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ORIGINAL ARTICLE

Molecular analysis of mutant *granule-bound starch synthase-I* (*waxy1*) gene in diverse waxy maize inbreds

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Abstract

Waxy corn is popular beacuse of its high amylopectin due to mutation in *granule-bound starch synthase-I* or *Waxy1* (*Wx1*) gene. Here, we characterized the *wx1* allele among 24 diverse waxy inbreds using gene-based markers. A total of 29 alleles with average of 1.81 alleles/locus were observed. Major allele frequency varied from 0.42 to 1.00, with mean of 0.74. The polymorphism information content ranged from 0.00 to 0.56 (average 0.24). Three simple sequence repeat markers, viz., *phi027, phi022* and *phi061* were more polymorphic in the study. The mean heterozygosity was 0.04, which indicated attainment of higher levels of homozygosity. Dissimilarity coefficient varied from 0.00 to 0.90 with average of 0.51. Seventeen diverse haplotypes of *wx1* allele were observed that was consistent with the pedigree. Cluster analyses grouped 24 genotypes into two main clusters each having sub-clusters. The information generated here possesses great potential for improvement of high amylopectin in maize through marker-assisted selection. This is the first report of molecular dissection of *wx1* gene among the novel waxy inbreds developed in India.

Keywords Amylopectin · Characterization · Haplotypes · Maize · Waxy inbreds

Introduction

Waxy maize possesses 95–100% of amylopectin compared to 70–75% in normal maize and has been abundantly used for cultivar development (Zhou et al. 2016). Waxy maize, also called as 'sticky maize' is a popular choice as food in China and other South Asian countries (Xiaoyang et al. 2017). It is also popular in North-Eastern parts of India as food prepared from waxy maize grains is widely preferred by the local people. Food prepared from waxy grains is easily digestible in human gut as compared to normal maize with higher amylose fractions (Fukunaga et al. 2002). Immature green ears of waxy maize are also gaining popularity as a breakfast item worldwide. Further, high viscosity of amylopectin makes the starch of waxy grains suitable for adhesive, paper and textile industries (Bao et al. 2012; Devi et al. 2017). Therefore, waxy maize possesses great potential as a high value crop (Tian et al. 2009).

Waxy maize was first discovered in 1909 at Yunnan–Guangxi region of China and subsequently disseminated to other Asian countries (Zheng et al. 2013). The coding region of *Waxy1* (*Wx1*) gene (located on chromosome 9) consists of 14 exons spanning 3718 bp (Zheng et al. 2013). In the endosperm, *Wx1* codes for the *granule-bound starch synthase-I* (GBSS-I), which catalyses amylose biosynthesis from ADP-glucose (Klosgen et al. 1986; Mason-Gamer et al. 1998). However, the mutant version which acts as a recessive allele (*wx1*) suppresses the action of GBSS-I that shifts ADP-glucose towards synthesis of amylopectin (Tsai 1974; Wessler et al. 1986; Bao et al. 2012; Zhang et al. 2013). The visual appearance of mutant waxy endosperm is distinct from normal maize endosperm, and can be easily identified through its cloudy appearance.

Waxy germplasm especially from China, Vietnam and Korea has been well characterized using simple sequence repeats (SSRs) distributed throughout the genome (Park et al. 2008; Hung et al. 2012; Zheng et al. 2013). However, molecular characterization of the waxy locus provides more useful information on variation of recessive *wx1*

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alleles present in the diverse germplasm. Fan et al. (2008) studied nucleotide diversity at 9–14 exons of waxy locus, while Zheng et al. (2013) analysed sequence variation at 8–14 exons of the *wx1* gene. So far, no study has been carried out to analyse the sequence variation of the entire *wx1* gene, primarily due to involvement of considerable cost in sequencing. Shin et al. (2006) used only four single-nucleotide amplified polymorphism (SNAP) markers that led to the identification of four SNPs at waxy locus. In the present study, we used gene-based markers to assess the genetic variation in the entire length of *wx1* locus in a panel of 24 diverse waxy inbreds developed at ICAR-Indian Agricultural Research Institute (IARI), New Delhi. The aims of present investigation were (1) to analyse the sequence variations in *wx1* allele present in diverse waxy inbreds; (2) to identify haplotype patterns of *wx1* allele, and (3) to study the genetic relationships among inbreds based on the polymorphism at *wx1* locus.

Materials and methods

Panel of waxy maize inbreds

The pedigree details of waxy inbreds (MGUWX-101 to 124) developed at ICAR-IARI, New Delhi, are described in Table 1. Selection from the populations—and introgression—of *wx1* strategy were employed for development of the waxy inbreds. Among the waxy inbreds, 15 were of white kernel colour, while nine inbreds of yellow kernel colour. The purity of inbreds was conserved through manual selfing.

Extraction of genomic DNA

Genomic DNA was isolated from the seeds using sodium dodecyl sulphate (SDS) extraction protocol (Dellaporta et al. 1983) and quality was checked on 0.8% agarose gel. The extracted DNA was quantified on UV-spectrophotometer (BT-UVS-SBA-E, G-Biosciences).

