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GENETIC VARIABILITY AMONG THE BROWN RUST RESISTANT AND SUSCEPTIBLE GENOTYPES OF SUGARCANE BY RAPD TECHNIQUE

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Abstract

Brown leaf rust in sugarcane is caused by *Puccinia melanocephala* (Syd. & P. Syd.), which is a major cause of cultivar withdrawal. We attempted to analyze the RAPD diversity of two discrete phenotypic classes, i.e., rust resistant (R) and rust susceptible (S) of six commercially available sugarcane elite genotypes in Pakistan. DNA samples were extracted from these cultivars. The genomic DNA was amplified with 160 decamer primers and 33 of them generated 216 trackable loci with the range of 150-2500 bp. The total polymorphic bands were 164 (76%) and these were used to cluster rust resistant and susceptible cultivars. UPGMA (Unweighted Pair Group Method with Arithmetic Mean) based on Nei's genetic distance was analyzed by NTSYSpc and showed that 6 genotypes were clustered into 3 groups with the range from 63% to 73% genetic distances. This observation indicated the significant correlation between genetic distance and disease. Ten of 33 decamers (30%) had shown 100% polymorphism with three polymorphic bands/loci, which were generated in two different discrete groups rust resistant and rust susceptible. In this study we have found 17 cultivars specific loci generated by 11 RAPD primers and cluster analysis revealed three main groups and two subgroups against rust susceptible genotypes to develop characterization of genotypes at molecular level. Additionally, this explored the genetic basis of discrete phenotypic classes in sugarcane genome for marker development.

Introduction

Sugarcane (*Saccharum* spp. hybrids) is an important tropical crop and is cultivated over 105 countries in the world as a main source to get sugar. In recent years sugarcane is used to produce a bio-fuel ethanol as a renewable energy source. Sugarcane is cultivated in more than 20 million hectares in tropical and subtropical regions of world, producing up to 1.3 billion metric tonnes sugar. Among the 105 countries of the world growing sugarcane, Pakistan ranks 5th in area, 15th in production and 60th in yield. Although, Pakistan happens to be the world's 5th largest grower of sugarcane it has perhaps the lowest yield in the world. The area under sugarcane cultivation in Pakistan is estimated at 1 million ha (2.5 million acres), producing about 53.6 million metric tonnes of canes MY 010/2011 (Anon., 2009a, b; Anon., 2011).

The reason for the decrease and low sugarcane yield in Pakistan involves many factors, one of the major ones being crop disease. Among the diseases, the rust is quite common. The common rust of sugarcane, recently renamed brown rust, is a fungal disease caused by *Puccinia melanocephala* (Syd. & P. Syd.) and is present in almost all sugarcane-growing areas. An infection of 10% in an area resulted in loss of 7.78% cane weight and 3.96% sugarcane recovery in the 2006 cultivating season in Pakistan. The loss from 50% infection in an area was 77.81% in cane weight and 39.71% in sugarcane recovery during 2006 cultivating season (Hussnain & Afghan, 2006). Durability of the brown rust resistance in cultivars after they are released in agriculture is the major objective to control the brown rust effect on yield loss because epidemic property of this disease is effective to minimize the potential durability of resistance (Hoy & Hollier, 2009).

The genome of modern sugarcane cultivars is complex and highly polyploid (≈12X ≈120) and interspecific origin (D'Hont, 2005). The key characteristics of this genome are the high level of ploidy, the aneuploidy, the bispecific origin of the chromosomes, the existence of structural differences between chromosomes of the 2 origins, and the presence of interspecific chromosome recombinants. In addition, due to its genetic complexity, this species has received very little research investment despite its economic importance, and molecular resources have just recently begun to be developed (Grivet & Arruda, 2002). Molecular cytogenetics (Piperidis & D'Hont, 2001; Cuadrado *et al*., 2004) and genetic mapping studies (Grivet *et al*., 1996; Hoarau *et al*., 2001) showed that modern cultivars typically display 70–80% more complex crops with a large genome such as bread wheat (34 Gb/2C) or sugarcane (10 Gb/2C; D'Hont, 2005).

