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Combining ability and heterotic grouping among tropical maize lines, and association mapping for plant stature in set of temperate-adapted maize lines

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ABSTRACT

PEREIRA, Gabriela dos Santos, D.Sc., Universidade Federal de Viçosa, July, 2025. **Combining ability and heterotic grouping among tropical maize lines, and association mapping for plant stature in set of temperate-adapted maize lines**. Adviser: Rodrigo Oliveira de Lima.

Maize is the most widely produced cereal in the world due to its versatile uses, which include human and animal consumption, biofuel production, and various industrial applications. The efficiency of maize breeding programs can be enhanced by understanding combining ability and heterotic grouping of inbred lines, as well as by the utilization of germplasm as a source of favorable alleles. Therefore, the objectives were to evaluate the combining ability of 15 tropical maize inbred lines, allocate them into heterotic groups, identify high-performing hybrids; assess genetic variance, and identify molecular markers associated with plant architecture traits in a panel of lines derived from the tropical maize population adapted to temperate environments, BS39. The 15 inbred lines were genotyped using SNP markers (3,713) and crossed in a complete diallel scheme, and the 105 hybrids were evaluated for plant architecture, grain morphology, and ear traits across four environments in Minas Gerais. Additionally, four sets of 96 inbred lines were developed from the BS39 population and from a cross between BS39 and A427, using DH and SSD methods. These lines were genotyped and evaluated at three locations in Iowa for plant height and anthesis-silking interval. For the tropical inbred lines, significant differences ($P < 0.05$) in combining ability were observed for all traits. Five inbred lines showed promise as parents for germplasm development. Four heterotic groups were defined based on SCA estimates for grain yield and SNP marker data. Eight experimental hybrids demonstrated potential for recommendation in Brazil. For the temperate-adapted lines, genetic variance was observed for all traits across all evaluated sets ($P < 0.05$). Lines developed through the SSD method showed greater genetic variance than those obtained through the DH method. Plant height traits exhibited moderate to strong positive correlations between inbred lines derived from BS39 and their testcrosses. Nine SNPs associated with plant height were identified in the DH lines, but none in the SSD lines.

Keywords: *Zea mays* L.; maize germplasm; diallel crosses; GWAS; panel of inbred lines; doubled haploid

RESUMO

PEREIRA, Gabriela dos Santos, D.Sc., Universidade Federal de Viçosa, julho de 2025. **Capacidade de combinação e agrupamento heterótico entre linhagens de milho tropicais, e mapeamento associativo para arquitetura da planta em um conjunto de linhagens de milho adaptadas ao clima temperado**. Orientador: Rodrigo Oliveira de Lima.

O milho é o cereal mais produzido no mundo devido à sua versatilidade de uso, que abrange a alimentação humana e animal, produção de biocombustíveis e diversas aplicações na indústria. A eficiência dos programas de melhoramento de milho pode ser potencializada pela compreensão da capacidade de combinação e agrupamento heterótico das linhagens, assim como pela utilização de germoplasmas fonte de alelos favoráveis. Dessa forma, os objetivos foram avaliar a capacidade de combinação de 15 linhagens de milho tropical, alocá-las em grupos heteróticos, identificar híbridos de alto desempenho; estudar a variância genética e identificar marcadores moleculares associados com caracteres de arquitetura de plantas em um painel de linhagens derivadas da população de milho tropical adaptada a ambientes temperados, BS39. As 15 linhagens foram genotipadas com 3,713 polimorfismos de único nucleotídeo (SNPs) e cruzadas em esquema de dialelo completo. Os 105 híbridos foram avaliados para arquitetura de planta, caracteres de grãos e espigas em quatro ambientes de Minas Gerais. Além disso, foram desenvolvidos quatro conjuntos de 96 linhagens derivadas da população BS39 e de um cruzamento entre BS39 e A427, por meio dos métodos DH e SSD. Essas linhagens foram genotipadas e avaliadas em três locais de Iowa quanto à estatura de planta e ao intervalo de florescimento. Para as linhagens tropicais, foram observadas diferenças significativas ($P<0.05$) nas capacidades de combinação para todos os caracteres. Cinco linhagens mostraram-se promissoras como genitores para o desenvolvimento de germoplasma. Quatro grupos heteróticos foram definidos com base nas estimativas de SCA para GY e nos marcadores SNP. Oito híbridos experimentais demonstraram potencial para recomendação em regiões do Brasil. Para as linhagens adaptadas ao clima temperado, foi observada variância genética ($P<0.05$) para todas as características em todos os conjuntos avaliados. As linhagens desenvolvidas pelo método SSD apresentaram maior variância genética em relação aquelas obtidas pelo método DH. As características da estatura de planta apresentaram correlações positivas, de moderadas a fortes, entre as linhagens derivadas de BS39 e seus testcrosses. Foram identificados nove SNPs associados à estatura de planta nas linhagens DH, mas nenhum nas linhagens SSD.

Palavras-chave: *Zea mays* L.; germoplasma de milho; dialelo; GWAS; painel de linhagens; duplo-haploide

SUMMARY

1.1 General introduction

Maize (*Zea mays* L.) is cultivated worldwide due to its extensive genetic diversity, which enables broad adaptation to diverse environmental conditions (Teixeira and Trindade 2021). Moreover, its versatility of use, mainly for human and animal nutrition, makes maize one of the most important crops in global agriculture (Môro and Fritsche-Neto 2017). During the 2024/2025 growing season, global maize production was approximately 1,22 billion tons (USDA 2025). The top three countries producers in the world were the United States (377.63 million tons), China (294.92 million tons), and Brazil (126 million tons; USDA 2025). In Brazil, maize is primarily used for animal feed, being the second most cultivated crop, with an estimated 21.3 million hectares planted during 2024/2025 season (Conab 2025).

In Brazil, approximately 90% of the maize cultivated area is planted with hybrids, mainly single and three-way hybrids (Souza 2018). Hybrid cultivars are derived from the crossing of genetically distinct inbred lines, aiming to exploit heterosis effects between them (Hallauer et al. 2010). These inbred lines are developed through successive self-pollinations or the double haploid (DH) method, starting from breeding populations derived from crosses between elite lines (line recycling) and/or advanced generations of commercial hybrids (Hallauer and Carena 2009; Guimarães et al. 2018). During the line development process, they are selected for traits with high heritability, based on their *per se* performance, and for traits with low heritability, mainly grain yield, based on their performance in crosses (Hosana et al. 2015). Identifying superior lines through crosses is one of the most important and costly stages in a hybrid maize breeding program (Abera et al. 2016; Guimarães et al. 2018). Line selection based on crosses can be performed through diallel or testcross methods. The testcross method involves evaluating lines crossed with a common tester and is generally used when a large number of lines need to be assessed (Miranda Filho 2018). Diallel involves all possible hybrid combinations among a set of genotypes, typically used when fewer lines are being evaluated (Miranda Filho 2018; Ribeiro 2021). The study of combining abilities of lines in crosses, using either diallel or testcrosses, aims to determine the potential of these genotypes for developing elite hybrids (Abera et al. 2016).

