Usefulness of Adapted Exotic Maize Lines Developed By Doubled Haploid and Single Seed Descent Methods

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Usefulness of adapted exotic maize lines developed by doubled haploid and single seed descent methods

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Declarations

Conflicts of interest

The authors declare that they have no conflict of interest.

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Author Contributions
ALV, UKF, TL design the project and performed the experiments. IGS, ALV, JE, NB, LTZ, LPMP, GNLF analyzed the data. IGS, ALV wrote the manuscript. All authors read and approved the final manuscript.

Key message
Spontaneous haploid genome doubling is not associated with undesirable linkage drag effects. The presence of spontaneous doubling genes allows maximum exploitation of variability from the temperate-adapted BS39 population.

Abstract
Adapted exotic maize (Zea mays L.) germplasm, such as BS39, provides a unique opportunity for broadening the genetic base of U.S. Corn Belt germplasm. In vivo doubled haploid (DH) technology has been used to efficiently exploit exotic germplasm. It can help to purge deleterious recessive alleles. The objectives of this study were to determine the usefulness of BS39-derived inbred lines using both SSD and DH methods, to determine the impact of spontaneous as compared to artificial haploid genome doubling on genetic variance among BS39-derived DH lines, and to identify SNP markers associated with agronomic traits among BS39 inbreds monitored at testcross level. We developed two sets of inbred lines directly from BS39 by DH and SSD methods, named BS39_DH and BS39_SSD. Additionally, two sets were derived from a cross between BS39 and A427 (SHGD donor) by DH and SSD methods, named BS39×A427_DH and BS39×A427_SSD, respectively. Grain yield, moisture, plant height, ear height, stalk lodging, and root lodging were measured to estimate genetic parameters. For genome-wide association (GWAS) analysis, inbred lines were genotyped using Genotype-by-Sequencing (GBS) and Diversity Array Technology Sequencing (DArTSeq). Some BS39-derived inbred lines performed better than elite germplasm inbreds and all sets showed significant genetic variance. The presence of spontaneous haploid genome doubling genes did not affect performance of inbred lines. Five SNPs were significant and three of them located within genes related to plant development or abiotic stresses. These results demonstrate the potential of BS39 to add novel alleles to temperate elite germplasm.

Keywords: spontaneous doubling, artificial doubling, doubled haploid, single-seed descent
Introduction

Genetic improvement in temperate maize breeding programs has been achieved using only 2 to 5% of the available races of maize. Using tropical germplasm is an effective way to broadening the genetic base of maize breeding programs (Goodman 2004). However, assessing the genetic merit of tropical germplasm in temperate environments by phenotypic evaluation is challenging. The maladaptive syndrome when growing non-adapted (exotic) materials masks the value of potentially favorable alleles (Hallauer and Carena 2014), which includes weak and variable seedling vigor, excessively late flowering, increased plant and ear heights, and poor standability.

Developing inbred lines from segregating exotic germplasm can be negatively impacted by genetic load because masked recessive deleterious alleles may be expressed (Stewart et al. 2017). DH technology can help to effectively purge lethal recessive alleles expressed at the haploid level (Strigens et al. 2013). Even though DH technology is widely used for line development, it is not well adopted in public and small maize breeding programs, especially in developing countries. The labor-intensive haploid genome doubling step during DH line production is a bottleneck for large-scale application of DH technology (Ma et al. 2018; Boerman et al. 2020). Artificial haploid genome doubling requires specific infrastructure for application of toxic colchicine or alternative chemicals, as well as for seedling cultivation and transplanting (Trampe et al. 2020).

Exploiting spontaneous haploid genome doubling to overcome artificial genome doubling would increase the efficacy of inbred line development at reduced costs (Wu et al. 2014). Haploids obtained by spontaneous haploid genome doubling no longer require greenhouse planting, chemical treatment, and transplanting (Boerman et al. 2020). Moreover, haploid plants carrying spontaneous haploid genome doubling alleles tend to produce more pollen than colchicine treated haploids, and consequently more seeds per cob.

In order to compare the value (or potential) of breeding populations, the usefulness concept has been developed (Utz et al. 2001). Usefulness criterion is equal to the expected genotypic mean of the selected individuals (Bernardo, 2020) and is indicative of the breeding potential of a population. The objectives of this study were to (i) to determine the usefulness (Utz et al. 2001) of BS39-derived inbred lines using both SSD and DH methods, (ii) to determine the impact of spontaneous as compared to artificial haploid genome doubling on genetic variance among BS39-derived DH lines, and (iii) to identify SNP markers associated with agronomic traits among BS39 inbreds monitored at testcross level potentially beneficial for 2nd cycling breeding of continuously improved BS39 germplasm.
Materials and methods

Genetic materials. DH and SSD breeding methods were used to derive inbred lines directly from BS39 and from the cross between BS39 and A427, respectively. BS39 is tropical germplasm adapted to temperate environments, and composed of five exotic accessions of Tusón germplasm, representing South American regions. It is a good source to expand the genetic base of temperate maize germplasm, potentially contributing useful and unique alleles (Hallauer and Carena 2016). A427 is a non-stiff stalk (NSS) public line developed at the University of Minnesota and was used as a source of spontaneous haploid genome doubling alleles (Ren et al. 2020; Trampe et al. 2020). The utilization of A427 as a parent in a breeding population can lead to approximately 46% of spontaneous haploid genome doubling in haploid plants across environments (De la Fuente et al. 2018; Trampe et al. 2020). BS39 and A427 were used to develop four sets of inbred lines as described below.