DNA based markers are valuable tools for a large number of applications such as genotype identification, population and pedigree analysis, the screening of segregating for linked markers, localization of a gene and improvement of plant varieties by marker-assisted selection (Fracaro *et al*., 2005; Khan *et al*., 2011). RAPD (Rapid Amplified Polymorphism) is the least expensive route for molecular marker development, which is based on the amplification of multiple random segment of genome using arbitrary primer. Decamer primers provide high polymorphism and can be used not only to differentiate between resistant and susceptible varieties but the genes responsible for disease resistance can also be located. The polymorphisms generated, function as genetic markers and can be used to construct genetic maps (Williams *et al*., 1990; Ghafoor *et al*., 2012). Quiros *et al*., (1991) have identified genome-specific markers in *Brassica* using RAPDs.

Because of size and complexity in DNA of sugarcane, it represents a major challenge to isolate agronomically important genes. In the present study, we performed the RAPD based assay to characterize and categorize the brown rust resistant and susceptible commercially growing cultivars by using arbitrary decamers primers. We also documented the genetic diversity of the response to brown rust resistance and susceptibility to the brown rust in sugarcane. This investigation on RAPD based DNA polymorphism provides an essential basis on which to plan future marker assistant selection for breeding programme.

Materials and Methods

Plant material: 6 genotypes of sugarcane were selected on the basis of disease resistance/susceptibility to rust (Table 1). All the data collection i.e., parentage brown rust rating about interested genotypes was conducted at Shakarganj Sugar Research Institute, Jhang. The rating system recommended by The International Society of Sugar Cane Technologists' (ISSCT) was used (Hutchinson & Daniels, 1972) as follows: 1-3, resistant; 4-6, intermediate; and 7-9, susceptible. Of 6 genotypes, we had made 2 discrete classes of trait Rust Resistant (R) and Rust Susceptible (S) to pool our interested gene.

Genomic DNA extraction: DNA was extracted from fresh leaves of six randomly selected plants of each experimental variety from the nursery grown in the field of SBS by the CTAB method (Doyle & Doyle, 1990). After purification, the quantity of DNA was estimated by measuring the absorbance at 260 nm using a spectrophotometer.

RAPD-PCR amplification: Genomic DNA was used for PCR amplification as described by Williams *et al*., (1990). A set of 160 arbitrary primers (S-series from Bio Basic Inc. and K, L and series M from Bio Neer) from different kits were used for the PCR amplification of extracted DNA. Amplification reactions were carried out in volumes of 25µl of the reaction mixture comprising 30ηg of genomic DNA as template DNA, 2.5µl of 10X PCR buffer, 2.5mM $MgCl_2$, 2mM dNTPs, 10pmol of primer and 1 unit of *Taq* DNA polymerase (Fermentas Life Sciences). DNA amplification was performed with Gene Amp (Applied Biosystems, PCR system 2700) for 45 cycles programmed as follows: 5.0 min at 94°C, 1 min at 37°C, 2 min at 72°C; followed by 44 cycles each of 1 min at 94°C, 1 min at 37°C, 2 min at 72°C and final extension cycle of 10 min at 72°C. The amplification products were resolved by electrophoresis on 1.5% agarose gel with 1X TAE , stained with ethidium bromide and visualized under UV light (Dolphin Gel Documentation system). The size of the amplicons was estimated from a 0.1 to 2.5 kb with DNA ladder mix (Fermentas cat# SM0403; Fermentas Life sciences). All the RAPD-PCR reactions were performed at least thrice, and only the consistently reproducible bands were taken into account.

Data analysis: The DNA fragment amplified by RAPD primers were characterized by size and intensity from all scorable bands (bp). All the data was recorded after scoring RAPD profiles the number of bands/DNA fragments as present (1) or absent (0) in our elite genotypes for cluster analysis. Those samples for which the DNA quality was insufficient for amplification were not included in the study. The data collected was used to estimate the similarity on the basis of the number of shared amplification products (Nei & Li, 1979**).** The similarity coefficients were utilized to generate dendrogram by using UPGMA (Unweighted Pair Group Method of Arithmetic means) through the programme, by NTSYSpc statistical software (version 2.02j) (Applied Biostatistics Inc.).