General combining ability (GCA) refers to the average performance of parental lines in crosses, while specific combining ability (SCA) corresponds to the deviation of hybrid performance from what would be expected based on the performance of the parents (Sprague and Tatum 1942; Hallauer et al. 2010). GCA is associated with the importance of genes exhibiting additive effects, whereas SCA is related to genes exhibiting non-additive effects, i.e., the genetic variance due to dominance deviations and epistasis (Sprague and Tatum 1942). Therefore, breeders aim for hybrid combinations that show high SCA effects among lines with high GCA (Guimarães et al. 2018). In addition, in breeding programs targeting the development of commercial hybrids, the inbred lines must be allocated into heterotic groups to exploit maximum heterosis among the genotypes.

The success of selecting inbred lines in crosses and, consequently, the development of heterotic and high-yielding maize hybrids is strongly associated with the knowledge of the heterotic groups in a maize breeding program. These groups can be defined as sets of individuals that when crossed within the same group, the hybrids do not exhibit heterosis, but when crossed with individuals from another group, the resulting hybrids show significant heterosis (Melchinger et al. 1989; Reif et al. 2005). Understanding the heterotic groups of breeding germplasm helps maize breeders to guide crosses in order to obtain heterotic and productive hybrids, saving time, labor, and money (Osorno and Carena 2008; Badu-Apraku et al. 2021). In temperate germplasm, heterotic groups were defined nearly a hundred years ago, such as Stiff Stalk and Non-Stiff Stalk, and have been successfully used in the development of hybrids in many maize-producing countries (Beckett et al. 2017). In contrast, tropical germplasm is highly diverse, has a complex genetic background, and only recently has begun breeding for heterotic groups. Currently, the Tuxpeño and non-Tuxpeño groups have been used to classify tropical maize germplasm (Vasal et al. 1999; Duvick et al. 2004; Trevisan 2018). In the case of germplasm with unknown heterotic grouping, classification can be carried out based on information from combining ability of genotypes and molecular markers (Parentoni et al. 2001; Delucchi et al. 2012; Bidhendi et al. 2012; Badu-Apraku et al. 2013; Fan et al. 2014; Grassi et al. 2015; Beckett et al. 2017; Faria et al. 2022; Zebire et al. 2022).

Estimates of combining ability among inbred lines can be obtained through crosses of the lines with elite testers or among themselves using a genetic mating design, such as diallel crosses. Diallel designs are widely used in maize breeding to estimate combining abilities among parents, identify testers, and determine the gene action of important agronomic traits. The diallel analysis model proposed by Griffing (1956), extensively applied in plant breeding, estimates the effects of GCA and SCA among lines. As SCA estimates are directly related to heterosis between parents (Vasal et al. 1992; Hallauer et al. 2010), SCA effects have been used as a measure of genetic diversity among inbred lines for their allocation into heterotic groups (Badu-Apraku et al. 2013; Fan et al. 2014; Suwarno et al. 2014; Badu-Apraku et al. 2015a; Elmyhun et al. 2020; Olayiwola et al. 2021; Patil et al. 2021). Positive SCA effects suggest that the lines belong to opposite heterotic groups, whereas negative SCA effects indicate that the lines are likely from the same group (Vasal et al. 1992; Wegary et al. 2013). Badu-Apraku et al. (2013) evaluated 190 single-cross hybrids derived from a diallel involving 20 extra-early maize inbred lines. In this study, GCA effects were predominant over SCA effects for traits related to *Striga hermonthica* infestation resistance and nitrogen stress tolerance. Furthermore, the authors classified the lines into four heterotic groups based on SCA effects, and into three groups based on molecular marker data and GCA effects. Patil et al. (2021) studied the combining abilities among 20 inbred lines crossed with three testers to allocate the lines into heterotic groups. Based on the SCA effects for grain yield, six inbred lines were assigned to heterotic group A, and seven to group B. Three inbred lines exhibited positive SCA effects with two testers and were assigned to a mixed heterotic group, AB, while four lines showed negative SCA effects with two testers and were not assigned to any group. To investigate the combining abilities of ten white maize inbred lines and classify them into heterotic groups, Olayiwola et al. (2021) evaluated 45 hybrids derived from a diallel among these lines. Based on SCA estimates for grain yield, the authors allocated the lines into three heterotic groups. Additionally, the lines were grouped based on GCA and SCA for grain yield, GCA for multiple traits, and genetic distance derived from Simple Sequence Repeat (SSR) and Single Nucleotide Polymorphism (SNP) markers. All methods classified the lines into three heterotic groups, however, the authors found greater grouping efficiency with methods based on SCA and GCA in combination with SCA estimates.

The allocation of lines into heterotic groups through population structure analysis using molecular markers has been widely employed, especially for germplasms with complex backgrounds such as tropical maize (Beckett et al. 2017; Trevisan 2018; Faria et al. 2022). Therefore, analyses based on molecular markers are viable alternatives for large-scale genetic diversity characterization, since evaluations through diallel or testcross designs are constrained by the number of possible crosses (Leng et al. 2019). Genetic distance is a measure of genetic divergence between lines or populations, based on the number of alleles shared among genotypes. Consequently, cluster analyses based on genetic distances obtained from molecular markers can be used to classify lines into heterotic groups, as genetically closer lines tend to cluster together (Leng et al. 2019; Faria et al. 2022). The study of population structure enables breeders to understand the genetic composition of a population, estimate migration and dispersion levels, support germplasm bank management and evolutionary diagnostics, and assist in the classification of lines into heterotic groups (Cruz et al. 2011; Faria et al. 2022).

According to Viana et al. (2013), most population structure analyses in plant breeding involve inbred lines, and their classification into subpopulations is generally based on geographic and climatic adaptation, pedigree information, germplasm origin, and heterotic grouping. Thus, the allocation of lines into heterotic groups has been based on estimates of GCA effects between parental lines, as well as on genetic diversity and/or population structure derived from molecular marker data (Badu-Apraku et al. 2013; Suwarno et al. 2014; Olayiwola et al. 2021).

The first commercial maize hybrid in Brazil was released in 1938 by the Universidade Federal de Viçosa (UFV), as a result of research carried out by professors Gladstone Drummond and Antônio Secundino. In 1945, these researchers founded the seed company Agroceres, which became a national reference in maize breeding and remained a leader in the hybrid seed market for many years (Môro 2018). It was only in the late 1990s that the UFV maize breeding program was reestablished. Today, it is the second-largest public maize breeding program in Brazil. Currently, the UFV program maintains a collection of tropical germplasm comprising 182 inbred lines. However, most of these genotypes were derived from the selfing of commercial hybrids or populations. As a result, these lines have a complex genetic background and consist of mixtures of heterotic groups. Furthermore, few studies have been published involving diallel crosses among tropical maize lines, making information on the heterotic grouping of tropical lines from Brazilian breeding programs scarce. As heterotic groups are not well-defined in Brazil, studies using diallel crosses among tropical maize lines are essential to support the definition and establishment of heterotic groups, aiming at the development of new commercial hybrids and increased genetic gains.