DH lines development. 648 individual plants of BS39 were pollinated with the maternal haploid inducer BHI201 (http://isurftech.technologypublisher.com/technology/19126), at the Iowa State University Agricultural Engineering and Agronomy Farm (ISU-AEA) in the summer of 2015. At physiological maturity, 520 ears were harvested, dried, and individually shelled. Putative haploids were sorted manually based on embryo and aleurone coloration ($R_1$-$nj$). Kernels expressing the $R_1$-$nj$ gene in the aleurone but not in the embryo were classified as haploid kernels (Chang and Coe 2009), contaminants were discarded. Ten haploid kernels were selected from each cob to compose a balanced bulk of BS39. In total, 5,200 haploid kernels were selected. During summer 2016, haploid seeds were planted in the greenhouse for colchicine treatment to promote haploid genome doubling. The colchicine treatment was performed in 4,353 seedlings following the protocol from Eder and Chalyk (2002). Briefly, haploid seedlings at the 3 - 4 leaf developmental stage were injected (approximately 3-5 mm above the apex) with 0.125% colchicine solution containing 0.5% dimethyl sulfoxide (DMSO). Haploid plants were then transplanted into the field, at the ISU-AEA. Putative DH plants shedding pollen were self-pollinated. At physiological maturity, 256 DH lines were harvested and shelled individually and number of seed per ear was recorded. Genome duplication was obtained by the number of DH plants shedding pollen while the average seed per cob was obtained from the harvested cobs. In summer 2017, ~16 seeds of each DH line were increased at ISU-AEA and all plants were self-pollinated. At physiological maturity, the DH lines were harvested and shelled individually. DH lines derived from 100% BS39 germplasm by artificial haploid genome doubling were named BS39_DH lines (Figure 1).

For spontaneous haploid genome doubling, 648 plants from the BS39 population were crossed with
A427 at ISU-AEA. At physiological maturity, 359 F₁ ears were harvested, dried, and individually shelled. A balanced bulk was prepared with a total of 750 F₁ seeds. The 750 F₁ plants were cross-pollinated with the maternal haploid inducer BHI201. At physiological maturity, 700 ears were harvested, dried, and individually shelled. Putative haploid kernels were classified as described before. Approximately 10 haploid kernels were selected from each cob to have a balanced bulk from the cross between BS39 and A427. In total, 7,128 haploid kernels were selected and directly sown in the field at ISU-AEA in summer 2016. Putative DH plants that spontaneously shed pollen were self-pollinated. In total, seed was harvested from 598 DH plants, which were shelled individually, and the seed number was recorded. In summer 2017, ~20 seeds of each DH line were increased at ISU-AEA. DH lines displaying substantial phenotypic variation were discontinued. For homogeneous DH lines all plants were self-pollinated, and the seed bulked. DH lines derived from the cross between BS39 and A427 were named BS39×A427_DH lines (Figure 1).

**Single-seed descent line development.** In winter 2015, 648 plants from BS39 were self-pollinated to produce S₁ seeds at Tuniche Seed Services in Graneros, Chile. At physiological maturity, 600 ears were harvested and shelled individually, then shipped to Iowa State University (ISU). Two kernels from each cob were taken to generate a balanced bulk of 1200 S₂ seeds. In summer 2016, a balanced bulk of the S₂ seeds was planted at ISU-AEA. At physiological maturity, 700 self-pollinated ears were harvested and shelled individually. One kernel from each cob was taken to generate an S₃ balanced bulk, which was sent to Chile in the winter of 2016. At physiological maturity, 300 ears were harvested and shelled individually, then sent to ISU. In Summer 2017 at ISU-AEA, 10 kernels from each of the 300 S₃ ears were planted in an individual row. The first plant of each row was self-pollinated and 120 rows were selected at physiological maturity. Individual ears were harvested and shelled. The same procedure was applied in winter 2017 in Chile with 120 S₃ ears. In summer 2018, seeds from 96 S₄ ears were planted at ISU-AEA for phenotypic evaluation. Inbred lines derived from this process were named BS39_SSD lines (Figure 1).

In order to obtain the fourth set of inbred lines 750 F₁ plants from the cross of BS39 and A427 were self-pollinated at Tuniche Seed Services during winter 2015. After Winter 2015, the same procedure as described for BS39_SSD was followed, including utilizing the same number of plants and selection intensity. In summer 2018, seeds of the best 96 F₆ ears were planted at ISU-AEA for phenotypic evaluation considering the same agronomic traits as BS39_SSD. Inbred lines derived from this process were named BS39×A427_SSD lines (Figure 1).

**Testcross assays.** For testcross evaluation, 96 inbred lines from each set of lines described above (BS39_DH,
BS39×A427_DH, BS39_SSD, BS39×A427_SSD), were crossed with the Ex-PVP inbred line LH195, derived from the stiff-stalk synthetic (SSS) heterotic group. In winter 2017, the 384 inbred lines were planted in an isolation plot at Tuniche Seed Services. Additionally, the ex-PVP inbred lines PHG29, PHG83, PHP76, PHN82, PHZ51, and the public line A427 were testcrossed as performance checks. Inbred lines from each set and the checks were sown in a 1-row plot of 25 kernels, to be used as female parents, and the LH195 tester was used as a male parent. Female rows were hand-emasculated, and wind pollinated by the tester to produce testcross seeds for the field trials.

The 96 testcross hybrids obtained for each set of inbred lines and checks were planted in Ames, Carrol, Crawfordsville, and Keystone, Iowa in 2018. Each location contained two replications in a split-plot design with the sets (BS39_DH, BS39×A427_DH, BS39_SSD, BS39×A427_SSD) as whole plot factor and individual lines within sets as subplots. Whole plots were laid out in a randomized complete block design. Subplots were randomized in a 9×12 resolvable row–column block design within each set (John and Williams, 1995). Row-column randomizations were generated by the software CycDesigN (Whitaker et al. 2002). An individual yield trial plot consisted of two rows, both 5.49 m in length with 0.76 m between rows. Seeds were machine planted at a density of 76,540 plants ha⁻¹. Cultivation practices were consistent with commercial maize production for central Iowa.