Table 1 Pedigree information of waxy inbred lines used in the study

S. No.	Genotype	Kernel colour	Pedigree
1.	MGUWX-101	Yellow	[{(K027 LOS BANOS (Waxy) × POOL 15 C35 TEWF)-2-⊗-⊗} × VQL1]-BC_1-5-⊗
2.	MGUWX-102	White	[{(K027 LOS BANOS (Waxy) × POOL 15 C35 TEWF]-2-⊗-⊗} × VQL1] -BC_1-2-⊗
3.	MGUWX-103	Yellow	[{K022 GLUTINOUS DMR COMP# 41-4 (Waxy) × POOL 15C35 TEWF}-5-⊗-⊗-1-⊗ × VQL2]-BC_1-11-⊗
4.	MGUWX-104	White	[{K022 GLUTINOUS DMR COMP# 41-4 (Waxy) × POOL 15C35 TEWF}-5-⊗-⊗-1-⊗ × VQL2] -BC_1-3-⊗
5.	MGUWX-105	Yellow	[{K011 (Waxy) × POOL 15 C35}-2-⊗-× V351]-F_2-34-⊗
6.	MGUWX-106	White	[{K022 GLUTINOUS DMR COMP# 41-4 (Waxy) × POOL 15C35 TEWF}-8-1-× BML10]- F_2-66-⊗
7.	MGUWX-107	Yellow	[(ARZM 07 141)-2-× V340]- F_2-15-⊗
8.	MGUWX-108	White	[(ARZM 07 141)-2-× V340]- F_2-34-⊗
9.	MGUWX-109	Yellow	[{K078 No. 34 (Waxy) × POOL 15 C35 TEWF]-3-1- × V345]- F_2-21-⊗
10.	MGUWX-110	Yellow	[{K078 No. 34 (Waxy) × POOL 15 C35 TEWF]-3-1- × V345]- F_2-43-⊗
11.	MGUWX-111	Yellow	[{K022 GLUTINOUS DMR COMP# 41-4 (Waxy) × POOL 15C35 TEWF}-5- × CM139]- F_2-30-⊗
12.	MGUWX-112	Yellow	[{K022 GLUTINOUS DMR COMP# 41-4 (Waxy) × POOL 15C35 TEWF}-5- × CM139]- F_2-4-⊗
13.	MGUWX-113	Yellow	[{K022 GLUTINOUS DMR COMP# 41-4 (Waxy) × POOL 15C35 TEWF}-8-1- × CM143]- F_2-27-⊗
14.	MGUWX-114	White	[{K022 GLUTINOUS DMR COMP# 41-4 (Waxy) × POOL 15C35 TEWF}-8-1-× CM143]- F_2-52-⊗
15.	MGUWX-115	White	[K078 No. 34 (Waxy) × POOL 15 C35 TEWF]-1-⊗
16.	MGUWX-116	White	[K027 LOS BANOS (Waxy) × POOL 15 C35 TEWF]-2-⊗
17.	MGUWX-117	White	[K021 PHILIPPINES GLUTINOUS SYN #20 (Waxy) × POOL15 C35]-4-⊗
18.	MGUWX-118	White	[K021 PHILIPPINES GLUTINOUS DMR COM # 41-4 (Waxy) × POL 23 C34 TLWF]-1-⊗
19.	MGUWX-119	White	[K011 (Waxy) × POOL 15 C35]-4-⊗-⊗-1-⊗
20.	MGUWX-120	White	[K027 LOS BANOS (Waxy) × POOL 15 C35 TEWF]-7-1-⊗-1-⊗
21.	MGUWX-121	White	[K027 LOS BANOS (Waxy) × POOL 15 C35 TEWF]-5-1-⊗
22.	MGUWX-122	White	[K027 LOS BANOS (Waxy) × POOL 15 C35 TEWF]-3-⊗
23.	MGUWX-123	White	[K022 GLUTINOUS DMR COMP# 41-4 (Waxy) × POOL 15C35 TEWF]-8-1-⊗-2-⊗
24.	MGUWX-124	White	[{K078 No. 34 (Waxy) × POOL 15 C35 TEWF}-3-1- × V345]- F_2-9-⊗

Primers used in the study

Markers designed by Shin et al. (2006), Liu et al. (2007) and Bao et al. (2012) were used to cover the major portion of *Wx1* gene, (Table 2). Fourteen primers were selected from the studies of Shin et al. (2006), Liu et al. (2007) and Bao et al. (2012), and three *Wx1*-based SSRs retrieved from maize genome database (http://www.maizegdb.org) were also used in the present study. Primer binding sites of selected markers in the *Wx1* gene are presented in Fig. 1. The purified and lyophilized form of oligonucleotide primers was synthesized from M/s. Macrogen. Final primer concentration of 10 µM in Milli-Q water was utilized.

Genotyping of *waxy1* locus

The PCR reactions of final volume 20 µl consisting of 1X One PCR™ mix (Ready-to-use PCR mix, Gene Direx), 50 ng of template DNA, 0.5 µM of forward and reverse primers, were performed using Applied Biosystems' Veriti96-thermal cycler. The amplification protocols were standardized for each of the primer-pairs as the size of amplicons varied from 70 to 1150 bp. Denaturation at 95 °C/5 min, 35 cycles consisting of denaturation at 95 °C/45 s, primer annealing ranged between 55 and 62 °C/45 s, primer extension at 72 °C/45–90 s and final extension at 72 °C/8 min was followed depending upon the amplicon size. The separation of PCR amplicons was performed using 4.0% agarose gel (Lonza, Rockland, ME USA).