Table 1. Trait specific characteristic and parentage of sugarcane genotypes.

Genotype	Parentage	Distinctive characteristics
NSG-555	CP63-588 x Mo/F	R
Co-1148 (Commercial)	PoJ-2878 x Co-299	R
NSG-311	N19 x Mo	R
U-27	?	S
SPF-234	SP71-8210 x SP71-6180	S
CoJ-84	Co-1148x?	S

R=Resistant, S=Susceptible

Results

Six commercially growing genotypes were selected. All the selected genotypes were grouped into R and S on the basis of rust resistance and susceptibility (Table 1). One hundred and sixty RAPD makers were selected and applied against the genome of two pooled DNA samples of resistant and susceptible genotypes. Thirty three markers produced polymorphism between 20% to 100% and generated 216 detectable loci in our selected DNA samples with the mean of 6.5 loci per primer (Table 2). The number of amplified loci by each primer was 2 to 14, with the approximate range from 150 to 2500 bp. Primers S31 (GAATCGCCGT) and L-15 (AAGAGAGGGG) had amplified the least number of bands/loci i.e. 2, while S84 (AGCGTGTCTG) had amplified maximum number of bands/loci i.e. 14. The RAPD markers had shown 164 (76%) polymorphic loci and 52 (24%) monomorphic loci in all 6 cultivars. Several loci had also been observed specific to a single cultivar either resistant or susceptible (Table 2). Twenty five (75%) markers had 50 or more than 50 percent polymorphism but 13 decamers (39%) i.e., L09, L10, L11, L15, M08, M09, M10, M18, S23, S30, S31, S90 S93 had shown 100% polymorphism (Table 2).

Table 2. The detail of polymorphic and monomorphic bands produced by 33 RAPD primers in six sugarcane genotypes.

Primer names	Sequences	TB	MB	PB	PP	Band size range as bp
K19	CACAGGCGGA	6	3	3	50%	400-1100
K20	GTGTCGCGAG	8	1	7	88%	400-1500
L01	GGCATGACCT	10	3	7	70%	350-1800
L04	GACTGCACAC	7	1	6	86%	600-1500
L05	ACGCAGGCAC	4	1	3	75%	450-950
L06	GAGGGAAGAG	6	1	5	83%	850-2000
L07	AGGCGGGAAC	8	6	2	25%	250-1500
L08	AGCAGGTGGA	7	5	2	29%	300-1500
L09	TGCGAGAGTC	7	0	7	100%	500-2500
L10	TGGGAGATGG	8	0	8	100%	500-1200
L11	ACGATGAGCC	8	0	8	100%	400-1500
L12	GGGCGGTACT	9	1	8	89%	400-1500
L14	GTGACAGGCT	5	4	1	20%	450-1000
L15	AAGAGAGGGG	2	0	2	100%	750-800
M03	GGGGGATGAG	4	3	1	25%	700-1000
M04	GGCCGTTGTC	3	1	2	67%	500-1000
M05	GGGAACGTGT	8	4	4	50%	250-1100
M06	CTGGGCAACT	8	4	4	50%	200-1100
M07	CCGTGACTCA	7	3	4	57%	400-1200
M08	TCTGTTCCCC	5	0	5	100%	400-1300
M09	GTCTTGCGGA	6	0	6	100%	200-1000
M10	TCTGGCGCAC	3	0	3	100%	150-400
M12	GGGACGTTGG	4	2	2	50%	200-400
M18	CACCATCCGT	11	0	11	100%	200-2500
M20	AGGTCTTGGG	6	4	2	33%	200-1200
S12	CCTTGACGCA	4	2	2	50%	500-1200
S23	AGTCAGCCAC	7	0	7	100%	500-1200
S30	GTGATCGCAG	4	0	4	100%	200-700
S31	GAATCGCCGT	2	0	2	100%	800-900
S76	CACACTCCAG	12	1	11	92%	200-1500
S84	AGCGTGTCTG	14	2	12	86%	500-2500
S90	AGGGCCGTCT	8	0	8	100%	400-1600
S93	CTCTCCGCCA	5	0	5	100%	400-2200

Abbreviations: TB = Total Bands; MB = Monomorphic Bands; PB = Polymorphic Bands; PP = % of polymorphism.