**Phenotypic data collection.** Number of haploid kernels, loss after transplanting, number of false positive haploids, genome duplication, and average number of seed per cob was recorded for DH lines produced by artificial and spontaneous haploid genome doubling. Haploid kernels represented the number of seeds used to develop the BS39_DH seedlings or the number of seeds sown in the field (BS39×A427_DH). Loss after transplanting is the number of seedlings that died after transplanting. After transplanting or planting of haploids in the nursery, vigorous false-positive heterozygous plants were eliminated by roguing. For the haploid plants grown to maturity, pollen production was scored visually, and the number of seed harvested per DH plant determined.

Prior to harvest, the following agronomic traits were measured: stand count (number of plants per plot); plant height (cm), measured as the distance between the soil surface and the uppermost leaf collar; ear height (cm), measured as the distance between the soil surface and the uppermost ear node, root lodged and stalk lodged plants per plot at maturity. Data were collected on an individual plot basis in the testcrosses for grain yield (grain yield, adjusted to 15.5% grain moisture). Plots were harvested with a Sperry New Holland TR88 combine modified for automatic acquisition of plot weight, moisture, and test weight with an Almaco Seed.
**Statistical Analyses.** To compare the mean of the four derivation processes a hierarchical analysis of variance (ANOVA) was carried out for each location according to the following model:

\[ Y_{ijk} = \mu + b_i + S_j + (G/S)_{jk} + \epsilon_{ijk} \quad \text{(model 1)} \]

Where \( \mu \) is the overall mean, \( b_i \) is the effect of block \( i \), \( S_j \) is the effect of set \( j \), \( (G/S)_{jk} \) is the effect of genotype \( k \) within set \( j \), and \( \epsilon_{ijk} \) is the effect of residuals. Coefficients of variation were estimated for each location as

\[ CV_e = \frac{S}{\bar{X}} \]

where \( S \) is the root mean square error and \( \bar{X} \) corresponds to the mean.

In order to get Best Linear Unbiased Predictors (BLUPs), variance component estimates were obtained by Restricted Maximum Likelihood procedure using the R package lmerTest (Kuznetsova et al. 2017). BLUPs were obtained for grain yield, grain moisture, plant height, ear height, root lodging, and stalk lodging, according to the model:

\[ Y_{ijkl} = \mu + L_i + (b/L)_{ij} + S_k + (G/S)_{ik} + GL_{ik} + \epsilon_{ijkl} \quad \text{(model 2)} \]

Where \( \mu \) is the overall mean, \( L_i \) is the fixed effect of location \( i \), \( (b/L)_{ij} \) is the block within location nested effect (random), \( S_k \) is the fixed effect of set \( k \), \( G_k \) is the random effect of genotype \( k \), \( GL_{ik} \) is the interaction between the genotype \( k \) and location \( i \), and \( \epsilon_{ijkl} \) is the random effect of residuals.

The estimation of variance components for each set was carried out according to the model for four locations using the same lmerTest package:

\[ Y_{ijk} = \mu + L_i + (b/L)_{ij} + G_k + GL_{ik} + \epsilon_{ijk} \quad \text{(model 3)} \]

Where \( \mu \) is the overall mean, \( L_i \) is the fixed effect of location \( i \), \( (b/L)_{ij} \) is the block within location nested effect (random), \( G_k \) is the random effect of genotype \( k \), \( GL_{ik} \) is the interaction between the genotype \( k \) and location \( i \), and \( \epsilon_{ijk} \) is the random effect of residuals. Random effects were tested by a Likelihood Ratio Test (LRT) at 5% probability. Trait heritability (\( H^2 \)) was estimated as follows for the four locations:

\[ H^2 = \frac{\sigma_G^2}{\left( \sigma_G^2 + \frac{\sigma_{GL}^2}{l} + \sigma_E^2/b \right)} \]

where \( \sigma_G^2 \) is the genotypic variance, \( \sigma_{GL}^2 \) is the genotype by environment variance, and \( \sigma_E^2 \) is the residual variance, \( l \) is the number of locations and \( b \) is the number of blocks. The quality of the model was checked by the root mean square error using the lmerTest R package. In order to compare sets, we estimated the predicted response from selection as follows:

\[ \Delta G(\alpha) = i(\alpha)H\sigma_G \]
where \( i(\alpha) \) is the selection intensity, \( H \) is the squared root of heritability, and \( \sigma_G \) is the genotypic standard deviation (Prigge et al. 2012). Responses were determined for a selected proportion of \( \alpha \) equals 5% (\( i = 2.06 \)), 10% (\( i = 1.76 \)), and 40% (\( i = 0.97 \)). To account for the differences in mean, genetic background and selection response, the usefulness criterion (Bernardo 2020) was then estimated for each set and each group as \( U(\alpha) = \mu + \Delta G \), where \( \mu \) is the mean of the respective set.

**SNP genotyping.** Genotyping of BS39_DH and BS39×A427_DH inbred lines was performed using genotyping by sequencing (GBS) according to Elshire et al. (2011). Leaf samples of 299 BS39_DH and 334 BS39×A427_DH lines were collected at the seedling stage and freeze-dried to send to Cornell Institute for Genomic Diversity for DNA extraction and genotyping. Genotypes were called using the Tassel 5 - GBS production pipeline, which used the ZeaGBSv2.7 (AGPv2) as a reference genome, as described by Glaubitz et al. (2014).