Tropical maize germplasm has an enormous potential to contribute with useful and unique alleles to the U.S. Corn Belt breeding programs and, consequently, expand their genetic base (Hallauer and Carena 2016). An example is the BS39 maize population which has contributed with novel alleles for economically important traits for temperate maize breeding programs (Santos et al. 2022). The temperate-adapted BS39 population is an open-pollinated accession, composed of five exotic accessions of Tusón germplasm, representing South American regions. The BS39 was developed by the maize breeding program at Iowa State University and it includes 100% tropical germplasm photoperiod adapted to temperate environments (Hallauer and Carena 2016).

DH technology has emerged as an efficient alternative to speed up the development of maize lines. The use of DH lines saves time in a breeding program, allows better use of genetic variation, and also increases response to selection by increasing heritability for both per se and testcross evaluation (Maqbool et al. 2020). One effective way to compare whether potential genetic variability is affected by the DH process is to compare DH lines with inbred lines developed by the single-seed descent (SSD) breeding method (Bordes et al. 2007).

Considering the foregoing, our objectives were to: i) assess the combining ability for a large set of traits among 15 elite inbred lines of tropical maize from UFV maize breeding program; ii) group them into heterotic groups and identify the high-yielding hybrids to be cropped for Brazilian maize growers; and iii) identify SNP markers and candidate genes associated with plant height, ear height and anthesis-silking interval in set of temperate-adapted maize lines developed by DH and SSD methods.

1.2 Literature review

1.2.1 Importance of maize

Maize (*Zea mays* L.) is a cereal of significant global importance due to its high energy content, relatively low cost, and broad adaptability to diverse edaphoclimatic conditions. Maize grains are primarily used as an energy source for animal feed production, human consumption, and ethanol manufacturing (Môro and Fritsche-Neto 2017). During 2024/2025, approximately 40% of U.S. maize production was allocated to ethanol and its byproducts (USDA 2025). In Brazil, around 56.3 million tons are used for animal feed, 25.6 million tons for industrial purposes, and only 2 million tons are allocated to human consumption (Abimilho 2025).

Brazil is the third-largest maize producer in the world, with an estimated 126 million tons harvested during the 2024/2025, across an area of 21.3 million hectares and an average yield of 5.853 kg ha^{-1} (Conab 2025). The low national average yield is mainly due to the wide range of cultivation conditions, as maize is grown in nearly all regions of the country under varying technological levels. The lack of technology adoption by farmers such as the use of uncertified seeds, inadequate fertilization, and non-improved cultivars directly impacts maize yield. In addition, abiotic stresses such as drought, soil acidity and aluminum toxicity, as well as nitrogen and phosphorus deficiencies, contribute to yield reductions worldwide (Môro and Fritsche-Neto 2017; Mundim et al. 2018). Despite these limiting factors, Brazil’s maize yield has grown at an average annual rate of approximately 5% over the past ten years (Embrapa 2021). During the same period, the cultivated area increased by an estimated 30%, and total production by 200%. This growth is attributed to continuous genetic progress through the development of maize hybrids with improved responsiveness to fertilizers and fungicides, as well as greater tolerance to drought and higher plant population densities (Duvick 2005; Andorf et al. 2019).

Currently, the seed market offers a wide range of maize cultivars with high yield potential and several technological traits. As maize is an allogamous, seed-propagated species with a high level of heterosis, mainly for yielding performance, hybrid cultivars are the most widely adopted. Based on the number of parental lines involved, maize hybrids can be categorized into different types. A single-cross hybrid results from the cross between two inbred lines; a double-cross hybrid is derived from the cross between two single-cross hybrids; and a three-way cross hybrid is developed by crossing a single-cross hybrid (used as the female parent) with an inbred line (used as the male parent). In the development of three-way cross hybrids, it is common for the inbred lines used in the initial single-cross to belong to the same heterotic group, while the pollinator line originates from a different group (Souza 2018). Single-cross hybrids are recommended for high-technology production systems due to their superior yield potential and greater genetic uniformity. Double-cross hybrids are better suited to medium-technology systems, offering increased genetic variability, which contributes to more stable performance. Three-way cross hybrids are appropriate for medium to high-technology systems, combining high yield potential with slightly reduced genetic uniformity compared to single-cross hybrids (Fritsche-Neto and Môro 2017). Nowadays, maize production in Brazil is predominantly carried out using single-cross and three-way cross hybrids, particularly transgenic cultivars with insect resistance and/or herbicide tolerance traits (Fritsche-Neto and Môro 2017; Souza 2018; Pereira Filho and Borghi 2022).

1.2.2 Maize germplasm

Maize breeding programs in Brazil typically utilize both tropical and temperate germplasm sources. Evidence suggests that tropical genetic materials offer superior grain quality and greater resistance or tolerance to the major pests and diseases affecting maize (Sharma and Carena 2012; Laude and Carena 2014; Samayoa et al. 2015; Kuki et al. 2018). In addition, tropical genotypes carry genes that offer enhanced tolerance to soil nutrient deficiencies and water stress (Kamara et al. 2014; Trachsel et al. 2016; Mengesha et al. 2017; Ribeiro et al. 2018; Mebratu et al. 2019; Nurmberg et al. 2022).

Over the years, breeding efforts for tropical germplasm have primarily focused on increasing tolerance to high plant population density. As a result, grain yield gains in tropical germplasm have been less pronounced compared to temperate germplasm (Edmeades et al.

2017; Cerrudo et al. 2020). Therefore, traits related to plant architecture such as reduced plant and ear height, narrower leaf angles and stalk angles, narrower leaves, and improved stalk strength are crucial for enhancing tolerance to high planting densities in tropical genotypes (Zheng and Liu 2013; Wei et al. 2018; Sandhu and Dhillon 2021). In contrast, breeding in temperate germplasm has led to significant improvements in both grain yield and tolerance to high plant densities with these genotypes also commonly exhibiting a high harvest index and earlier flowering (Di Matteo et al. 2016; Trachsel et al. 2016; Tokatlidis 2017).

In temperate germplasm, the main method for developing new inbred lines is through the recycling of lines derived from elite materials or breeding populations (Hallauer et al. 2010). In tropical germplasm, in addition to line recycling, it is also common to obtain new inbred lines by selfing commercial hybrids (Guimarães et al. 2018). The introduction of exotic germplasm into breeding programs can be a strategy to prevent the narrowing of the genetic base among the program’s lines. However, before exotic germplasm can be used for the development of new hybrids, a pre-breeding phase is required (Hallauer and Carena 2014). Pre-breeding involves the introduction, adaptation, evaluation, and improvement of germplasm sources for use in breeding programs (Hallauer and Carena 2009). In practice, commercial hybrids tend to have a narrower genetic base since breeders aim to develop new cultivars in less time; therefore, including a pre-breeding step is often not desirable, particularly in the private sector, which dominates the hybrid maize seed market in Brazil (Pereira Filho and Borghi 2022).

1.2.3 BS39 population

The lowland tropic landraces such as Cuban Flint, Suwan, Tusón, and Tuxpeño are the most widely used sources of exotic germplasm for introgression into temperate germplasm (Goodman, 1999). A new maize strain of Tusón germplasm adapted to the U.S Corn Belt environment is BS39, which can serve as a new and unique source for inbred line and population development (Hallauer and Carena 2016). This population was developed by the maize breeding program at Iowa State University and it is also undergoing a process of adaptation to short-season environments at North Dakota State University (Santos et al. 2022). Eleven cycles of stratified mass selection for early flowering were applied to this population to adapt it to temperate conditions. Throughout the selection cycles, date of silk emergence was the primary trait considered, but selection also considered plant and ear height, lodging, and absence to *Ustilago maydis* infection at time of silking (Hallauer and Carena 2016). According to Hallauer and Carena (2014), the evaluation of flowering time is very important for adapting germplasm, since this trait is one of the simpler traits for breeders to move maize to higher latitudes quickly and accurately with low cost.

