42 markers mapping to this group), LG5 and LG7. LG3 showed skewed markers favoring alleles from both parents in different portions of the linkage group.

QTL identification for rust resistance

General statistics

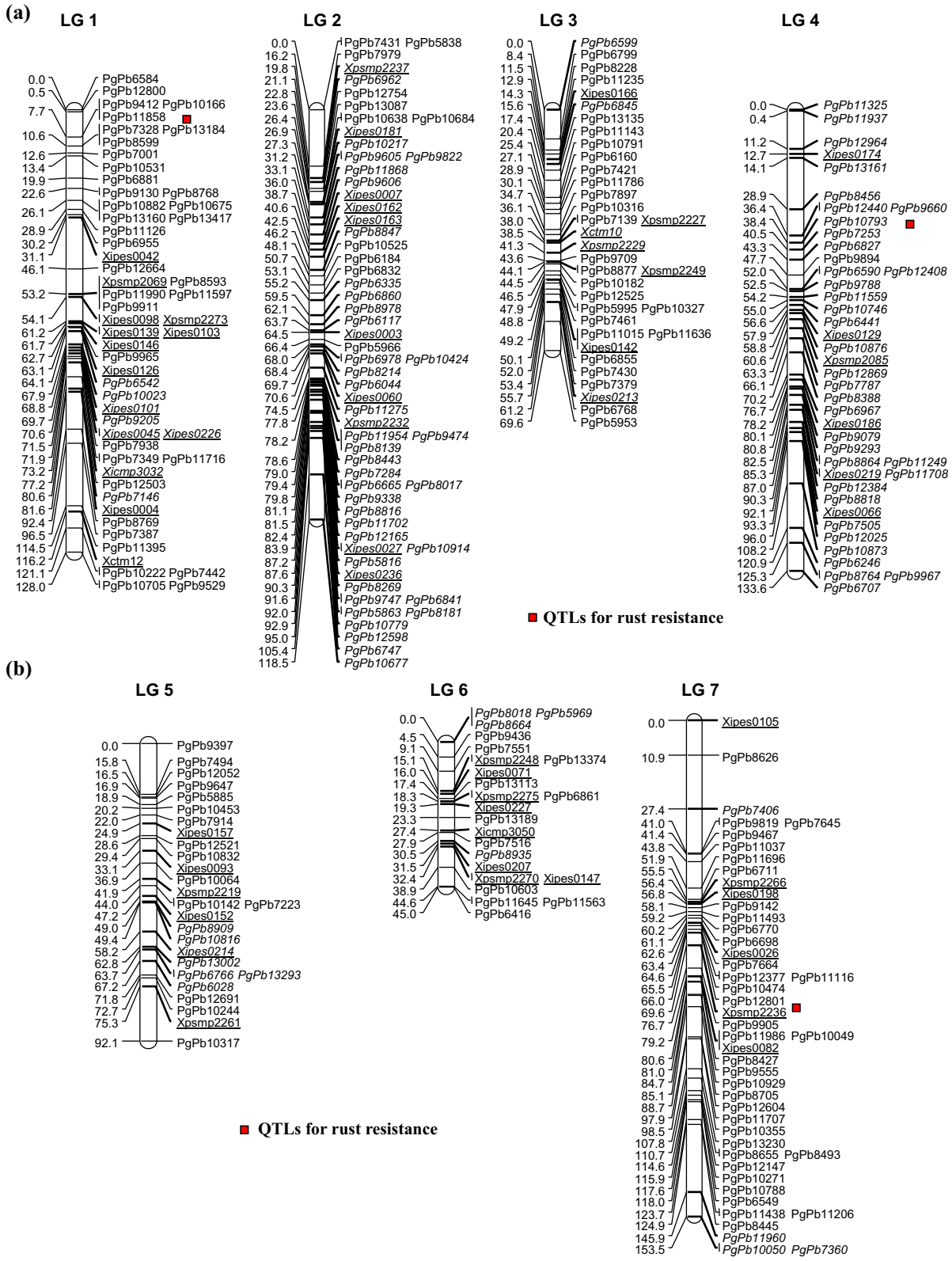
Rust severity (%) in the test lines ranged from 0 to 95 % in the RILs derived from the cross (81B-P6 × ICMP 451-P8). Highly significant differences were detected by ANOVA between individual RIL progenies. Mean rust severity (%) was calculated for each RIL using the data from four replications, and it ranged from 0.25 to 89.38 % (grand mean 35.0 %) with an operational heritability (repeatability) of 99 %, SE_m of 2.5 % and CV of 7.1 %. Parental line ICMP 451-P8 was resistant and exhibited mild symptoms in a few replications with a mean rust severity of 4.6 %, while parental line 81B-P6 was highly susceptible recording 77.8 % severity. Among various control entries, ICML 11 was moderately resistant (10.6 % rust severity), ICMB 89111 (55.4 %) was susceptible, and ICMB 06222 (83.5 %) was highly susceptible. Of the 167 RILs, 32 were resistant (≤10 % severity), 18 moderately resistant (11–20 % severity), 73 moderately susceptible (21–50 % severity) and 40 susceptible (51–75 % severity), and the remaining four lines were highly susceptible (>75 % severity), as shown in the histogram in Fig. 2.