In total, 955,690 SNPs were generated by GBS. SNPs with minor allele frequency below 0.05 and call rate below 0.50 were removed. For SSD lines, kernels from 120 BS39_SSD and 120 BS39×A427_SSD were harvested during winter 2017 and sent to the Genetic Analysis Service for Agriculture (SAGA) at International Maize and Wheat Improvement Center (CIMMYT, Mexico) for genotyping. SNPs were obtained using Diversity Arrays Technology (DArTSeq), according to (Jaccoud et al. 2001). SNP calling used the DArTsoft analytical pipeline (https://www.diversityarrays.com/technology-and-resources/initial-technology-microarray/data-analysis/dartsoft-one-3/) using B73 AGPv4 as a reference genome. A total of 32,930 SNPs were generated by DArTSeq, quality control parameters of DArTSeq SNPs were the same of DHL. SNPs with minor allele frequency below 0.05 and call rate below 0.50 were removed. Additionally, any DHL or SSD line with more than 5% heterozygosity in its genome, was discarded and the remaining heterozygotes within a line were considered missing data. Due to differences in SNP calling (B73 reference genome version 4 for GBS SNPs and version 2 for DArTSeq), GBS data were converted to AGPv4. The conversion was made based on the assembly Converter tool in Gramene (http://www.gramene.org/). After both data sets were aligned with the same B73 reference genome, they were merged using the option “merge genotype table” on Tassel (Bradbury et al. 2007). After merging, missing SNPs were imputed using Beagle 5.0 (Browning et al. 2009). After correction and imputation, 97,345 markers were retained in 90 BS39_DH, 95 BS39×A427_DH, 96 BS39_SSD, and 96 BS39×A427_SSD lines.

**Genome-wide association study methods.** GWAS was performed using the GBS data and the BLUPs estimated by the model (2) for grain yield, moisture, plant height, ear height, stalk lodging, and root lodging.
The fixed and random model Circulating Probability Unification (FarmCPU) (Liu et al. 2016) in the R package GAPIT (Lipka et al. 2012) was used. The first three principal components, obtained by GAPIT were included as covariates in the model. The kinship matrix was automatically estimated in FarmCPU. The False Discovery Rate (FDR) control (Benjamini and Hochberg 1995), implemented in the procedure, determined a significance threshold to account for multiple testing.

**Results**

*Genetic parameters obtained for different sets of inbred lines.* The comparison between artificial and spontaneous haploid genome doubling approaches showed a higher rate of genome doubling using spontaneous haploid genome doubling (Table 1). We observed a loss of 20.5% of BS39_DH plants due to transplantation, a non-existent step in spontaneous haploid genome doubling process. BS39_DH has 100% exotic germplasm, 17.8% of false positives were observed in BS39_DH versus 1.1% in BS39×A427_DH. Rate of genome doubling reached 22.6% in BS39×A427_DH, compared to 12.7% in BS39_DH. The average seeds per cob in BS39×A427_DH was 28 versus 18 in BS39_DH.

Crawfordsville was the only location where sets did not differ (model 1) (Table 2). In Keystone, the BS39_SSD mean was higher (11 Mg ha⁻¹) than for other sets. This yield was significantly different (p=0.05) from BS39_DH and BS39×A427_SSD. In Carroll, BS39_SSD had the highest mean (9.4 Mg ha⁻¹), which did not significantly differ (p=0.05) from BS39×A427_DH (8.87 Mg ha⁻¹), and BS39×A427_SSD (8.8 Mg ha⁻¹). This location presented the lowest overall mean (8.8 Mg ha⁻¹). Regardless of the location, sets containing A427 genome did not differ from each other.

The grain yield mean across environments (model 2) was significantly (p=0.05) higher for BS39_SSD (9.66 Mg.ha⁻¹) (Table 3), compared to the other three sets (9.03 – 9.51 Mg.ha⁻¹) which did not differ significantly. For moisture, BS39×A427_DH and BS39×A427_SSD had 19.74% and 19.73%, respectively, and were significantly lower from sets with 100% BS39 background (20.44 % and 20.39% for BS39_DH and BS39_SSD, respectively). The lowest plant height and ear height were observed for BS39×A427_DH (240.92 and 122.59 cm, respectively) and BS39×A427_SSD (242.8 and 123.57 cm, respectively). These two sets were statistically not different but differed from BS39_DH and BS39_SSD. All sets had very similar means for stalk lodging. BS39_SSD had the lowest mean (16.76%), which was not significantly different from BS39_DH (20.62%) and BS39×A427_DH (21.16%). However, BS39×A427_SSD showed significantly (p=0.05) increased stalk lodging (25.46%). BS39×A427_DH and BS39×A427_SSD showed a significantly (p=0.05) lower percentage of root lodging (2.37% and 2.82%, respectively; Table 3) from BS39_SSD and BS39_DH, which did
not differ (p=0.05) and ranged from 7.08% to 8.98%, respectively. Estimates of trait heritability ranged from 0.72 to 0.80 for grain yield, 0.23 to 0.51 for moisture, and 0.28 to 0.46 for stalk lodging (Table 3). The highest heritabilities among all traits were found for grain yield and ear height, most of the values were above 0.5. Root lodging had the lowest heritability values, ranging from 0.08 (BS39xA427_SSD) to 0.18 (BS39_DH).