1.2.4 Maize inbred lines

The choice of germplasm for the development of new inbred lines is a key factor for the success of a maize breeding program. The main sources for extracting superior lines include elite materials such as biparental populations derived from elites inbred lines within a heterotic group, synthetic populations, and commercial hybrids (Guimarães et al. 2018). Line recycling involves the use of elite inbred lines from the breeding program itself to form new populations that exhibit high mean performance for agronomically important traits, while maintaining genetic variability. To prevent a narrowing of the genetic base among recycled lines, the introduction of temperate or tropical germplasm into breeding programs is necessary (Hallauer and Carena 2009; Guimarães et al. 2018). Furthermore, in Brazil, legislation permits the use of any commercial hybrid as a source of variability for the development of new cultivars. As a result, conventional commercial hybrids serve as germplasm sources for the extraction of new inbred lines. However, according to the Brazilian Cultivar Protection Law (Brasil 1997), it is not permitted to use third-party cultivars as direct parents of new cultivars, and transgenic events may not be incorporated without prior agreements or contracts with their owners (Guimarães et al. 2018).

Until the late 2000s, maize breeding programs commonly employed successive self-pollinations to develop inbred lines, as this strategy enables rapid allele fixation (Hallauer et al. 2010; Guimarães et al. 2018). In this traditional process, the advancement of inbreeding increases genetic variability among individuals within the population and enhances the efficiency of selecting superior genotypes, since plants carrying unfavorable recessive alleles can be eliminated once they become homozygous at those loci (Hallauer et al. 2010). However, this method is both time-consuming and costly, requiring six to eight generations of selfing to achieve approximately 99% homozygosity (Prigge and Melchinger 2012; Guimarães et al. 2018). Consequently, from the mid-2000s onward, DH technology stood out in breeding programs, particularly among multinational companies (Prasanna 2012b; Guimarães et al. 2018; Chaikam et al. 2019).

DH technology enables the development of inbred lines in a shorter time compared to the traditional method and, consequently, allows for the faster development of new hybrids.

According to Prigge and Melchinger (2012), this technology involves four main steps: crossing with a haploid inducer to obtain haploid seeds; identifying the haploid seeds; chromosome doubling in these genotypes; and seed multiplication through self-pollination of the resulting plants. However, this method has limitations related to operational costs and low embryo germination rates (Humphreys and Knox 2015). Furthermore, DH technology is not widely employed in tropical maize breeding programs within the public sector in countries like Brazil, mainly due to limited financial resources and the lack of well-adapted haploid inducers for tropical growing conditions (Prasanna 2012b).

One of the main bottlenecks in DH production is the artificial genome doubling (AGD) step, which typically involves the use of chemical agents such as the highly toxic colchicine (Melchinger et al. 2013; Chaikam et al. 2019). AGD introduces additional stages to the DH pipeline, including sowing of putative haploids in a greenhouse, chemical treatment, and subsequent transplanting. An alternative strategy involves the use of genotypes with spontaneous haploid genome doubling (SHGD) capacity, which can simplify and accelerate DH line development (Wu et al. 2014; Boerman et al. 2020). However, genomic regions responsible for SHGD may be linked to undesirable traits, potentially causing linkage drag in derived DH lines. One effective way to compare whether potential genetic variability is affected by the DH process is to compare the DH lines with inbred lines developed by SSD breeding method (Bordes et al. 2007).

Hybrids result from crosses between genetically distinct inbred lines, as inbred lines are homozygous individuals with approximately 100% of their loci fixed in homozygosity. The primary objective of hybrid development is to harness heterosis, as hybrids are highly stable under high-yielding conditions and more responsive to management practices and input applications (Hallauer et al. 2010). Thus, the development of agronomically superior hybrids requires the use of elite inbred lines that outperform their parental lines, belong to well-defined and complementary heterotic groups, and are supported by a germplasm bank with broad genetic diversity (Guimarães et al. 2018; Fan et al. 2018). Breeding programs based on germplasm with a broad genetic base have the potential to contribute favorable alleles to cultivar development in the face of climate change and biotic stress (Carena and Bari 2018; Prasanna 2012a).

The development of inbred lines is one of the essential steps in breeding programs aimed at producing commercial hybrids. The history of maize hybrid development can be described according to the origin of the parental lines: lines derived directly from local populations between the 1930s and 1950s (Lu and Bernardo 2001); lines derived from artificial crosses between selected inbred lines during the 1950s to 1980s (Hallauer et al. 1988; Troyer 1990); and, from the 1980s onward, lines originating from elite inbred lines used commercially in hybrid formation (Troyer 1999). Therefore, the development of superior inbred lines from an appropriate germplasm source is directly related to the success of a breeding program focused on the production of commercial maize hybrids. Depending on the available resources, it is also important to adopt strategies that increase efficiency throughout this process, from the selection of parental lines for the formation of base populations to the identification of the best-performing inbred lines.

1.2.5 Heterotic groups

Heterotic groups are collections of germplasm that tend to exhibit higher levels of heterosis when crossed with germplasm from a different group, compared to crosses within the same group (Melchinger et al. 1989; Reif et al. 2005). Heterosis can be defined as the superior performance of hybrids relative to their parental lines (East 1908; Shull 1908). Maximum heterosis is typically expressed when crossing genetically contrasting inbred lines from different heterotic groups. Inbred lines that show high heterotic responses in crosses are allocated to different heterotic groups, whereas lines that do not exhibit significant heterosis are assigned to the same group (Hallauer et al. 2010). Therefore, identifying heterotic groups within maize breeding programs is fundamental for the development of heterotic and consequently, highly productive hybrids.

A heterotic pattern refers to the cross between previously characterized genotypes that exhibit high levels of heterosis, and it specifically denotes pairs of genotypes that express heterosis when crossed (Carena and Hallauer 2001; Reif et al. 2005). These patterns have been established based on the relationship between the parental origin and the heterosis expressed in their crosses (Hallauer et al. 1988). The correct assignment of an inbred line to a heterotic group is a prerequisite for the efficient use of germplasm, as it guides the selection of crosses between lines to obtain high-performing hybrids. Thus, the success of maize breeding aimed at developing single-cross hybrids is intrinsically linked to the classification of germplasm into heterotic groups and the identification of high-yielding heterotic patterns (Li et al. 2022).