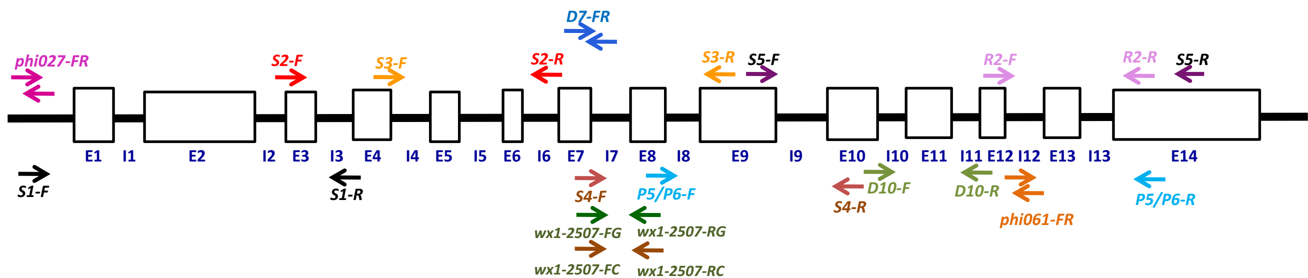

Fig. 1 *Waxy1* gene structure with 14 exons depicting locations of selected primer pairs, E: exon and I: intron, 14 exons depicted as white boxes, F: forward, R: reverse, single primer pair is depicted with same colour

Table 2 Details of primers used in genotyping assay of 24 waxy mutants

S. No.	Marker	References	Position (start–end)	Exon (E)/intron (I)
1.	*R2*	Bao et al. (2012)	3829–4532	E12–E14
2.	*D7*	Bao et al. (2012)	2131–NA	At the junction of exon 7–intron 7
3.	*D10*	Bao et al. (2012)	3223–3848	E10–E12
4.	*phi027*	http://www.maizegdb.org	217–374	I1 (regulatory factor binding site-GC stretch)
5.	*phi022*	http://www.maizegdb.org	NA	NA
6.	*phi061*	http://www.maizegdb.org	3877–3954	E12–I12
7.	*S1FR*	Bao et al. (2012)	209–1880	Upto E4
8.	*S2FR*	Bao et al. (2012)	1592–2498	I2–E7
9.	*S3FR*	Bao et al. (2012)	1932–2962	E4–E9
10.	*S4FR*	Bao et al. (2012)	2478–3379	E7–E10
11.	*S5FR*	Bao et al. (2012)	3066–4463	E9–E14
12.	*P3/P4*	Liu et al. (2007)	NA	NA
13.	*P1/P2*	Liu et al. (2007)	NA	NA
14.	*P5/P6*	Liu et al. (2007)	2651–4258	E8–E14
15.	*S1/S2*	Liu et al. (2007)	NA	NA
16.	*wx1-2507-F/RC*	Shin et al. (2006)	2467–2745	E7–E8
17.	*wx1-2507-F/RG*	Shin et al. (2006)	2467–2745	E7–E8

NA not available

Analysis of molecular data

The various genetic parameters (polymorphism information content, gene diversity, heterozygosity, total number of alleles, major allele frequency) were estimated by PowerMarker V3.0 (Liu and Muse 2005). DARwin-6.0 software (Perrier et al. 2003) was used for hierarchical clustering-based dendrogram construction. Jaccard's coefficient was used to calculate dissimilarity matrix. The principal coordinate analysis (PCoA) was also performed to supplement the clustering pattern to further illustrate the diversity of the inbreds (Perrier et al. 2003).

Results and discussion

Genetic variation in *wx1* locus

Of the 17 markers employed in the study, S2FR marker showed monomorphic pattern across the genotypes and therefore was not considered for data analysis. A total of 29 alleles within *wx1* locus were detected among the 24 waxy maize inbreds, with mean of 1.81 alleles/locus (Table 3). Number of alleles/locus ranged from 1 to 3 among the inbreds. In contrast, higher numbers of alleles were identified in studies where waxy inbreds were characterized utilizing markers spread across the maize genome and not linked to *wx1*. For example, Yu et al. (2012) reported 60 alleles with a mean of 2.73 alleles/locus and a range of 2–4 alleles/locus. Similarly, Park et al. (2008), Hung et al. (2012) and Zheng et al. (2013) reported much higher values studying genetic characterization of waxy maize genotypes, viz., 127, 117 and 104 alleles; 4.20 alleles/locus, 3.26 alleles/locus and 5.20 alleles/locus; and range of 2–9 alleles/locus, 1–6 alleles/locus and 2–8 alleles/locus, respectively. Markers in non-coding region tend to show more polymorphism, as they are not likely to affect the fitness of the organism. The extent of polymorphism within any specific gene is low, as nucleotide variation affecting the fitness is generally lost and only beneficial or neutral mutations are retained in the populations. In the quality protein maize (QPM) inbreds, Babu et al. (2012a) also reported higher number of alleles/locus (3.35) using genome-based SSRs, while it was low (2.75 alleles/ locus) for lysine- and tryptophan-biosynthesis pathway-specific candidate gene-based SSR analyses (Babu et al. 2012b). Among the 16 polymorphic markers identified in the study, five markers [*S1FR* (1130 bp), *P1/P2* (1070 bp), *P5/P6* (1110 bp), *S1/S2* (450 bp) and *wx1-2507-F/RC* (300 bp)] showed presence–absence polymorphism, eight markers showed co-dominant behaviour, while 3 markers exhibited both dominant and co-dominance pattern (Table 3). Two alleles were revealed by nine markers while three alleles were amplified with two markers (Fig. 2). Shin et al. (2006) analysed the diversity of *wx1* gene using single nucleotide-amplified polymorphic (SNAP) markers, and showed allele-specific polymorphisms within waxy locus in maize. It is well established that molecular structure of waxy locus in rice and foxtail millet possesses co-linearity with that of maize (Fukunaga et al. 2002). Van et al. (2008) has reported 17 single nucleotide polymorphic (SNP) markers in non-coding and three SNPs in coding region of waxy locus of foxtail millet. Nucleotide variation in waxy locus of rice has also been reported by Chrungoo and Devi (2016). However, in our study, of the 16 polymorphic markers, three were SSRs which produced 2.67 alleles/locus compared to 1.61 alleles/locus for the rest 13 markers. The high level of allelic polymorphism detected by SSRs is possibly due to reasons like recombination errors, unequal crossing over and replication slippage at the SSR locus (Tautz and Schlotterer 1994).