Genotypic variances were significant (p=0.05) for all sets and traits (model 3) (Table 3). BS39_DH had the highest genotypic variances for all traits. As expected, DH lines carrying A427 genome had smaller genetic variances compared to those with 100% BS39 for all the traits. For grain yield and moisture, for example, BS39_DH had a genotypic variance of 1.49 and 1.08 versus 1.09 and 0.47, for BS39xA427_DH, respectively. The same pattern was observed for SSD derived sets. Except for stalk lodging, BS39_SSD showed smaller genotypic variance than BS39xA427_SSD. For grain yield and moisture, for example, BS39_SSD had genotypic variance of 0.94 and 1.06 versus 0.86 and 0.35 for BS39xA427_SSD, respectively. Root mean square estimate was higher in BS39xA427_SSD for stalk lodging (23.46). For grain yield, moisture, plant and ear height, and root lodging the higher estimates were found within BS39_DH (1.01, 1.50, 19.89, 14.62, 18.15, respectively).

Genotype x location interaction variances were significant (model 3) (p=0.05) for most sets and traits (Table 3). BS39_DH had the highest estimates for plant height, ear height, stalk lodging, and root lodging. In general, genotypic variances were higher than the genotype x location interaction, the only exceptions were the variances of moisture in BS39xA427_DH, and root lodging in BS39_DH and BS39_SSD sets.

The predicted gain from selection (ΔG) was higher in BS39_DH for all the selection intensity values followed by BS39xA427_DH, BS39_SSD, and BS39xA427_SSD (Table 4). Usefulness (U) criteria were also higher for BS39_DH under 5%, 10% or 40% selection intensity. The lowest U value was observed in BS39xA427_SSD (9.79) at 40% selection intensity. The breeding potential of BS39 germplasm is reflected by the distribution of testcross yields across sets and locations (Figure 2). The overall mean grain yield across sets and locations was 9.4 Mg.ha^{-1}. 105 lines had a better or similar yield as compared to five ex-PVP temperate inbred lines used as checks in the testcross assays. None of inbred lines yielded higher than the commercial check MBS5787HXT (12.52 Mg.ha^{-1}). The highest yielding lines were BS39_DH_167 (11.39 Mg.ha^{-1}), BS39_SSD_199 (11.19 Mg.ha^{-1}), BS39xA427_DH_237 (11.17 Mg.ha^{-1}), and BS39_DH_067 (11.14 Mg.ha^{-1}). The overall mean moisture across sets and locations was 20%. None of the lines showed values for moisture below the commercial check MBS5787HXT (17.91%). The lowest estimates were 18.21% for
GWAS analysis. GWAS analyses for all traits were conducted using the BLUPs from testcross trials (from model 2), using the FarmCPU model for balancing false positives and negatives. Population structure was accounted for by the inclusion of three principal components in the model. No SNPs were found to be significantly associated with grain yield, moisture, ear height and stalk lodging (Figure 3). Three significant SNPs were found for plant height on chromosome 4, S4_46050389 bp, \( p \)-value: 6.62E-09), chromosome 7, S7_108845044 (112016019 bp, \( p \)-value: 3.06E-08), and on chromosome 1, S1_304720322 (304720322 bp, \( p \)-value: 9.67E-08) (Figure 3c and Table 6). For root lodging, two significant SNP markers were detected: one on chromosome 4, S4_13418182 (14318810 bp, \( p \)-value: 2.40E-08), and one on chromosome 9, S9_150676939 (150676939 bp, \( p \)-value: 9.02E-09) (Figure 3e, Table 7).

S1_304720322 is located within the gene Zm00001d034887 that codes for phosphoglycolate phosphatase, which is fundamental in the photorespiration pathway (Li et al. 2020). S7_108845044 is within the gene Zm00001d20409, which codes for TIFY6B. TIFY6B has been shown to be responsive to abiotic stresses including drought, salinity, and low temperature in rice (Ye et al. 2009). S9_150676939 is within the gene Zm00001d048137, which codes for snowy cotyledon 3, required for chloroplast and etioplast biogenesis in seedlings of Arabidopsis (Albrecht et al. 2010).

Discussion

Application and relevance of spontaneous haploid genome doubling in a breeding program. DH technology has been used in connection with exotic germplasm to effectively purge landraces of genetic load (Smelser et al. 2016). Only the more vigorous haploids survive the stressful steps of genome doubling by colchicine treatment and transplanting of the seedlings. Respective effects of DH production were observed in tropical germplasm using temperate inducers under non temperate conditions (Prigge et al. 2011) and through single crosses and open-pollinated populations (Prigge et al. 2012). Despite evidence that DH production does purge genetic load at the haploid level and success rates in DH production are generally lower compared to elite germplasm, only a few significant changes in allele frequencies from the tropical base population were observed (Melchinger et al. 2017).

This study compares sets of inbred lines obtained by DH and SSD approaches, which differ considerably in the amount of field activities and resources needed to produce them. Deriving inbred lines directly from BS39 germplasm by SSD required self-pollination of around 3,000 individual plants per generation for at least six generations (BS39_SSD), plus winter nursery costs. In comparison, assuming the
exotic germplasm used, and the procedures used by the ISU DH Facility, 639 hand pollinations of haploid plants were required to produce 256 inbred lines (BS39_DH). Even though induction and genome duplication required additional effort, this still results in considerably less work than establishing SSD lines. Haploids can be economically produced in inducer isolation fields at a large scale followed by a single generation of self-pollination, whereas developing SSD inbred lines requires at least six generations of self-pollinations.

In addition, spontaneous haploid genome doubling produced twice as many haploid lines as artificial doubling and can be considered an efficient alternative to improve DH production. Finally, haploid kernels with spontaneous haploid genome doubling ability can be sown directly in the field, eliminating greenhouse planting, colchicine treatment and transplanting. It is important to mention that unless exotic germplasm does carry spontaneous haploid genome doubling alleles, a cross between exotic germplasm and a spontaneous haploid genome doubling donor like A427 inbred line is necessary. The first step for applying spontaneous haploid genome doubling in breeding programs is the introgression of these genes into the germplasm for the subsequent DH process. Since a major spontaneous haploid genome doubling QTL was identified on chromosome 5 (Trampe et al. 2020) without obvious linkage drag associated with it (this study), the effort to introgress this QTL into exotic germplasm of interest by backcrossing or forward breeding approaches prior to DH line development seems feasible.