Meanwhile the polymorphism and total number of bands were also observed in the sugarcane cultivars to see variable pattern of dominant RAPD loci (Table 3). Overall 474 (57.2%) polymorphic loci were produced by 33 decamers in 6 cultivars. The maximum number of loci (161) were amplified in NSG-311 with 105 (63.69%) polymorphic loci. More than 50% loci had been amplified by 33 RAPD primers in rust resistant group (R) with high polymorphism. On an average 5 polymorphic loci per primer were observed in 33 decamer primers against 6 sugarcane genotypes in this study (Table 3).

Table 3. Total Bands (TB), Polymorphic Bands (PB) and Percentage of Polymorphism (PP) in six genotype of sugarcane produced by 33 decamer primers.

Genotypes	TB	PB	PP	Per Primer
NSG-555	140	83	59.00	4.24
CO-1148	135	79	58.50	4.10
NSG-311	161	105	63.19	4.78
U-27	138	81	58.20	4.18
SPF-234	130	71	54.11	3.90
COJ-84	112	55	49.00	3.39
Overall Total	816	474	342	

The genotype specificity of primers was observed as shown in Table 4. The specificity of RAPD loci indicated that markers could be used to identify specific cultivar, resistance/susceptible against rust. Eleven RAPD marker had shown specificity with 4 cultivars i.e. NSG-311,U-27, CO-1148 and NSG-555 (Table 4) by producing 17 loci with the range of 200 bp to 2200 bp. Primers L01, L06, M04, M05, M09 and M10 were only having specificity with NSG-311 and produced nine detectible loci ranging between 200 bp and 1200 bp. S90 (AGGGCCGTCT) had shown very good specificity with NSG-555 by producing three loci with the range of 400-1600 bp while S93 (CTCTCCGCCA) had produced only one locus with size of 2200 bp in NSG-555. L10 (TGGGAGATGG) produced 2 specific loci (700 bp and 1000 bp) against CO-1148. Primer L08 (AGCAGGTGGA) had produced only one 1000 bp locu against rust susceptible genotype U-27. In the maximum number of genotype specific loci were produced against rust resistant cultivars while only one marker L08 had shown specificity with rust susceptible cultivar (Table 4).

Table 4. Genotype specificity of primers against rust resistant (R) and rust susceptible (S) genotypes.

Primers	Sequence	Bands (bp)	Specific to	R/S
L01	GTGTCGCGAG	1200	NSG-311	R
L06	GACTGCACAC	850	NSG-311	R
		1200	NSG-311	R
L08	AGCAGGTGGA	1000	U-27	S
L10	TGGGAGATGG	700	CO-1148	R
		1000	CO-1148	R
L14	GTGACAGGCT	900	CO-1148	R
M04	AGCCTGAGCC	500	NSG-311	R
M05	GGGGGATGAG	600	NSG-311	R
M09	GTCTTGCGGA	700	NSG-311	R
		1000	NSG-311	R
M10	TCTGGCGCAC	200	NSG-311	R
		350	NSG-311	R
S90	AGGGCCGTCT	400	NSG-555	R
		1000	NSG-555	R
		1600	NSG-555	R
S93	CTCTCCGCCA	2200	NSG-555	R

RAPD banding pattern were scored for the presence (scored 1) or absence (scored 0) of specific amplicon. Data were analyzed using NTSYSpc statistical software (version 2.02j; Applied Biostatistics Inc.). Based on the divergence matrix and using the neighbor-joining method (NJ), we constructed a dendrogram showing the differences among 6 objects (genotypes) as determined with 33 primers from 216 binary characters (loci) of the amplicon presence–absence pattern (Saitou & Nei, 1987). The Nei's (1978) genetic distance based on UPGMA (Unweighted Pair Group Method with Arithmetic Mean) classified 6 sugarcane genotypes into 3 major groups with 2 subgroups (Fig. 1).