The major allele frequency varied from 0.42 (*phi027*) to 1.00 (*S1FR, P1/P2, P5/P6, S1/S2, wx1-2507-F/RC*) with mean of 0.74 (Table 3). The range for gene diversity was from 0.00 (*S1FR,P1/P2, P5/P6, S1/S2, wx1-2507-F/RC*) to 0.64 (*phi061*), while the mean was 0.30. Polymorphism information content (PIC) among the markers varied from 0.00 (*S1FR, P1/P2, P5/P6, S1/S2, wx1-2507-F/RC*) to 0.56 (*phi061*) with an average of 0.24 (Table 3). Eight markers exhibited PIC > 0.30. The mean PIC of markers present within *wx1* locus is lower than PIC of genome-based SSRs observed in waxy germplasm. For example, Hung et al. (2012) and Hao et al. (2015) reported PIC of 0.46 and 0.31 in waxy germplasm, respectively. The higher PIC values, 0.70 and 0.62 among waxy maize germplasm were observed by Zheng et al. (2013) and Sa et al. (2015), respectively. Low PIC is an indicator of lower diversity of the gene. This may be due to beneficial nucleotide variation fixed through strong positive selection for waxy locus. Whitt et al. (2002) also found low level of nucleotide diversity or 'selective sweep' at *wx1* locus in maize. Similar observation in *wx1* locus was also reported by Fan et al. (2008) where 25 Chinese glutinous maize accessions were characterized. Their results suggested that a genomic region of 53 kb or longer, was affected by selective sweep in the waxy maize germplasm. The phenomenon of selective sweep has also been described in *teosinte branched1* (*tb1*) and *yellow1* (*y1*) genes in maize (Wang et al. 1999; Palaisa et al. 2004). However, PIC among SSRs ranged from 0.37 to 0.56, compared to 0.00 to 0.38 in other markers, thereby suggesting the hyper-variability of SSR locus.

The lower heterozygosity (range 0.00–0.23; mean 0.04) among the set of inbreds indicates that the repeated selfing of inbreds has led to higher degree of homozygosity (Table 3). However, some primers, viz., *R2* (0.23), *D7* (0.23), *D10* (0.13), *phi022* (0.04) and *wx-2507-F/RG* (0.04) detected heterozygosity among the 24 waxy inbreds. Since maize is a highly cross-pollinated crop, residual heterozygosity at few