Currently, some heterotic groups within maize germplasm are well established. In the United States and Canada, the predominant heterotic groups are Stiff Stalk (SS) and Non-Stiff Stalk (NSS) (Duvick et al. 2004). In the tropical germplasm, two major groups have been proposed: the non-Tuxpeño group, which includes races such as Cuban Flint, Coastal Tropical Flints (Caribbean Flint), Tuson, and ETO; and the Tuxpeño group (Vasal et al. 1999). In tropical regions, the most widely explored heterotic pattern by breeders is the Flint × Dent cross (Hallauer et al. 2010). Parentoni et al. (2001) proposed the first classification of heterotic groups in Brazil based on specific combining ability data from 28 open-pollinated varieties. Several methods have been reported in the literature for classifying inbred lines into heterotic groups, including the use of molecular markers and genetic diversity analyses (Grassi et al. 2015; Beckett et al. 2017; Faria et al. 2022), testers (Delucchi et al. 2012; Patil et al. 2021; Zebire et al. 2022), pedigree analysis (Boakyewaa Adu et al. 2019), and diallel analysis (Parentoni et al. 2001; Bidhendi et al. 2012; Badu-Apraku et al. 2013; Fan et al. 2014).

The number of required crosses limits the use of diallel analysis, thus this method is more commonly applied when only a few inbred lines with unknown heterotic group affiliations are being evaluated. When numerous populations or parental lines need to be assessed, testcrosses are more commonly employed (Fritsche-Neto et al. 2018). In this approach, lines are evaluated by crossing them with representative genotypes from each heterotic group, and classification is based on the performance of the resulting testcross hybrids. However, to implement this strategy, the breeding program must have representative testers for each heterotic group. When such groups are not well established, the program may use germplasm with a broad genetic base that has previously been identified as an effective discriminator of heterotic groups, or genotypes with high SCA (Miranda Filho 2018).

Molecular markers are widely used to assess genetic relatedness, population structure, and genetic diversity among inbred lines in many maize breeding programs worldwide (Grassi et al. 2015; Mengesha et al. 2017; Boakyewaa Adu et al. 2019; Belalia et al. 2019; Faria et al. 2022). However, the use of markers for assigning lines to heterotic groups has shown varying levels of success. The use of a limited number of markers hampers the discrimination of closely related lines, as such a small marker set may not include all loci responsible for heterotic divergence (Beckett et al. 2017). Nevertheless, combining genetic distance analyses based on molecular marker data with specific combining ability analyses and principal component analyses can be effective in identifying heterotic groups in breeding programs (Suwarno et al. 2014).

Ertiro et al. (2013) investigated the genetic purity, relatedness, and population structure of 265 maize inbred lines using 220,878 SNP markers. Based on marker data, the lines were classified into two heterotic groups, consistent with a classification previously established by the International Maize and Wheat Improvement Center (CIMMYT) using pedigree information and combining ability effects. Additionally, the authors recommended the use of high-density molecular markers in future studies for heterotic group assignment. Akinwale et al. (2014) evaluated the combining ability of 28 tropical inbred lines to classify them into heterotic groups. Based on SCA and GCA effects, the authors identified four heterotic groups under *Striga hermonthica*-infested conditions and five groups under non-infested conditions. Moreover, the heterotic groupings based on molecular markers were highly correlated with those based on specific combining ability under the non-infested environment. Fan et al. (2014) assessed combining abilities and reciprocal cross effects in a diallel involving twelve maize lines derived from temperate and tropical germplasm. The study showed that including reciprocal crosses in the diallel influenced both GCA and SCA effects. Furthermore, heterotic group classification was affected by the inclusion of reciprocal effects. Ultimately, the twelve lines were classified into three heterotic groups. Similarly, Badu-Apraku et al. (2015b) evaluated 91 hybrids derived from a diallel involving 14 early-maturing, quality protein maize inbred lines to study combining abilities and compare heterotic group assignment methods. Based on SCA effects, as well as GCA and SCA effects for grain yield, the lines were classified into three heterotic groups. However, grouping based on GCA effects for multiple traits and genetic distance from SNP markers resulted in two groups. There was strong agreement between methods regarding the genotypes assigned to the same group. Additionally, SNP markers proved effective in identifying representative testers for each heterotic group. Patil et al. (2021) conducted a testcross analysis involving twenty tropical maize inbred lines and three genetically divergent testers to evaluate combining abilities and assign lines to heterotic groups. In this study, based on SCA effects for grain yield, thirteen lines were classified into two distinct groups, three lines were assigned to a mixed group due to positive SCA effects with two testers, and four lines were not assigned to any group because they exhibited negative SCA effects with both testers.

Therefore, the use of genetic designs such as diallel crosses is an effective strategy for identifying parental lines with a high frequency of favorable alleles and the potential to generate hybrid combinations with high heterosis (Guimarães et al. 2018). Also, the parental lines identified in the best hybrid combinations can serve as standards for new heterotic groups or as testers for evaluating new germplasm.

1.2.6 Diallel crosses

Diallel crosses are genetic designs widely used in maize for studying the combining abilities of inbred lines and the inheritance of traits (Sughroue and Hallauer 1997; Cruz et al. 2012). The methodologies proposed by Hayman (1954), Griffing (1956), and Gardner and Eberhart (1966) are the most commonly used in plant breeding. Hayman (1954) introduced a method that provides estimates of the genetic values of the parents as well as insights into the genetic control of the traits under study. Gardner and Eberhart (1966) proposed a model for estimating genetic effects in varieties, while Griffing's (1956) method estimates general and specific combining ability (GCA and SCA) effects among inbred lines.

Diallel analyses as proposed by Griffing (1956) can be conducted depending on the inclusion of parental lines and/or reciprocal F_1 hybrids. Therefore, this methodology is classified into four methods: i) includes all possible combinations among genotypes; ii) excludes reciprocal F_1 hybrids; iii) excludes the parents; and iv) excludes both the parents and the reciprocal F_1 hybrids. Furthermore, this method provides information on the frequency of additive gene effects, which assists in identifying parental lines with a high frequency of favorable alleles to be used in breeding programs (Cruz et al. 2012).

According to Sprague and Tatum (1942), GCA refers to the average performance of an inbred line in hybrid combinations, while SCA corresponds to the performance of hybrids relative to what would be expected based on the average performance of their parents. According to Cruz et al. (2012), when GCA estimates are high either positively or negatively, the parent is either much superior or much inferior to the other parents included in the diallel, in terms of the average hybrid performance. In contrast, low GCA estimates indicate that the parent does not differ significantly from the overall mean of the diallel crosses. SCA estimates are associated with the importance of genes exhibiting non-additive effects. Therefore, positive SCA estimates indicate the presence of dominance deviations and heterosis in hybrid combinations between divergent parents. The greater the absolute value of the SCA, the greater the heterotic effect expressed in the hybrids (Cruz et al. 2012). The most desirable hybrid combinations are those that show high SCA effects between parents and exhibit high GCA effects (Guimarães et al. 2018).

Badu-Apraku et al. (2013) evaluated 190 single-cross hybrids derived from a diallel with 20 extra-early maize inbred lines. The authors observed that GCA effects were predominant over SCA effects for the traits studied, indicating that additive gene action was more important than non-additive effects. In addition, the inbred lines were classified into four heterotic groups based on SCA effects and into three groups based on molecular marker data and GCA effects. Werle et al. (2014) investigated combining ability and heterosis effects for grain yield, oil content, and protein content in tropical and temperate maize inbred lines. The significance of GCA and SCA effects indicated that both additive and non-additive genetic effects were involved in the control of the evaluated traits; however, estimates of the quadratic components revealed that non-additive effects were predominant.