QTL mapping

QTL analysis was performed with PlabQTL using the integrated DArT-SSR genetic map. A major QTL with a LOD value of 27 (Fig. 3) was mapped near the top of the LG1 (Fig. 1), explaining 58 % of the observed phenotypic variation in rust reaction of the RIL progenies (Table 3). At this locus the allele of resistant parent ICMP 451-P8 conferred resistance. In addition to this major QTL, two modifiers were also detected, one each on LG4 and LG7, explaining 9.0 and 8.3 % of the observed phenotypic variation, respectively (Table 3). The favorable allele for the LG4 modifier was inherited from susceptible parent 81B-P6, whereas that for the LG7 modifier was inherited from ICMP 451-P8.

Discussion

DArT technology enabled identification of many markers with relatively high polymorphism content [i.e., 256 polymorphic DArT markers having 100 %


(a)
LG 1
0.0 PgPb6584
0.5 PgPb12800
7.7 PgPb9412 PgPb10166
PgPb11858
10.6 PgPb7328 PgPb13184
12.6 PgPb8599
13.4 PgPb7001
19.9 PgPb10531
22.6 PgPb6881
26.1 PgPb9130 PgPb8768
PgPb10882 PgPb10675
PgPb13160 PgPb13417
28.9 PgPb11126
30.2 PgPb6955
31.1 Xipes0042
46.1 PgPb12664
53.2 Xpsmp2069 PgPb8593
PgPb11990 PgPb11597
PgPb9911
54.1 Xipes0098 Xpsmp2273
61.2 Xipes0139 Xipes0103
61.7 Xipes0146
62.7 PgPb9965
63.1 Xipes0126
64.1 PgPb6542
67.9 PgPb10023
68.8 Xipes0101
69.7 PgPb9205
70.6 Xipes0045 Xipes0226
71.5 PgPb7938
71.9 PgPb7349 PgPb11716
73.2 Xicmp3032
77.2 PgPb12503
80.6 PgPb7146
81.6 Xipes0004
92.4 PgPb8769
96.5 PgPb7387
114.5 PgPb11395
116.2 Xctm12
121.1 PgPb10222 PgPb7442
128.0 PgPb10705 PgPb9529
LG 2
0.0 PgPb7431 PgPb5838
16.2 PgPb7979
19.8 Xpsmp2237
21.1 PgPb6962
22.8 PgPb12754
23.6 PgPb13087
26.4 PgPb10638 PgPb10684
26.9 Xipes0181
27.3 PgPb10217
31.2 PgPb9605 PgPb9822
33.1 PgPb11868
36.0 PgPb9606
38.7 Xipes0007
40.6 Xipes0162
42.5 Xipes0163
46.2 PgPb8847
48.1 PgPb10525
50.7 PgPb6184
53.1 PgPb6832
55.2 PgPb6335
59.5 PgPb6860
62.1 PgPb8978
63.7 PgPb6117
64.5 Xipes0003
66.4 PgPb5966
68.0 PgPb6978 PgPb10424
68.4 PgPb8214
69.7 PgPb6044
70.6 Xipes0060
74.5 PgPb11275
77.8 Xpsmp2232
78.2 PgPb11954 PgPb9474
PgPb8139
78.6 PgPb8443
79.0 PgPb7284
79.4 PgPb6665 PgPb8017
79.8 PgPb9338
81.1 PgPb8816
81.5 PgPb11702
82.4 PgPb12165
83.9 Xipes0027 PgPb10914
87.2 PgPb5816
87.6 Xipes0236
90.3 PgPb8269
91.6 PgPb9747 PgPb6841
92.0 PgPb5863 PgPb8181
92.9 PgPb10779
95.0 PgPb12598
105.4 PgPb6747
118.5 PgPb10677
LG 3
0.0 PgPb6599
8.4 PgPb6799
11.5 PgPb8228
12.9 PgPb11235