S. No.	Marker (Allele Size)	MGUWX-101	MGUWX-102	MGUWX-103	MGUWX-104	MGUWX-105	MGUWX-106	MGUWX-107	MGUWX-108	MGUWX-109	MGUWX-110	MGUWX-111	MGUWX-112	MGUWX-113	MGUWX-114	MGUWX-115	MGUWX-116	MGUWX-117	MGUWX-118	MGUWX-119	MGUWX-120	MGUWX-121	MGUWX-122	MGUWX-123	MGUWX-124
1	*R2 (700)*	■	■	□	□	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
2	*R2 (420)*	□	□	□	□	□	□	□	□	■	■	□	□	□	□	■	■	□	■	□	□	□	□	□	□
3	*D7 (400)*	■	■	■	■	■	■	■	■	□	□	■	■	■	■	■	■	■	■	■	■	■	■	■	■
4	*D7 (360)*	□	□	□	□	□	□	□	□	□	□	■	■	□	□	■	■	□	■	□	□	□	□	□	□
5	*D10 (650)*	□	□	□	□	□	■	□	□	□	□	□	□	■	■	■	□	□	□	□	□	□	□	■	□
6	*D10 (640)*	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	■
7	*phi027 (160)*	■	■	□	□	■	□	□	□	□	□	□	□	□	□	□	■	□	□	□	□	□	□	□	□
8	*phi027 (150)*	□	□	■	■	□	■	□	□	□	□	■	■	■	■	■	□	□	■	□	□	□	□	■	□
9	*phi027 (140)*	□	□	□	□	□	□	■	■	■	■	□	□	□	□	□	□	■	□	■	■	■	■	□	■
10	*phi022 (180)*	□	□	■	■	□	□	■	■	■	■	□	□	□	□	□	□	■	□	■	■	■	■	□	■
11	*phi022 (140)*	■	■	□	□	■	■	□	□	□	□	■	■	■	■	■	■	■	■	□	□	□	□	■	□
12	*phi061 (90)*	■	■	□	□	□	□	□	□	□	□	■	■	■	■	■	■	■	■	■	□	□	□	□	□
13	*phi061 (80)*	□	□	□	□	■	■	□	□	■	■	□	□	□	□	□	□	□	□	□	■	■	■	■	□
14	*phi061(70)*	□	□	■	■	□	□	■	■	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	■
15	*S1 (1130)*	■	■	■	■	■	■	□	□	□	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
16	*S3 (1030)*	□	□	■	■	□	□	□	□	■	■	□	□	□	□	□	□	■	□	■	■	■	■	■	■
17	*S3 (1020)*	■	■	□	□	■	■	■	■	□	□	■	■	■	■	■	■	□	■	□	□	□	□	□	□
18	*S4 (950)*	□	□	■	■	□	□	■	■	■	■	□	□	□	□	□	□	■	□	■	■	■	■	□	■
19	*S4 (900)*	■	■	□	□	■	■	□	□	□	□	■	■	■	■	■	■	□	■	□	□	□	□	■	□
20	*S5 (1150)*	■	■	■	■	■	■	□	□	■	■	□	□	□	□	□	□	□	□	■	■	■	■	□	■
21	*S5 (1100)*	□	□	■	■	□	□	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
22	*P3/P4 (1020)*	□	□	■	■	□	□	■	■	■	■	□	□	□	□	■	□	■	□	■	■	■	■	□	■
23	*P3/P4 (1000)*	■	■	□	□	■	■	□	□	□	□	■	■	■	■	■	■	□	■	□	□	□	□	■	□
24	*P1/P2 (1070)*	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	■	■	■	■	■	■
25	*P5/P6 (1110)*	■	■	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■	□	■	■	■	■	■
26	*S1/S2 (450)*	□	□	■	■	■	■	■	■	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■
27	*wx1-2507-F/RC (300)*	□	□	□	□	□	□	■	■	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□
28	*wx1-2507-F/RG (300)*	□	□	■	■	□	□	□	□	□	■	□	□	□	□	□	□	■	□	■	■	■	■	□	■
29	*wx1-2507-F/RG (250)*	■	■	□	□	■	■	□	□	□	□	■	■	■	■	■	■	□	■	□	□	□	□	■	□

Fig. 2 Haplotype of *wx1* alleles using 16 markers; each column represents the waxy line and rows represent the allele for a given marker, black box colour: presence of DNA band, white box colour: absence of DNA band

loci remains and continues to perpetuate as breeders select lines based on morphological uniformity. This could be the possibility of heterozygosity at *wx1* gene, although waxy phenotype in different lines may look similar. Several maize researchers have reported residual heterozygosity in maize inbreds despite of continuous inbreeding (Choudhary et al. 2015; Muthusamy et al. 2015; Zunjare et al. 2015; Mehta et al. 2017). Complete homozygosity across the genome is achieved through doubled haploid technique which requires 1–2 generations compared to 6–7 generations of selfing in conventional inbred development programme (Choudhary et al. 2015).

Haplotype variation in *wx1* locus

A total of 17 haplotypes of recessive *wx1* gene were detected among the 24 inbreds using 16 gene-based markers (Fig. 2). MGUWX-120, -121, -122 possessed same haplotype of waxy locus inherited from the parent germplasm, K027LOSBANOS (waxy). Similarly, (1) MGUWX-101 and MGUWX-102; (2) MGUWX-111 and MGUWX-112; (3) MGUWX-113 and MGUWX-114; (4) MGUWX-107 and MGUWX-108, and (5) MGUWX-103 and MGUWX-104, also had the same haplotype. Shin et al. (2006) working with 40 waxy accessions identified two haplotypes for *wx1* allele using four SNAP markers. While three and four haplotypes in wild-type *Wx1* gene were observed in 26 dent and 15 sweet corn accessions, respectively. Van et al. (2008) reported 23 SNP haplotypes in waxy locus in a set of 113 landraces of foxtail millet adapted in Korea. The haplotype patterns generated here, serve as the tool for the identification of specific *wx1* alleles present in the waxy germplasm. The expression analyses of diverse *wx1* would help in identifying the most desirable allele as it would possess the highest amylopectin. A combination of markers specific to a haplotype of the most desirable allele can be

Table 3 Summary statistics of genotyping assay of 24 inbred lines

S. No.	Primer	Major allele frequency	Number of alleles	Gene diversity	Heterozygosity	PIC	Type of the marker
1.	*R2*	0.89	2.00	0.20	0.23	0.18	Dominant/co-dominant
2.	*D7*	0.89	2.00	0.20	0.23	0.18	Dominant/co-dominant
3.	*D10*	0.85	2.00	0.25	0.13	0.22	Co-dominant
4.	*phi027*	0.42	3.00	0.63	0.00	0.55	Co-dominant
5.	*phi022*	0.52	2.00	0.50	0.04	0.37	Co-dominant
6.	*phi061*	0.46	3.00	0.64	0.00	0.56	Co-dominant
7.	*S1FR*	1.00	1.00	0.00	0.00	0.00	Dominant
8.	*S3FR*	0.54	2.00	0.50	0.00	0.37	Co-dominant
9.	*S4FR*	0.50	2.00	0.50	0.00	0.38	Co-dominant
10.	*S5FR*	0.73	2.00	0.39	0.00	0.31	Co-dominant
11.	*P3/P4*	0.52	2.00	0.50	0.00	0.37	Co-dominant
12.	*P1/P2*	1.00	1.00	0.00	0.00	0.00	Dominant
13.	*P5/P6*	1.00	1.00	0.00	0.00	0.00	Dominant
14.	*S1/S2*	1.00	1.00	0.00	0.00	0.00	Dominant
15.	*wx1-2507-F/RC*	1.00	1.00	0.00	0.00	0.00	Dominant
16.	*wx1-2507-F/RG*	0.57	2.00	0.49	0.00	0.37	Dominant/co-dominant
	Mean	0.74	1.81	0.30	0.04	0.24	–
	Range	0.42–1.00	1.00–3.00	0.00–0.64	0.00–0.23	0.00–0.56	–