Pádua et al. (2016) investigated the inheritance of resistance to fumonisin contamination in a set of hybrids derived from a diallel involving 13 inbred lines from the EMBRAPA breeding program. In this study, SCA effects were more relevant than GCA effects, indicating that dominance effects predominated in the expression of resistance to fumonisin contamination in tropical germplasm. In another study, aiming to evaluate disease resistance, Hung and Holland (2012) assessed combining abilities in a diallel involving 18 inbred lines. In this case, both GCA and SCA effects were important, but GCA played a more significant role in explaining hybrid resistance. Additionally, the *per se* performance of the inbred lines for resistance to *Fusarium* and fumonisin contamination showed high correlations with their GCA values in hybrid combinations. Therefore, evaluating inbred lines *per se* is a valuable reference for selecting parents in a breeding program.

Begum et al. (2018) conducted a diallel using seven inbred lines derived from CIMMYT to study the combining ability of various traits. In this study, substantial differences in GCA and SCA were detected for all traits except for one-thousand kernel weight. Murtadha et al. (2018) evaluated 15 hybrids derived from a diallel to determine GCA and SCA effects under different environmental conditions. This study also highlighted the importance of both additive and non-additive gene effects in controlling the evaluated traits. Moreover, the authors emphasized the importance of including at least one parent with high GCA effects to develop promising hybrids.

Olayiwola et al. (2021) assessed the combining abilities of 45 hybrids derived from white maize inbred lines. The results showed that additive gene action played a more important role than non-additive effects in the inheritance of those traits. The authors emphasized that genetic gains could be achieved more efficiently through recurrent selection for traits governed by additive effects, whereas heterosis-based breeding would be more appropriate for traits controlled by non-additive gene action.

1.2.7 Association mapping

Association mapping is an approach that accounts for thousands of polymorphisms to evaluate the effects of quantitative trait loci (QTL). It is an important instrument for identification of alleles and new genes as well as dissection of complex traits (Flint-Garcia et al. 2005; Ibrahim et al. 2020). This technique has been widely used to identify candidate genes involved in the genetic control of important traits in maize (Xiao et al. 2017). Using two bi-parental populations, Li et al. (2016) detected 21 QTL for plant height and ear height by QTL mapping, in which a QTL on Chr.1 and Chr.6 might be the candidate gene for plant height and ear height, respectively. Fei et al. (2022) identified 11 QTL for plant height and 13 QTL for ear height in F2:3 and recombinant inbred lines (RIL) populations of maize. The authors found three candidate genes within the three consistent QTL regions, all associated with the pathway of maize growth. Yang et al. (2014) used three independent RIL genotyped with SNP markers to conduct QTL analysis for kernel width, ear length and kernel number per row, which are traits related to yield. They reported six candidate genes for kernel width, one candidate gene for ear length and four candidate genes for kernel number per row. The flowering time variation is the result of the cumulative effect of several small QTL (Buckler et al. 2009; Dell'Acqua et al. 2015). Based on the literature, a total of 919 candidate genes, including cloned genes and homologs of other plants, have been reported for maize flowering time (Danilevskaya et al. 2008; Chen et al. 2012; Dong et al. 2012; Hung et al. 2012).

The QTL regions and candidate genes are effective starting points to unravel complex and important traits, and therefore SNPs associated with these traits can be used in genome prediction. Currently, phenotyping is a limiting factor to the efficiency of breeding conventional, as well as a bottleneck so that the molecular improvement can achieve its full potential. Thus, accurate characterization of germplasm under different field conditions combined with genomic data can improve prediction accuracy, resulting in increased selection gains in breeding programs.

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2. Chapter one

Combining ability and heterotic grouping among elite inbred lines among tropical maize from a public breeding program in Brazil

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Abstract

Diallel crosses are genetic designs widely used to estimate the general combining ability (GCA) and specific combining (SCA), identify superior parents and understand the inheritance of traits. Also, estimates of SCA effects for grain yield (GY) might be used to allocate breeding germplasm of maize into heterotic groups. Therefore, our maize proposal was to estimate GCA and SCA effects for a large set of traits among 15 elite inbred lines of tropical maize, allocate these inbred lines in heterotic groups based on single nucleotide polymorphism (SNP) markers and combining ability, and identify the high-yielding hybrids for tropical environments in Brazil. Fifteen elite maize inbred lines were crossed using a complete diallel design. The 105 hybrids and five checks were evaluated across four environments in the Minas Gerais in an alpha-lattice design with three replications. We evaluated a large set of traits related to plant architecture, yield components and grain morphology, and GY. The 15 inbred lines were genotyped with 3,713 SNPs and after quality control, 3,083 SNPs were selected for genetic diversity analysis. The genetic distance between the genotypes was estimated by the Roger distance modified by Wright, and a dendrogram was constructed using the unweighted pair-group method using an arithmetic average (UPGMA) cluster analysis. Moreover, a dendrogram was constructed using the UPGMA method based on estimates of SCA for GY. We found differences for GCA and SCA ($P<0.05$) for all traits across environments. Estimates of additive genetic variance component ($\hat{\sigma}^2_A$) were much greater than estimates of dominance genetic variance component ($\hat{\sigma}^2_D$) for all plant architecture, grain morphology traits, and some yield components. Conversely, the $\hat{\sigma}^2_D$ values were greater than $\hat{\sigma}^2_A$ for number of kernels per ear (NKE), ear length (EL) and GY. Therefore, for all plant architecture and grain morphology traits, we need to use approaches that capitalize on the additive effects of the parents, such as recurrent selection, while for NKE, EL and GY we need to adopt hybridization strategies to exploit heterosis. The inbred lines VML016, VML062, VML081, VML083 and VML131 could be promising parents to develop breeding populations, new hybrids and open-pollinated varieties of tropical maize targeting tropical environments. We identified four groups based on SCA estimates for GY and SNP markers, with a strong concordance regarding the inbred lines of each group. Finally, we identify eight experimental hybrids with potential to be recommended to crop in Southeastern Brazil.

Keywords *Zea mays* L.; Diallel crosses; Genetic diversity; Tropical maize germplasm.

2.1 Introduction

Maize (*Zea mays* L.) is a major global cereal crop, essential for human nutrition, livestock feed and biofuel production, and Brazil is the third largest maize producer worldwide after the United States and China (Serna-Saldivar 2023; USDA 2025). In Brazil, even though maize is grown across tropical and temperate environments, more than 75% of the production occurs under tropical conditions (CONAB 2025). In these environments, maize production is substantially constrained by biotic and abiotic stress, such as diseases, pests, heat, drought, salinity and soils with mineral deficiencies, especially nitrogen (N) and phosphorus (Vergutz et al. 2017; Tesfaye et al. 2018; Prasanna et al. 2021; Von Pinho et al. 2022). Furthermore, these factors frequently occur in combination, intensifying the negative impact on maize production. Consequently, Brazilian maize yield (5.65 Mg ha^{-1}) remains low compared to the United States (11.26 Mg ha^{-1}), where maize is produced in temperate environments (USDA 2025). Thus, the maize breeding programs in Brazil has focused their efforts on the development well adapted cultivars to stress conditions for increasing maize yield across tropical environments, and consequently, ensuring the security of food production in developing tropical countries (Rezende et al. 2020; Prasanna et al. 2021; Yousaf et al. 2022).