14.3 Xipes0166
15.6 PgPb6845
17.4 PgPb13135
20.4 PgPb11143
25.4 PgPb10791
27.1 PgPb6160
28.9 PgPb7421
30.1 PgPb11786
34.7 PgPb7897
36.1 PgPb10316
38.0 PgPb7139 Xpsmp2227
38.5 Xctm10
41.3 Xpsmp2229
43.6 PgPb9709
44.1 PgPb8877 Xpsmp2249
44.5 PgPb10182
46.5 PgPb12525
47.9 PgPb5995 PgPb10327
48.8 PgPb7461
49.2 PgPb11015 PgPb11636
50.1 Xipes0142
52.0 PgPb6855
53.4 PgPb7430
55.7 PgPb7379
61.2 Xipes0213
69.6 PgPb6768
PgPb5953
LG 4
0.0 PgPb11325
0.4 PgPb11937
11.2 PgPb12964
12.7 Xipes0174
14.1 PgPb13161
28.9 PgPb8456
36.4 PgPb12440 PgPb9660
38.4 PgPb10793
40.5 PgPb7253
43.3 PgPb6827
47.7 PgPb9894
52.0 PgPb6590 PgPb12408
52.5 PgPb9788
54.2 PgPb11559
55.0 PgPb10746
56.6 PgPb6441
57.9 Xipes0129
58.8 PgPb10876
60.6 Xpsmp2085
63.3 PgPb12869
66.1 PgPb7787
70.2 PgPb8388
76.7 PgPb6967
78.2 Xipes0186
80.1 PgPb9079
80.8 PgPb9293
82.5 PgPb8864 PgPb11249
85.3 Xipes0219 PgPb11708
87.0 PgPb12384
90.3 PgPb8818
92.1 Xipes0066
93.3 PgPb7505
96.0 PgPb12025
108.2 PgPb10873
120.9 PgPb6246
125.3 PgPb8764 PgPb9967
133.6 PgPb6707
QTLs for rust resistance
(b)
LG 5
0.0 PgPb9397
15.8 PgPb7494
16.5 PgPb12052
16.9 PgPb9647
18.9 PgPb5885
20.2 PgPb10453
22.0 PgPb7914
24.9 Xipes0157
28.6 PgPb12521
29.4 PgPb10832
33.1 Xipes0093
36.9 PgPb10064
41.9 Xpsmp2219
44.0 PgPb10142 PgPb7223
47.2 Xipes0152
49.0 PgPb8909
49.4 PgPb10816
58.2 Xipes0214
62.8 PgPb73002
63.7 PgPb6766 PgPb13293
67.2 PgPb6028
71.8 PgPb12691
72.7 PgPb10244
75.3 Xpsmp2261
92.1 PgPb10317
LG 6
0.0 PgPb8018 PgPb5969
PgPb8664
4.5 PgPb9436
9.1 PgPb7551
15.1 Xpsmp2248 PgPb13374
16.0 Xipes0071
17.4 PgPb13113
18.3 Xpsmp2275 PgPb6861
19.3 Xipes0227
23.3 PgPb13189
27.4 Xicmp3050
27.9 PgPb7516
30.5 PgPb8935
31.5 Xipes0207
32.4 Xpsmp2270 Xipes0147
38.9 PgPb10603
44.6 PgPb11645 PgPb11563
45.0 PgPb6416
LG 7
0.0 Xipes0105
10.9 PgPb8626
27.4 PgPb7406
41.0 PgPb9819 PgPb7645
41.4 PgPb9467
43.8 PgPb11037
51.9 PgPb11696
55.5 PgPb6711
56.4 Xpsmp2266
56.8 Xipes0198
58.1 PgPb9142
59.2 PgPb11493
60.2 PgPb6770
61.1 PgPb6698
62.6 Xipes0026
63.4 PgPb7664
64.6 PgPb12377 PgPb11116
65.5 PgPb10474
66.0 PgPb12801
69.6 Xpsmp2236
76.7 PgPb9905
79.2 PgPb11986 PgPb10049
Xipes0082
80.6 PgPb8427
81.0 PgPb9555
84.7 PgPb10929
85.1 PgPb8705
88.7 PgPb12604
97.9 PgPb11707
98.5 PgPb10355
107.8 PgPb13230
110.7 PgPb8655 PgPb8493
114.6 PgPb12147
115.9 PgPb10271
117.6 PgPb10788
118.0 PgPb6549
123.7 PgPb11438 PgPb11206
124.9 PgPb8445
145.9 PgPb11960
153.5 PgPb10050 PgPb7360
QTLs for rust resistance