used in the marker-assisted breeding programme. Marker-based on single polymorphism is having less probability to be polymorphic between recipient and donor, than based on combination of markers. The haplotypes are the result of accumulation of different mutation types, viz., insertion of transposable elements, retroposons and fragments of few nucleotides and deletion of nucleotides in the *wx1* allele (Bao et al. 2012; Devi et al. 2017). Reports suggest different types of spontaneous mutations including addition of transposable elements and few nucleotides into genic and intergenic regions of *wx1* locus exist in maize germplasm. This may lead to (1) disruption of coding regions; (2) modified transcripts; (3) premature termination of polypeptide due to occurence of stop codon or alteration of amino acids in protein domain, and (4) alternative splicing and/or decreased expression of *wx1* allele (Wessler and Varagona 1985; Okagaki et al. 1991; Marillonnet and Wessler 1997; Liu et al. 2007; Tian et al. 2008; Ding et al. 2009).

Relationships among waxy inbreds

Genetic dissimilarity among the waxy inbreds ranged from 0.00 to 0.90 with an average of 0.51. Cluster diagram grouped 24 genotypes into two major clusters, viz., – A and – B (Fig. 3, Table S1). The genetic dissimilarity comparisons of the 276 pairs revealed presence of genetic variation in waxy locus. 23% of total pair of inbreds belonged to 0.70–0.79 dissimilarity thereby suggesting higher levels of genetic divergence among those inbreds (Fig. 4). Cluster-A had 12 genotypes with two sub-clusters (– A1 and – A2). Sub-cluster-A1 comprised 10 inbreds, viz., MGUWX-122, MGUWX-121, MGUWX-120, MGUWX-124, MGUWX-119, MGUWX-117, MGUWX-104, MGUWX-103, MGUWX-110, and MGUWX-109. Sub-cluster-A2 possessed only two inbreds, MGUWX-106 and MGUWX-107. Cluster-B had 12 genotypes with two sub-clusters, viz., -B1 and -B2 (Fig. 3). The B1 sub-cluster consisted of 10 waxy inbreds, viz., MGUWX-114, MGUWX-113, MGUWX-115, MGUWX-112, MGUWX-111, MGUWX-118, MGUWX-116, MGUWX-102, MGUWX-101, and MGUWX-105; while sub-cluster-B2 had only two inbreds (MGUWX-123 and MGUWX-106). Shin et al. (2006) analysed 81 maize accessions including 40 waxy types from South Korea using SNAP markers present in *wx1* and other genes, viz., *shrunken2 (sh2), brittle2 (bt2), sugary1 (su1) and amylose extender (ae1)*. Waxy germplasm existed in clusters I–I, I–II, II–I and II–II, and generally clustered away from the dent and sweet corn accessions. Fan et al. (2008) also analysed 25 Chinese waxy accessions along with normal maize and wild maize using *wx1*-based markers, and observed close relationship between waxy genotypes with cultivated maize but diverse from *Coix lacryma-jobi*. Their results suggested that domestication of cultivated maize from wild species occurred first followed by selection of *Wx1* gene for glutinous characteristics. The clustering pattern observed in the study was also supported by PCoA (Fig. 5). The PCoA revealed genetic diversity for waxy locus as the inbreds were spread across the quadrangles.