In tropical maize germplasm, heterotic patterns are still unclear compared to the well-established heterotic pattern of Stiff Stalk and Non-Stiff Stalk found in temperate maize germplasm (Dinesh et al. 2016; Wu et al. 2016; Ertiro et al. 2017; Trevisan 2018; Akinwale 2021; Pal 2024). Most of the tropical maize inbred lines were originally derived from broad germplasm pools, without prioritizing the preservation of heterotic patterns (Kim et al. 1999). Furthermore, the self-pollination of commercial hybrids, allowed in several countries, has been a strategy widely adopted for generating new inbred lines around the world, particularly in Brazil (Guimaraes et al. 2018; Trevisan 2018). Consequently, tropical maize inbred lines have a complex and mixed genetic background, which makes it difficult to allocate them to heterotic groups. Also, the classification of tropical maize inbred lines into heterotic groups began only three decades ago by the International Maize and Wheat Improvement Center (CIMMYT) based on the increasing maize hybrid adoption in tropical environments (Wu et al. 2016; Ertiro et al. 2017). Currently, hybrid maize occupies over 95% of the maize production area in Brazil, leading all national breeding programs to focus their efforts on the development of hybrid cultivars.

Information on the combining ability of inbred lines enables breeders to assess and compare the performance of new lines in hybrid combinations, while also contributing to a better understanding of the gene action involved in the inheritance of traits. Therefore, estimates of general combing ability (GCA) and specific combining ability (SCA) have been used for genetic diversity evaluation, inbred line selection, heterotic pattern classifications, heterosis estimation and hybrid development (Akinwale et al. 2014, Annor and Badu-Apraku 2016, Badu-Apraku et al. 2016, Gichuru et al. 2016, Zhou et al. 2018, Ribeiro et al. 2020, Faria et al. 2022; Luz et al. 2024, Bhatla et al. 2025).The GCA and SCA effects are primarily used as key parameters for selecting maize inbred lines in breeding programs. Gichuru et al. (2016) evaluated twelve tropical maize inbred lines using a half-diallel mating design and reported a predominance of non-additive gene effects in the inheritance of grain yield. The study also revealed strong correlations between genetic distance and both SCA and heterosis estimates, and, consequently, they observed a high concordance among inbred lines grouping based on SCA estimates, molecular markers and heterosis effects. In another study, Badu-Apraku et al. (2016) used the combining ability estimates to identify the best inbred lines and high-yielding hybrids under multiple stress environments. The authors found two inbred lines that could be used as testers for grouping other lines in tropical maize hybrid breeding programs, and five outstanding hybrids in yield and stability that could be commercialized in the subregion of Africa. Recently, Bhatla et al. (2025) also investigated the combining ability, heterosis and heterotic group of Indian maize inbred lines to enhance hybrid performance. They found two testers suitable for classifying other Indian maize inbred lines and emphasized that the heterotic grouping can help breeders avoid unnecessary test crosses, thus saving time and resources.

The classification of inbred lines into heterotic groups is essential to the success of maize hybrid breeding programs since it guides breeders to plan crosses between lines from distinct groups, aiming to develop high-yielding hybrids. Heterotic grouping can be performed through several methods, including pedigree information, genetic diversity based on molecular markers and phenotypic traits, population structure analysis, as well as combining ability estimates obtained from some genetic designs like diallel crosses, topcrosses, line-by-tester and North Carolina Design II (Aslam and Zafar 2020; Akinwale 2021; Faria et al. 2022; Li et al. 2022; Pal 2024). Since the beginning of heterotic group classification, pedigree analysis has been used to group inbred lines based on their ancestral relationships and genetic backgrounds. The Iowa Stiff Stalk Synthetic and Lancaster Sure Crop groups, the most extensively utilized heterotic groups, were initially defined based on pedigree and geographical analysis (Meena et al. 2017). Phenotypic clustering holds significant importance, especially when integrated with pedigree information and combining ability analysis (Mosa et al. 2017). The mating designs line-by-tester and diallel are prominent for assigning parental lines to heterotic groups through estimates of GCA and SCA of inbred lines (Akinwale 2021). In breeding programs with validated testers, the line-by-tester design is commonly adopted, however, when validated testers are unavailable the diallel design becomes a more suitable alternative. Also, population structure analysis is an efficient method for identifying the origin of complex or unknown maize germplasm provided that a sufficient number of molecular markers is used (Zhang et al. 2016). The use of molecular markers has provided a more efficient, less labor-intensive, and cost-effective alternative for heterotic grouping (Aslam and Zafar 2020; Akinwale 2021). Currently, SNPs are highly recommended for several molecular studies due to reduced chances of genotyping error, high genomic abundance, ability of high-throughput analysis and cost efficiency (Wu et al. 2016). In conclusion, no single approach provides a complete understanding of heterotic grouping. Therefore, the combination of pedigree information, combining ability analysis, phenotypic and genotypic clustering is the most effective approach for developing and identifying heterotic groups (Aslam and Zafar 2020; Akinwale 2021; Pal 2024).

In the early 1930s, the Universidade Federal de Viçosa (UFV), located in Viçosa (lat. 20°45’14"S; long. 42°52’55"W; alt. 648 m a.s.l.), Minas Gerais, started its maize breeding program. In 1938, UFV released the first commercial maize hybrid in Brazil, based on the research of Professors Gladstone Drummond and Antônio Secundino. In 1945, both researchers resigned from UFV to establish Agroceres, a private Brazilian seed company that was a leader in the national market in commercial maize hybrids for several decades (Moro 2018). After a long period of inactivity, the UFV maize breeding program was reestablished in the late 1990s, becoming the second largest public maize breeding program nowadays in Brazil (Faria et al. 2022). Over the past two decades, the program has developed a germplasm collection comprising 182 inbred lines, which reflect the current genetic base of the UFV breeding pool. Most of those lines were derived from commercial hybrids previously cultivated in the region, and, consequently, they represent a mixture of heterotic groups with a complex genetic background. To elucidate the heterotic group relationships among inbred lines in the UFV breeding program, an initial set of 15 inbred lines was selected. Hallauer et al. (2010) suggest that testcrosses are a more efficient strategy when evaluating a large number of populations or parental lines. Since our program includes 182 inbred lines to be evaluated, Ribeiro (2021) performed testcrosses using the elite testers VML024, VML090, and VML144. These testers were selected for their well-defined grain types, VML024 with flint kernels, and VML090 and VML144 with dent kernels, as well as their high general combining ability for grain yield and good pollen production, desirable traits in testers. Therefore, the 15 inbred lines were selected based on both their testcross performance and *per se* evaluation.

Our main proposal was to assess the combining ability for a large set of traits among 15 elite inbred lines of tropical maize from UFV maize breeding program and group them into heterotic groups. Thus, specific objectives were to: i) estimate the general and specific combining abilities for a large set of traits among 15 elite inbred lines across tropical environments in Brazil; ii) identify inbred lines with good combining ability for hybrids and breeding populations development; iii) investigate the traits associations based on means of hybrids and GCA effects; iv) group 15 elite inbred lines into heterotic groups using estimates of SCA effects of grain yield and SNP-based methods v) assess the performance of inbred lines in hybrid combinations and identify the high-yielding hybrids to be cropped by Brazilian growers maize.