◀**Fig. 1** **a** Linkage groups LG 1 thru LG 4 of the genetic linkage map for the (81B-P6 × ICMP 451-P8)-based pearl millet RIL population. Map distances (Haldane cM) and marker names are shown on the *left* and *right side* of each linkage group, respectively. SSR markers are *underlined*, and DArT marker names begin with the prefix PgPb. Markers that showed distorted segregation are shown in *italics*. QTL positions for rust resistance are shown on LG1 and LG4. **b** Linkage groups LG 5 thru LG 7 of the genetic linkage map for the (81B-P6 × ICMP 451-P8)-based pearl millet RIL population. Map distances (Haldane cM) and marker names are shown on the *left* and *right side* of each linkage group, respectively. SSR markers are *underlined*, and DArT marker names begin with the prefix PgPb. Markers that showed distorted segregation are shown in *italics*. QTL position for rust resistance is shown on LG7

repeatability were segregating in the (81B-P6 × ICMP 451-P8)-derived mapping population] in a cost-effective manner in comparison to SSRs. Thus, the pearl millet DArT platform proved useful for application in genome-wide screening for QTL discovery. It can also be expected to prove useful for recurrent parent background recovery in marker-assisted backcrossing, for isolation of genes via map-based cloning, for comparative mapping and for genome organization studies. The availability of better-saturated molecular maps that are achievable using DArT, GBS-SNPs and other approaches will certainly provide breeders and geneticists with a much-wanted tool to identify various genomic regions of interest, which in turn will increase the efficiency of marker-assisted breeding (Moumouni et al. 2015).

A well-saturated pearl millet genetic linkage map was constructed spanning 740.3 cM with an average adjacent-marker distance of 2.7 cM and smaller marker intervals than any previously constructed maps with RFLPs and/or SSRs. The high level of genome coverage achieved in this map will be particularly useful to select markers for use in whole-genome breeding strategies and to saturate genomic regions of interest in other mapping populations. The distribution of markers was reasonably uniform including the distal regions of all chromosome arms because of inclusion of DArT and EST-SSRs. These markers typically show improved genome coverage compared to anonymous (non-coding) SSRs or AFLPs, which are characteristically clustered around the centromeric regions (Ramsay et al. 2000). The processes used to develop each type of marker accounts for this difference in genome coverage. Anonymous SSRs are usually developed from random genomic libraries in which microsatellites located in the heterochromatic regions are overrepresented (Röder et al. 1998), and the development of EST-SSRs from genic regions reduces the representation of regions that are rich in repetitive DNA (Parida et al. 2006).

In this study, a high proportion of DArT markers showed clustering in distal regions of several of the 14 chromosome arms, and such clustering of DArT markers was more frequent than that of SSRs, which was expected as DArT markers were over four times more abundant than the SSRs in the data sets (and the SSRs included both genic and genomic SSRs), and the endonuclease *Pst*I was used in the preparation of the reduced representation libraries used for pearl millet DArT clone development. It appears that DArT markers prepared using *Pst*I may have a stronger tendency than genomic SSR and AFLP markers in particular to map to such gene-rich regions (Vuylsteke et al. 1999), which may be due to use of the methylation-sensitive restriction enzyme *Pst*I in the complexity reduction of

Table 2 Linkage group details of the DArT- and SSR-based genetic map for a pearl millet RIL population based on cross (81B-P6 × ICMP 451-P8)