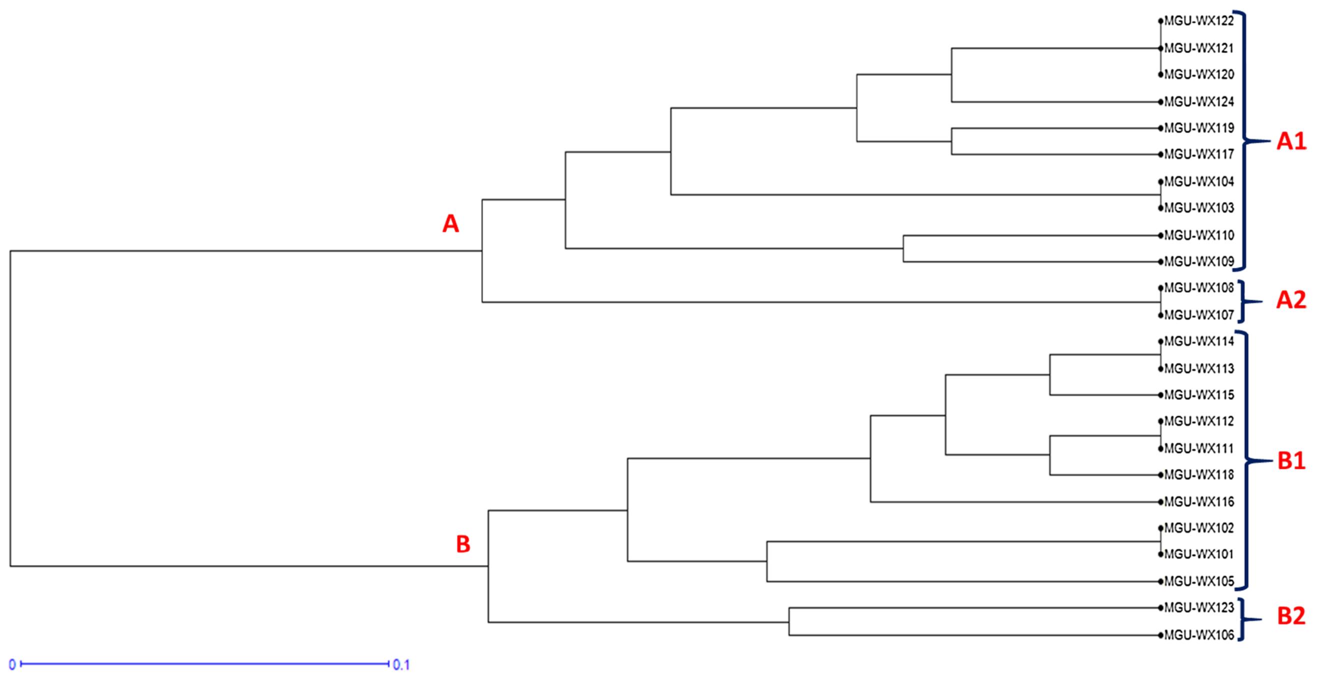

Fig. 3 Cluster diagram of 24 waxy maize inbreds based on waxy1 gene-based markers, A and B: two major clusters

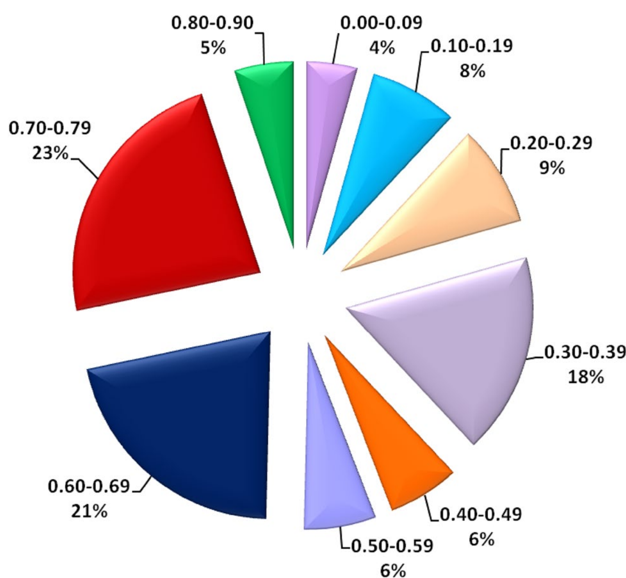

Fig. 4 Percent distribution of the genetic dissimilarity coefficient calculated between pair of inbreds. Different colour indicates different class intervals of genetic dissimilarity

Utilization of waxy inbreds in breeding programme

In a conventional waxy breeding programme, breeders rely upon presence of cloudy phenotypic expression in the endosperm for developing the waxy inbreds. Generally, breeders consider that waxy trait in the germplasm is due to one *wx1* allele and various haplotypes of *wx1* present in different germplasms. The study revealed that there are 17 haplotypes of *wx1* allele which provides great advantage in the breeding programme. Among the 17 markers used, three markers, viz., *R2, D7* and *wx1-2507-F/RG* showed both co-dominant as well as dominant nature, eight markers, viz., *D10, phi022, phi027, phi061, S3FR, S4FR, S5FR* and *P3/P4* behaved as co-dominant marker, while, five markers, viz., *S1FR, P1/P2, P5/P6, S1/S2* and *wx1-2507-F/RC* exhibited only dominant behaviour (Table 3). The co-dominance is due to insertion and deletion of nucleotide(s) within the amplicon generated by the marker, and is an ideal choice in marker-assisted selection (MAS), as it can differentiate the homozygotes from heterozygotes (Hossain et al. 2018). In a situation, where a normal maize hybrid is to be improved for amylopectin content, the parental lines can be introgressed with *wx1* allele using marker-assisted backcross breeding (MABB) (Sarika et al. 2018). MABB is an accelerated method of breeding and requires only two generations of backcrossing compared to 6–7 generations in conventional methods (Muthusamy et al. 2014; Zunjare et al. 2018). As a first step, the recurrent parents and waxy donors should show polymorphism between *Wx1* and *wx1* alleles. Of the 16 gene-based markers, the ones that differentiate the *Wx1* (from recurrent parent) and *wx1* (from donor parent) can be used in the MAS programme. If the marker shows co-dominance, then heterozygotes (*Wx1*/*wx1*) can be identified from dominant homozygotes (*Wx1*/*Wx1*) in backcross generations (BC_1F_1 or BC_2F_1) under the MABB. In BC_2F_2,