Efficiency of DH breeding method based on artificial versus spontaneous haploid genome doubling. One of the reasons the DH breeding has not been adopted in various public or small maize breeding programs is the specialized and labor-intensive genome doubling step in haploid plants during DH line production. Spontaneous haploid genome doubling has the potential to enhance the efficiency of DH line development at low costs, without particular greenhouse or laboratory requirements. One of the challenges of using spontaneous haploid genome doubling within commercial DH breeding programs is the introgression of spontaneous haploid genome doubling into breeding germplasm (Boerman et al. 2020). Until recently, it was not known whether major genes are involved in spontaneous haploid genome doubling. With the identification of a major QTL on chromosome 5 (Ren et al. 2019; Trampe et al. 2020), utilization of spontaneous haploid genome doubling appears to be a more straightforward backcrossing or forward breeding problem. However, it is not known whether the major spontaneous haploid genome doubling QTL on chromosome 5 is effective in exotic germplasm. A higher haploid genome doubling rate (22.59% in BS39×A427_DH versus 12.72% in BS39_DH) and a higher number of kernels per cob that were observed in inbred lines with A427 genome (28 in BS39×A427_DH versus 18 in BS39_DH) suggest that this QTL may be effective in non-temperate germplasm. Since each harvested ear in a
haploid field constitutes a homozygous line, a higher number of kernels per cob is highly desirable because it
can optimize the next breeding step. When we have a great number of seeds, we can multiply part of them and
make crosses in the same generation.

Linkage drag associated with major spontaneous haploid genome doubling QTL may penalize their use
for DH line development, if for example grain yield is reduced. Absence of significant differences between
BS39xA427_DH and BS39xA427_SSD lines for all traits in individual environments or across locations
(Tables 2 and 3) suggest that spontaneous haploid genome doubling was not associated with undesirable linkage
drag effects. Verzegnazzi et al. (2021) found a significant enrichment of the A427 haplotype in the spontaneous
haploid genome doubling QTL region on chromosome 5, which is consistent with strong selection for the major
spontaneous haploid genome doubling QTL from A427 (Ren et al. 2020; Trampe et al. 2020) in this region.
Thus, no linkage drag was detected for the major QTL for spontaneous haploid genome doubling on
chromosome 5 in our materials.

All four sets of inbred lines showed significant genotypic variance. The ratio between variance
components ($\sigma_g^2/\sigma_{ge}^2$) was, in most cases, higher than 1, which means the genotypic component is more
important than the genotype by environment interaction. For traits such as grain yield, plant height, and ear
height, the genotypic variance of BS39xA427_DH was greater than the sets derived from SSD. These results
indicate that the presence of spontaneous doubling genes does not limit the selection of BS39-derived lines and
allows maximum exploitation of variability from the BS39 population.

Usefulness of inbred lines developed by SSD vs DH method. Due to the considerable performance gap that
exists when tropical elite germplasm is evaluated in temperate environments (Lai et al. 2017), BS39-derived
lines are unlikely to be directly used as a parent in a competitive hybrid combination. However, BS39
germplasm seems to be a promising source for broadening the narrow genetic base of U.S. Corn Belt germplasm
without major yield penalty. Testcrosses of some BS39-derived lines yielded the same or more than testcrosses
of Ex-PVP lines in our study (Figure 2).

BS39 is a highly heterogeneous population. Donor genotypes respond differently to the DH process,
both with regard to inducibility (De la Fuente et al. 2018; Trampe et al. 2020) and haploid genome doubling (De
la Fuente et al. 2018; Trampe et al. 2021). This may lead to selection and perhaps fixation of particular
haplotypes in genome regions contributing to DH line development, and thus narrow the diversity among DH
lines compared to SSD lines. Based on allele frequencies, genetic differentiation, and heterozygosity,
Verzegnazzi et al. (2021) showed that both SSD and DH breeding methods capture most of the genetic
variability present in the BS39 population.

Even though the DH method may narrow genetic diversity due to haplotype selection, Verzegnazzi et al. (2021) suggest that the genetic diversity in BS39 is equally well represented in BS39_SSD, BS39_DH, or BS39×A427_DH. BS39_DH had 57.5% of its loci with the same allele frequency (P=0.05) with BS39, whereas BS39×A427_DH 62.1% loci showed the same allele frequency, while for BS39×A427_DH and BS39_SSD the value was 52% and 31.9%, respectively. Only BS39×A427_SSD showed reduced genetic diversity compared to BS39, which can be attributed to reduced sample size.

The estimated heritabilities for grain yield for the BS39_DH and BS39_SSD sets were 0.74, while the means across locations were significantly different with 9.82 Mg ha\(^{-1}\) and 9.28 Mg ha\(^{-1}\), respectively. The highest yielding lines within these two sets were BS39_DH_167 (10.5 Mg ha\(^{-1}\)) and BS39_SSD_199 (10.4 Mg ha\(^{-1}\)), which performed better or the same than the ex-PVP temperate inbred lines used as checks in the testcross assays (10.4 Mg ha\(^{-1}\) on average). This is consistent with Jumbo et al. (2011), who showed that both SSD and DH methods are efficient to develop high performing lines from GEM breeding crosses. Bordes et al. (2007) compared SSD and DH methods and concluded that for important agronomic traits such as grain yield both methods resulted in similar levels of genetic variation.