The first major group comprised 2 rust resistant genotypes i.e. NSG-555. NSG-311 and Co-1148 also a resistant genotype was placed in II group. The third major cluster comprised 3 rust susceptible genotypes i.e. U-27, SPF-234 and COJ-84. Three major clusters were observed with genetic distance matrix. The IA group included the genotypes NSG-555 and NSG-311 with 71% genetic distance while the U-27 and SPF-234 were included in group IIIA with 73 % genetic distance (Fig. 1). But Coj-84 was included in group IIIB with 65.20% genetic distance. The genetic diversity among the sugarcane genotypes was showing different pattern according the relationship of genetic distances and polymorphism of RAPD markers.

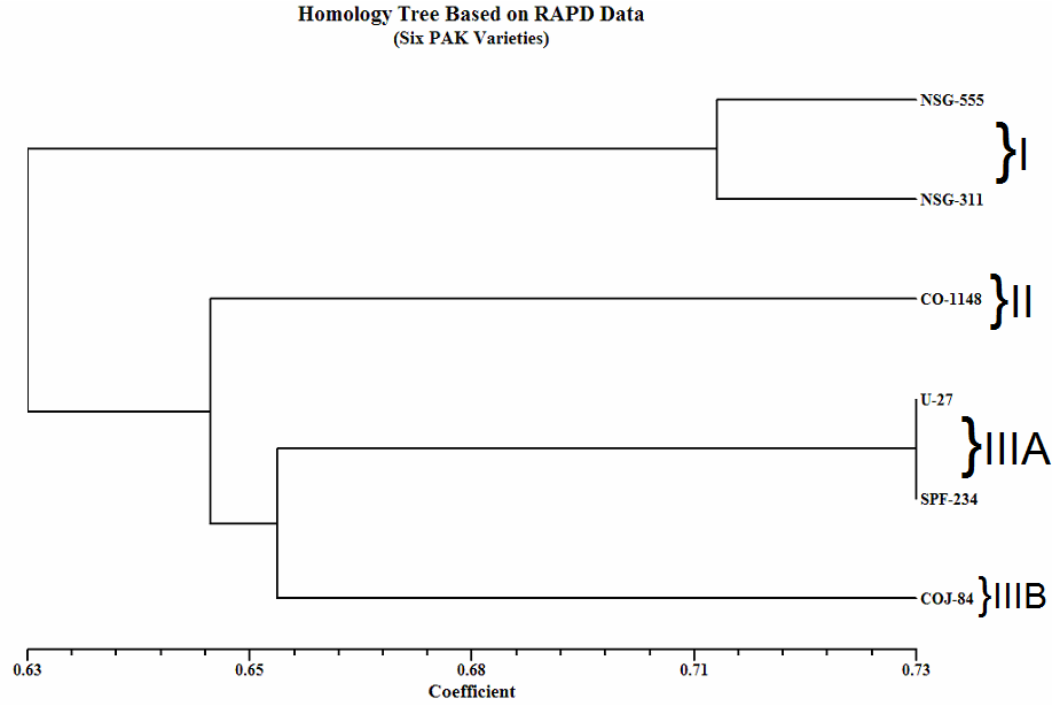

Fig. 1. Homology dendrogram constructed showing the genetic similarity among sugarcane genotypes by NTSYSpc based on Nei's (1978) identities/distances.