Linkage group	DArT marker loci	SSR marker loci	Total marker loci	Length (cM)	Adjacent-marker interval (cM)	Density (markers/cM)
LG1	40	14	54	128.0	2.42	0.42
LG2	49	10	59	118.5	2.04	0.50
LG3	28	7	35	69.6	2.05	0.50
LG4	36	6	42	133.6	3.26	0.31
LG5	21	6	27	92.1	3.54	0.29
LG6	15	8	23	45.0	2.05	0.51
LG7	40	6	46	153.5	3.41	0.30
Total	229	57	286	740.3	2.65	0.39

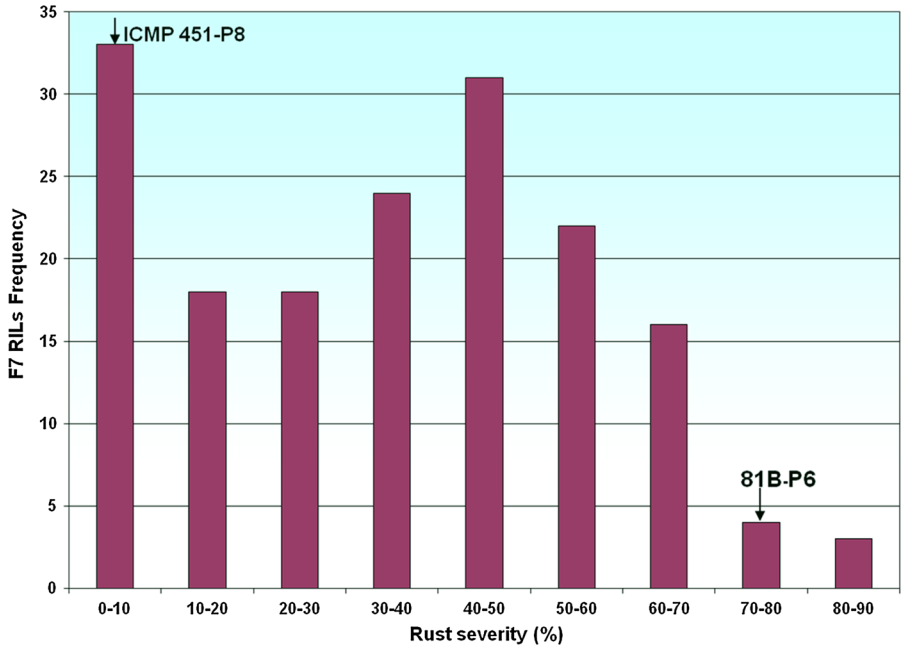


Fig. 2 Frequency distribution of rust severity (%) among F_7 RIL progenies from the pearl millet cross (81B-P6 × ICMP 451-P8)

the initial library. The occurrence of DArT marker clusters in distal regions of chromosome arms was observed in previous DArT mapping studies on barley (Wenzl et al. 2004) and wheat (Akbari et al. 2006; Semagn et al. 2006). Similar clustering in distal regions was also found in tetraploid wheat using *Pst*I-based AFLP markers (Peng et al. 2000), which is related to the trend of *Pst*I-based markers to map in gene-rich, hypomethylated regions of the genome (Langridge and Chalmers 1998; Moore 2000), although it could also be a consequence of the presence of redundant clones on the arrayed genomic representation (Semagn et al. 2006). Almost all types of markers illustrate clustering around centromeres due to centromeric suppression of recombination (Tanksley et al. 1992; Korol et al. 1994). The high proportion of DArT markers clustering away from the centromeres may therefore be indicative of gene-rich regions, and it is an additional advantage of DArT markers as they can be helpful for fine mapping of genes/QTLs residing in gene-rich regions, thereby facilitating positional cloning. Of course, genotyping-by-sequencing SNPs identified using reduced representation libraries constructed using *Pst*I will have similar advantages. However, DArT marker data sets have inherently lower frequencies of missing data points than do GBS-SNP data sets unless higher than normal sequencing densities are used for GBS.