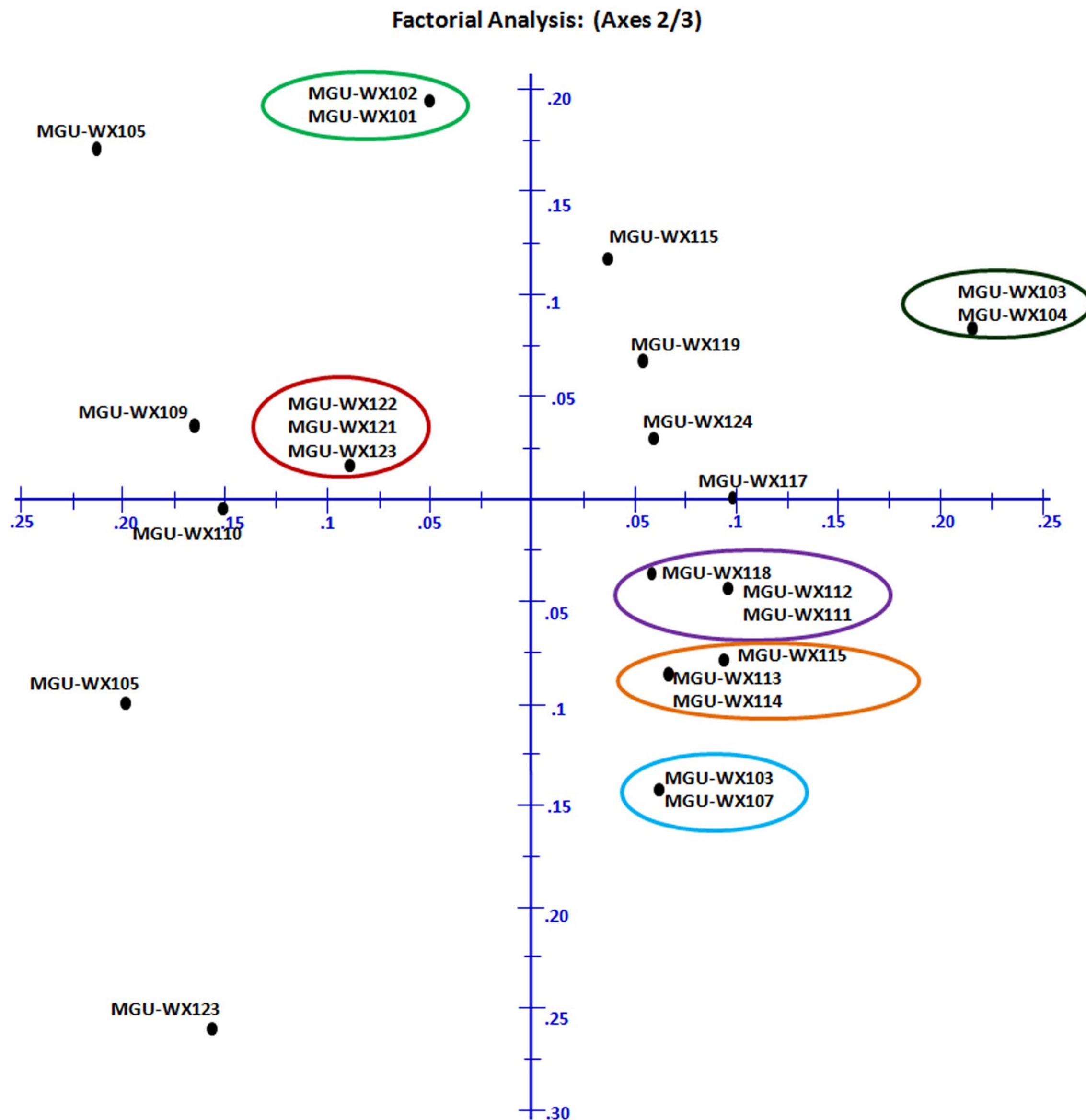

Fig. 5 Principal coordinate analyses depicting the genetic relationship among inbreds. The closely similar inbreds grouped with circle of same colour

the recessive homozygotes (*wx1*/*wx1*) can also be easily identified and selected for further line derivation through repeated selfing. The dominant behaviour of the markers is primarily due to change in nucleotide sequence at the primer binding site, thereby showing presence and absence of polymorphism. The dominant markers can also be used in MABB programme, if the waxy donor produces a band, while recurrent parent shows absence of band. In BC_1F_1 or BC_2F_1, the heterozygotes can be selected by presence of allele. In BC_2F_2, the desirable homozygotes (*wx1*/*wx1*) can be selected by phenotype of the seeds having cloudy expression compared to glossy expression in wild type (*Wx1*/*wx1* or *Wx1*/*Wx1*) seeds.

Conclusion

The present investigation is the first report on characterization of *wx1* allele present in Indian waxy maize germplasm. It revealed the presence of 17 diverse haplotypes of *wx1* which fall into two major clusters. Five of the markers showed dominant behaviour, eight markers showed co-dominant behaviour, while the rest three behaved in both co-dominant and dominant pattern in the present study. The results of our analysis can be effectively used in the breeding programme dealing with the improvement of amylopectin content in maize grains.

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Author contributions Design of experiment: FH; development of waxy inbreds: FH; compilation of marker information from public domain: SKJ; genotyping: RC and ELD; data analysis: FH and RC; field evaluation and maintenance of lines: RUZ; drafting of manuscript: FH and VM.

Compliance with ethical standards

Conflict of interest The authors declare that no conflict of interest exists.

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