Discussion

Molecular markers offer a powerful tool for precise assessment of diversity and deriving phylogenetic relationships among different species and related genera. The main goal of this work was to determine the feasibility of using RAPD markers as a tool to estimate diversity in agronomical important traits i.e.. rust resistance or susceptibility in sugarcane genotypes. The RAPD markers were used effectively for determining the phylogenetic relationships among the sugarcane genotypes having 2 different pools of rust resistance and susceptibility.

In the present work, based on 33 markers, 164 RAPD loci had shown 76% polymorphism among 6 genotypes of sugarcane to reveal the genetic similarity and genetic distances among rust resistant and susceptible genotypes. The polymorphism per primer was detected from 3.39 to 4.78 with the mean of 4.1 among 6 genotypes of sugarcane. Kawar *et al*., (2009) while analyzing genetic relatedness among 17 sugarcane varieties showed 44.9% polymorphism. Fernandez *et al*., (1999) detected 77 (72.6%) polymorphic RAPD fragments in the sugarcane varieties of Cuba by using 18 decamers. The wide range of size (150-2500 bp) and 100% polymorphism of RAPD primers were studied as a comparable and detectable by many authors in sugarcane (Nair *et al*., 2002; Khan *et al*., 2009; Khan *et al*., 2011), blackgram (Ghafoor *et al*., 2012) and potato (Milbourne *et al*., 1997). Various studies indicated that specific linked loci to genotypes within same population can be used to identify commercially growing cultivars of sugarcane for breeding (Alvi *et al*., 2008; Kawar *et al*., 2009; Nawaz *et al*., 2010; Chen *et al*., 2012).

In the present study, the 11 RAPD markers were observed as a genotype specific with 17 polymorphic loci, that is a potential use of RAPD-PCR for identifying *Saccharum spp.* hybrids and clones (Pan *et al*., 2004). Lee *et al*., (1998) observed nine cultivars specific RAPD markers and concluded that RAPD can be applied to identify cultivars, study genetic relationship of cultivars and determine sweetness in watermelon. The sugarcane cultivars specific markers-fragments were reported by Kawar *et al*., (2009) for genetic relatedness among sugarcane genotypes. Barnes and Botha (1998) had identified 2 RAPD marker linked to rust resistance in sugarcane variety NCo-376 on the basis of reproducible polymorphism of loci in rust susceptible clones, while we had ten markers showing polymorphism in rust resistant genotypes and only one marker in rust susceptible genotypes. Many RAPD markers were identified in different crops linked to rust resistance for gene isolation and potential use in the breeding for crop improvement (Park *et al*., 2003; Mumtaz *et al*., 2009). Butterfield *et al*., (2003) reported that identification of DNA markers linked to particular phenotypes, and mapping of the set of markers was done in structured sugarcane populations consisted of either bi-parental crosses or selfed progeny. Raboin *et al*., (2003) determined a quantitative Trait Locus (QTL) mapping to analyze sugarcane smut resistance. One thousand and 300 polymorphic AFLP markers were generated on a population of 200 individuals derived from a cross between R570 (resistant) and MQ 76/53 (highly susceptible).

Our study has further revealed that genetic diversity varied among 6 hybrid varieties and ranged from 63% to 73% which is commonly measured by genetic distances or genetic similarity. This study suggested that there was very limited genetic diversity within this group rust resistant (R) and rust susceptible, especially as the calculated values were skewed towards maximum variation. From the above mentioned results, it is concluded that high value of genetic similarity reflects that genotypes were from the similar genetic parentage. Alvi *et al*., (2008) reported genetic similarity ranging between 67.15 to 83.25% among the Pakistan commercially growing sugarcane genotypes towards red rot and Pan *et al*., (2004) also arrived at the same conclusion for *Saccharum spontaneum* and elite accessions.

In this study, the cluster analysis among the 6 genotypes showed noteworthy relationship with a specific character (Rust resistance and Susceptibility). Franco *et al*., (2001) revealed the possibility to obtain a relevant marker-fragment (loci) that can be used to compare only the continuous or only the discrete variable to discriminate the genotypes. Selvi *et al*., (2003) concluded that success of a breeding programme depends on the understanding of the extent of variation existing in the available gene pool.

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