The marker orders of SSRs from the present study were compared with those from maps based on SSRs only (Rajaram et al. 2013) and were almost identical except for swapping of some marker orders within several blocks on a few linkage groups (data not shown). Such differences in marker order among genetic maps is not unexpected, as genetic mapping only gives an indication of the relative positions and genetic distances of the markers to each other (Sourdille et al. 2004), and structural rearrangements of chromosomes are relatively common in pearl millet (Varshney et al. submitted). Moreover, inconsistency in the map position of these few SSRs could be explained by the presence of closely linked DArT loci. The order of loci was also compared with an integrated DArT-SSR pearl millet map based on cross (H 77/833-2 × PRLT 2/89-33) (Supriya et al. 2011), which was also very similar with limited levels of marker position swapping. Seventy-eight markers representing all seven linkage groups of pearl millet were mapped in both populations, which will permit the development of a better-saturated pearl millet consensus linkage map combining DArT and SSR markers. In this study, segregation distortion was observed for 38 % of the total marker loci analyzed, which is similar to the report of Supriya et al. (2011).

The high-density linkage map for the RIL population based on cross 81B-P6 × ICMP 451-P8 was

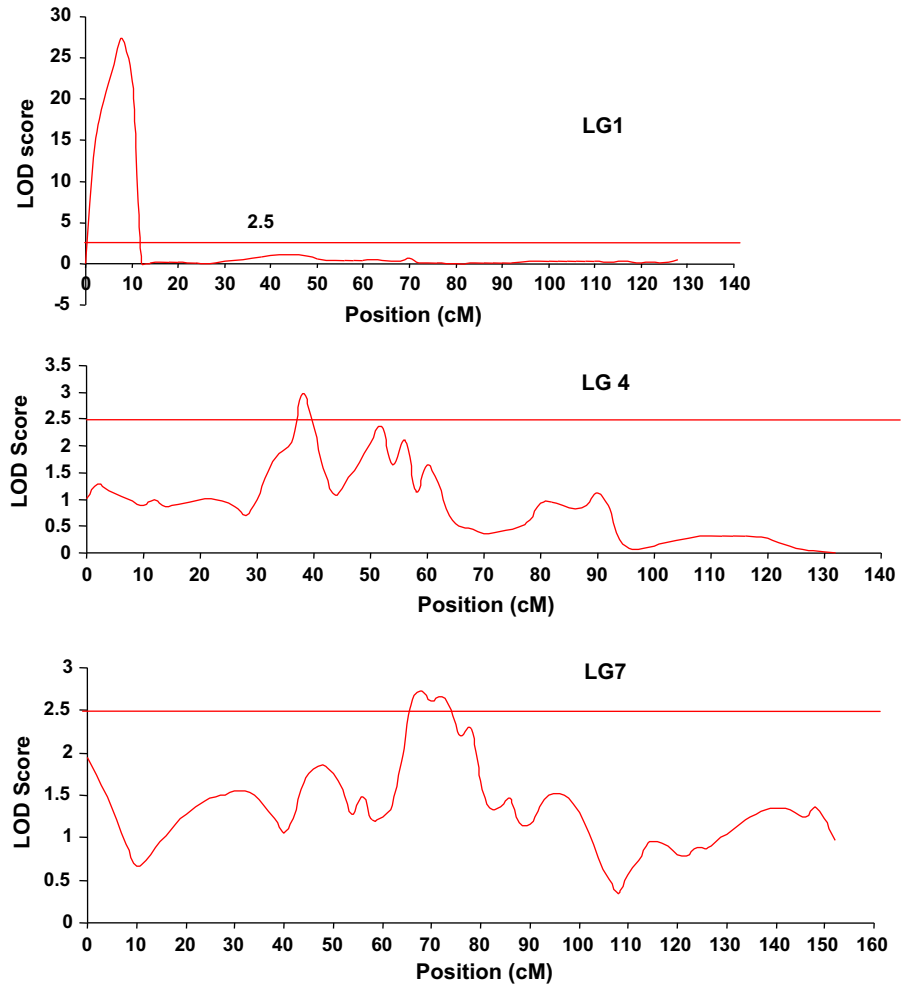


Fig. 3 Logarithm of odds (LOD) profiles for LG1, LG4 and LG7 for rust resistance QTLs segregating in the (81B-P6 × ICMP 451-P8)-based pearl millet RIL population. The *horizontal line* across each graph indicates the threshold level (LOD = 2.5) used for QTL identification