Usefulness or breeding potential is appropriate to compare types of source germplasm. It combines information on the mean and the predicted selection response and, thus depends on properties of source germplasm and on the selection intensity (Melchinger et al. 2010; Utz et al. 2001). SSD sets included high yielding lines such as BS39_SSD_199 with 11.19 Mg ha\(^{-1}\) and BS39×A427_SSD_117 with 11 Mg ha\(^{-1}\). However, usefulness criteria suggest that even if BS39_DH or BS39×A427_DH lines had a lower mean for grain yield compared with BS39_SSD and BS39×A427_SSD lines, respectively, this was compensated by selection responses. For example, the usefulness values for BS39_DH were 11.67 (\(\alpha = 5\%\)), 11.36 (\(\alpha = 10\%\)), and 10.53 (\(\alpha = 40\%\)) versus 11.37 (\(\alpha = 5\%\)), 11.12 (\(\alpha = 10\%\)), and 10.47 (\(\alpha = 40\%\)) for BS39_SSD, respectively, despite of a higher mean for BS39_SSD (9.66Mg ha\(^{-1}\)) compared with BS39_DH (9.51 Mg ha\(^{-1}\)). Usefulness of 100% BS39 set was higher than the respective SSD set considering identical selection intensities.

**SNPs associated with candidate genes responsible for agronomic traits in maize within exotic germplasm.**

Exotic germplasm is the primary source of novel alleles, and methods such as GWAS can help to identify and selectively introduce new and beneficial gene variants into elite germplasm. Successful application of molecular markers and GWAS in exotic germplasm has been previously reported (Hao et al. 2015; Romay et al. 2013; Tanksley and Nelson 1996; Zalapa et al. 2007). SNPs significantly associated with plant height (S1_304720322,
homologues of genes responsible for plant development and abiotic stress in rice and Arabidopsis. If these gene functions can be validated in maize, they could be used for marker-assisted selection. GWAS results from this study reinforce the potential of the BS39 population to add novel alleles into elite temperate germplasm.

For 2nd cycle breeding in this material, genomic selection (GS) may be more promising than selecting for individual significant SNPs detected by GWAS. Genotypic and phenotypic data from the current GWAS study can be used as training panel. We can select parents for crossing and obtain the next generation, and then select within segregating populations. Because GS breeding values are estimated based on all available marker information, breeding progenies can accumulate favorable alleles from both major and minor QTL. Shikha et al. (2017) stated that marker-assisted selection is limited to major QTL, which leads to a loss of genetic gain. GS overcomes this limitation because it also addresses the effect of small genes (Srivastava et al. 2020).

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Table 1. Field observation of doubled haploid production by artificial haploid genome doubling (BS39DH) and spontaneous haploid genome doubling (BS39×A427_DH).

|                                | BS39_DH | %  | BS39×A427_DH | %  |
|--------------------------------|---------|----|--------------|----|
| Haploid kernels                | 4992    | -  | 7128         | -  |
| Loss after transplanting       | 1025    | 20.53 | -          | -  |
| False Positive haploids        | 890     | 17.83 | 76         | 1.07|
| Genome duplication             | 635     | 12.72 | 1610       | 22.59|
| Average seeds per cob          | 18      | -  | 28          | -  |
Table 2. Testcross grain yield means of sets of inbred lines derived from doubled haploid (BS39_DH and BS39xA427_DH) and single seed descent (BS39_SSD and BS39xA427_SSD) breeding methods in four locations.

| Location     | Grain yield mean (Mg ha\(^{-1}\)) | Mean | CV\(_e\) |
|--------------|-----------------------------------|------|----------|
|              | BS39_DH  | BS39x427_DH | BS39_SSD | BS39x427_SSD | Mean | CV\(_e\) |
| Ames         | 9.50 bc  | 8.99 ab     | 9.62 c   |            | 8.84 a          | 9.22 | 12.1    |
| Carroll      | 8.76 a   | 8.87 ab     | 9.40 b   |            | 8.80 ab         | 8.96 | 14.6    |
| Crawfordsville| 9.51 ab  | 9.49 ab     | 9.69 ab  |            | 9.21 a          | 9.48 | 9.9     |
| Keystone     | 10.20 a  | 10.70 ab    | 11.00 b  |            | 10.40 a         | 10.6 | 12.1    |

*Different letters in a same row indicate significant differences among sets according to a Tukey test at the 0.05 probability level. CV\(_e\) is the coefficient of variation of each experiment.
Table 3. Mean, genotypic variance (\( \hat{\sigma}_g^2 \)), genotype x environment interaction variance (\( \hat{\sigma}_{ge}^2 \)), heritability (H²), and root mean square error (RMSE) for grain yield (GY, Mg ha\(^{-1}\)), grain moisture (MO, %), plant height (PHT, cm), ear height (EHT, cm), root lodging (RLG, %), and stalk lodging (SLG, %) on testcrosses of sets of inbred lines derived from doubled haploid (BS39_DH and BS39xA427_DH) and single seed descent (BS39_SSD and BS39xA427_SSD) breeding methods.