successfully used to identify QTLs for rust resistance, and this is the first report on QTL mapping for rust resistance in pearl millet from India. The only prior report of rust resistance mapping (Morgan et al. 1998) reported QTLs for resistance to pathogen populations present in the southeastern USA, and these mapped to LG3 and LG4. In contrast, in the present study a major QTL effective against an Indian population of *P. substriata* was detected on LG 1, along with two QTL modifiers (one each on LG4 and LG7), explaining 58 % of the observed phenotypic variation in rust reaction among the RIL progenies (Table 3). Highly significant differences detected by ANOVA between individual progenies and high operational heritability of 0.99 demonstrated that resistance was segregating in the population and that much of the observed variation in the rust reaction phenotype was attributable to genetic variation. The host rust reaction was continuously distributed in the population (Fig. 2). However, this does not necessarily imply that the inheritance of rust reaction is complex and that many genes are segregating. In fact, as the frequency distribution of the RILs showed two peaks, it was anticipated that a large portion of the variation would prove to be attributable to a single genomic region of large effect, and this was indeed the outcome of the QTL analysis. Andrews et al. (1985), Hanna et al. (1985), and Wilson (1993a) have previously reported

Table 3 Summary of QTLs for pearl millet rust resistance detected using PlabQTL and data from RILs derived from cross (81B-P6 × ICMP 451-P8)

Linkage group	Flanking markers	Position (cM)	LOD	Variance explained (%)	Additive effect	Inheritance
LG1	*PgPb9412-PgPb7328*	8.0	27.30	57.8	16.9	Major QTL; ICMP 451-P8 allele contributes to resistance
LG4	*PgPb12440-PgPb10793*	38.0	2.97	9.0	−1.1	QTL modifier; 81B-P6 allele contributes to resistance
LG7	*PgPb12801-Xpsmp2236*	68.0	2.73	8.3	2.6	QTL modifier; ICMP 451-P8 allele contributes to resistance

that pearl millet rust resistance is conferred by single dominant genes (individually named *Rpp1*, *Rr1*, *Rr2* and *Rr3*, respectively) and susceptibility by their recessive alleles. The major rust resistance gene mapped in the present study is also expected to be genetically dominant, although this was not tested. Further, unlike the Rr_1 gene reported by Hanna et al. (1985), it has proven durable, as it is still effective >20 years after its initial large-scale deployment in India in 1986 in dual-purpose pearl millet hybrid ICMH 451 (MH 179) = 81A × ICMP 451 (i.e., a commercial hybrid having the same nuclear genotype as the F1 from which the RIL population used in the present study was generated). This study will help to assess the role of this rust resistance locus in providing a framework for MAS and positional cloning of resistance genes in pearl millet.

The results obtained from the present study indicate that DArT provides high-quality markers that can be used to construct medium-density genetic linkage maps for plants even when no sequence information is available. The development of a reasonably well-saturated genetic linkage map of the RIL population could be useful for precise and fine QTL mapping as compared to earlier studies based on SSRs only. It is anticipated that this DArT array will also prove useful for background genotyping in marker-assisted back-crossing programs to speed up recovery of elite recurrent parent genetic backgrounds on genomic regions outside that targeted for introgression of donor parent alleles. The rust resistance locus identified on LG1 is a novel report (although its presence there was previously suspected, as it often accompanies marker-assisted introgression of a downy mildew resistance QTL that maps to the same chromosome arm of mapping population parent ICMP 451-P6, which was used as a donor in marker-assisted breeding of the male parent of pearl millet hybrid "HHB 67 Improved" (Hash et al. 2006) and will be useful for providing a framework for more effective MAS and cloning of such resistance genes. An additional advantage is that DArT clones can be sequenced readily and thus provide information for their conversion into PCR-based markers (Fiust et al. 2015). This can be advantageous in cases when there are not yet any inexpensively assayed markers closely flanking a potential target QTL that could be used in foreground selection for the favorable allele. In addition, in comparison with a DArT assay, the other highly parallel genotyping tool available is GBS and SNP. This approach has also been proven to be significantly efficient while not requiring any prior marker discovery work in the form of array development. Moumouni et al. (2015) demonstrated the usefulness of such a GBS approach to quickly produce a genetic map densely populated with SNP markers for pearl millet. They further reported that GBS can rapidly and efficiently provide high-quality, codominant SNP markers that can be used to construct densely populated genetic maps even in the absence of a reference genome, which will certainly be helpful for breeders and geneticists. The host plant resistance QTLs detected in the present study are likely to have longer economic life spans if deployed in heterogenous cultivars such as those created using a multiline approach (Witcombe and Hash, 2000; Hash and Witcombe 2002) or dynamic multiline approach (Wilson et al. 2001).

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