| Traits | Set              | Mean   | \( \hat{\sigma}_g^2 \) | \( \hat{\sigma}_{ge}^2 \) | \( \hat{\sigma}_e^2 \) | H²    | RMSE  |
|--------|------------------|--------|-------------------------|-------------------------|-------------------------|-------|-------|
| GY     | BS39_DH          | 9.51 a | 1.49                    | 0.04\(^{ns}\)           | 1.03                    | 0.74  | 1.01  |
|        | BS39xA427_DH     | 9.24 a | 1.09                    | 0.05                    | 0.53                    | 0.80  | 0.72  |
|        | BS39_SSD         | 9.66 b | 0.94                    | 0.07                    | 0.64                    | 0.74  | 0.80  |
|        | BS39xA427_SSD    | 9.03 a | 0.86                    | 0.03\(^{ns}\)           | 0.67                    | 0.72  | 0.81  |
| MO     | BS39_DH          | 20.44 b| 1.08                    | 0.29\(^{ns}\)           | 2.26                    | 0.47  | 1.50  |
|        | BS39xA427_DH     | 19.74 a| 0.47                    | 0.48                    | 2.23                    | 0.28  | 1.49  |
|        | BS39_SSD         | 20.39 b| 1.06                    | 0.39                    | 1.84                    | 0.51  | 1.35  |
|        | BS39xA427_SSD    | 19.73 a| 0.35                    | 0.34\(^{ns}\)           | 2.18                    | 0.23  | 1.47  |
| PHT    | BS39_DH          | 263.26 c| 170.9                   | 69.3                    | 395.7                   | 0.44  | 19.89 |
|        | BS39xA427_DH     | 240.92 a| 154.8                   | 59.27                   | 240.4                   | 0.53  | 15.50 |
|        | BS39_SSD         | 252.94 b| 84.05                   | 62.79                   | 340.01                  | 0.31  | 18.43 |
|        | BS39xA427_SSD    | 242.80 a| 74.26                   | 51.84                   | 359.82                  | 0.28  | 18.96 |
| EHT    | BS39_DH          | 136.95 c| 149.65                  | 42.51                   | 213.87                  | 0.56  | 14.62 |
|        | BS39xA427_DH     | 122.59 a| 97.76                   | 26.16                   | 147.84                  | 0.55  | 12.15 |
|        | BS39_SSD         | 129.21 b| 74.56                   | 27.37                   | 125.54                  | 0.52  | 11.20 |
|        | BS39xA427_SSD    | 123.57 a| 58.09                   | 39.98                   | 178.4                   | 0.37  | 13.35 |
| SLG    | BS39_DH          | 20.62 ab| 204.11                  | 123.52                  | 410.86                  | 0.46  | 20.26 |
|        | BS39xA427_DH     | 21.16 ab| 102.33                  | 54.27                   | 446.51                  | 0.30  | 21.13 |
|        | BS39_SSD         | 16.76 a | 86.64                   | 50.28                   | 351.48                  | 0.32  | 18.74 |
|        | BS39xA427_SSD    | 25.46 a | 109.74                  | 47.59                   | 550.56                  | 0.28  | 23.46 |
| RLG    | BS39_DH          | 8.98 b  | 42.72                   | 114.96                  | 329.55                  | 0.18  | 18.15 |
|        | BS39xA427_DH     | 2.37 a  | 3.75                    | 0                      | 76.68                   | 0.09  | 8.75  |
|        | BS39_SSD         | 7.08 b  | 22.34                   | 116.73                  | 224.36                  | 0.14  | 14.97 |
|        | BS39xA427_SSD    | 2.83 a  | 4.61                    | 0                      | 107.76                  | 0.08  | 10.38 |

Different letters in a same column indicate significant differences among sets according to a Tukey test at the 0.05 probability level. **, *, ns Significant according to a LRT test at the 0.01 and 0.05 probability level, and not significant, respectively.
Table 4. Predicted gain from selection ($\Delta G$) and usefulness criteria (U) at selection intensity $\alpha$ for grain yield of sets of inbred lines derived from doubled haploid (BS39_DH and BS39xA427_DH) and single seed descent (BS39_SSD and BS39xA427_SSD) breeding methods based on the average of the means obtained for four locations.

| Set           | $\alpha = 5\%$ |        | $\alpha = 10\%$ |        | $\alpha = 40\%$ |        |
|---------------|-----------------|--------|-----------------|--------|-----------------|--------|
|               | $\Delta G$      | U      | $\Delta G$      | U      | $\Delta G$      | U      |
| BS39_DH       | 2.16            | 11.67  | 1.85            | 11.36  | 1.02            | 10.53  |
| BS39xA427_DH  | 1.92            | 11.16  | 1.64            | 10.88  | 0.90            | 10.14  |
| BS39_SSD      | 1.71            | 11.37  | 1.46            | 11.12  | 0.81            | 10.47  |
| BS39xA427_SSD | 1.62            | 10.65  | 1.38            | 10.41  | 0.76            | 9.79   |
Table 5. Summary of GWAS analyses.

| SNP         | Chr | Position  | MAF     | P.Value     | Effect  | Gene stable ID   |
|-------------|-----|-----------|---------|-------------|---------|------------------|
| Plant height|     |           |         |             |         |                  |
| S1_304720322| 1   | 304720322 | 0.127869| 9.67E-08    | 2.947476| Zm00001d034887   |
| S4_46056056 | 4   | 48050389  | 0.445902| 6.62E-09    | -2.4416 | x                |
| S7_108845044| 7   | 112016019 | 0.062295| 3.06E-08    | -5.01254| Zm00001d020409   |
| Root lodging|     |           |         |             |         |                  |
| S9_150676939| 9   | 150676939 | 0.065574| 9.02E-09    | 1.008246| Zm00001d048137   |
| S4_13418182 | 4   | 14318810  | 0.022951| 2.40E-08    | -1.65646| x                |
Figure 1. Breeding scheme used to derive DH and SSD inbred lines from BS39 and from the cross between BS39 and A427.
Figure 2. Histogram of testcross means of testcrosses inbred lines across sets and locations in comparison with Ex-PVP and commercial check hybrids (arrows) for grain yield (ton ha$^{-1}$).
Figure 3. Manhattan plot (left) and QQ-plot (right) of the FarmCPU results for grain yield, moisture, plant height, ear height, root lodging, and stalk lodging.