2.2 Material and methods

2.2.1 Genetic materials

Fifteen tropical maize inbred lines were selected based on their *per se* performance for the cycle, plant architecture traits, disease and pest tolerance and also their yield performance on testcrosses with three elite testers: the inbred lines VML024, VML090 and VML144 (Ribeiro 2021; Table 1). These 15 selected inbred lines were crossed using a diallel mating design according to Griffing's method 4 (Griffing 1956) during the 2018/19 season. Each inbred line was used as both male and female, and seeds of reciprocal crosses were bulked to form 105 F_1 crosses. All inbred lines belong to Programa Milho®, the maize breeding program of the Department of Agronomy of UFV, Minas Gerais, Brazil.

Table 1. Description of the 15 elite inbred lines of tropical maize used in our study

Inbred line	Population source	Subpopulation[1]	Testers
VML081	A3663	2	VML024
VML083	30F53	3	
VML134	BRS1010	3	
VML154	DKB390	3	
VML176	5011	2	
VML055	P3041	2	VML090
VML062	Z8480	1	
VML105	C333	3	
VML140	Z8420	1	
VML157	A3663	3	
VML004	Balu184	2	VML144
VML016	DKB435	1	
VML066	P30F90	1	
VML115	AL30	1	
VML131	Z8447	1	

[1] Subpopulations based on STRUCTURE software using SNPs markers (Faria et al. 2022).

2.2.2 Trial management and experimental design

The 105 experimental hybrids obtained from diallel cross of the 15 inbred lines along with five checks commercial hybrids (total of 110 maize hybrids) were evaluated in the Experimental Station of UFV in Viçosa (20°45'S, 42°49'W, 661 m asl) during the 2021/22 summer season and in the Experimental Station of UFV in Coimbra (20°50'S, 42°48'W, 713 m asl) during the 2019/20, 2020/21 and 2021/22 summer seasons (total of four environments in southeastern Brazil). All experiments were sown under no-tillage conditions. Fertilizer rates were the same and, in all environments, 100 kg ha^{-1} of P_2O_5, 60 kg ha^{-1} of K_2O, and 20 kg ha^{-1} of N were applied before sowing. Urea (43% N) was applied as a top-dressing fertilizer at a rate of 380 kg ha^{-1} around the V4 and V6 growth stages (Abendroth et al. 2011). The experiments were conducted in rain-fed conditions, and no irrigation was applied. In all experiments, the weeds were controlled with pre-emergence herbicide followed by post-emergence herbicide applications using standard agronomic practices. Other trials management were the same for all experiments. All maize seeds were treated similarly with CropStar® (imidacloprid, thiocarb) and Maxim Advanced® (metalaxyl-M, thiabendazole, fludioxonil).

In all environments, the 110 maize hybrids were laid out in an 11 x 10 alpha lattice incomplete block design with three replications. Each plot was a single 4.0 m row, with rows spaced 0.8 m apart. Seeds were sown in late October 2019, 2020 and 2021. Initially, plots were over-seed with hand planters and then thinned at the V4 stage to stands of 62,500 plants ha^{-1}.

2.2.3 Trait measurements

We evaluated a large set of traits related to plant architecture, yield components and grain morphology, and finally grain yield. For plant architecture, we measured eight traits: days to pollen (DTP), days to silking (DTS), ear leaf area (LA, cm^2), plant height (PH, cm), ear height (EH, cm), stalk diameter (SD, mm), and above and below ear node number (AENN and BENN, respectively). For the yield components, we measured five traits: ear length (EL, cm), number of kernel rows (NKR), number of kernels per ear (NKE), ear diameter (ED, mm), and one thousand-kernel weight (TKW, g) as the weight of 1,000 kernels adjusted to 145 g kg^{-1} moisture. For the grain morphology, we measured five traits: grain perimeter (GP, mm), grain area (GA, mm^2), grain length (GL, mm) and grain width (GW, mm). These last set of traits were measured by ImageJ (Schneider et al. 2012) using a sample of one hundred kernels of each plot. Finally, grain yield (GY) was recorded for all ears in the plots at physiological maturity. The ears were shelled, the grain weight and grain moisture percentage were recorded, and GY (kg ha^{-1}) was calculated at 145 g kg^{-1} moisture.

2.2.4 Molecular markers

For molecular characterization, we genotyped the 15 inbred lines used in the diallel cross along with other inbred lines belonging to UFV maize breeding (Faria et al. 2022). Briefly, we obtained leaf tissue samples from the bulk of five plants for each inbred line and we sent them to DuPont Pioneer® Company, where DNA extraction and genotyping were performed. All inbred lines were genotyped using the GoldenGate platform (Illumina, San Diego, CA, USA), containing 3,713 single SNPs distributed across 10 chromosomes (Fan et al., 2013). Out of the 3,713 SNPs genotyped, a set of 3,083 SNPs with a minor allele frequency (MAF) greater than 0.01 and a 90% call rate was selected for genetic characterization analysis of tropical maize inbred lines from UFV maize breeding (Faria et al. 2022).

2.2.5 Statistical analysis

2.2.5.1 Analysis of hybrid performance

We performed a mixed model analysis for each agronomic traits across the four environments using the R package "ASReml" (Butler et al. 2018). Hybrid, environment and replication were considered fixed effects, whereas blocks within replications were considered random effects. The phenotypic values were modeled according to following Equation: $y_{ijkl} = \mu + g_i + e_l + ge_{il} + r_{(j)l} + b_{(k)jl} + \varepsilon_{ijkl}$, where y_{ijkl} is the phenotypic value of i^th^ hybrid in kth block within jth replication at lth environment; μ is the general mean; g_i is the fixed effect of ith hybrid; e_l is the fixed effect of lth environment; ge_{il} is the fixed effect of genotype-by-environment interaction; $r_{(j)l}$ is the fixed effect of jth replication within lth environment; $b_{(k)jl}$ is the random effect of kth block within jth replication at lth environment, with $b_{(k)jl} \sim N(0, \sigma_b^2)$; and ε_{iljk} is the random effect of error, with $\varepsilon_{iljk} \sim N(0, \sigma_\varepsilon^2)$. The Wald test was used to test fixed effects via the chi-square statistic (Gouriéroux et al. 1982). We estimated Pearson correlations between pairs of adjusted means of experimental hybrids for the traits using the R package "stats" (R Core Team 2022).

2.2.5.2 Diallel analysis

We fitted a model for genomic best linear unbiased prediction (GBLUP) based on SNP average effects of substitution (α) and dominance deviations (δ) according to Equation: $y_{ij} = \mu + Z_{\alpha ij}\alpha + Z_{\delta ij}\delta + \varepsilon_{ij}$, where y_{ij} is the genotypic value of SNP genotype A_iA_j (i,j = 1,2); μ is the general mean; $Z_{\alpha 11} = 2 - 2p$ for A_1A_1, $Z_{\alpha 12} = 1 - 2p$ for A_1A_2, and $Z_{\alpha 22} = -2p$ for A_2A_2; $Z_{\delta 11} = -2q^2$ for A_1A_1, $Z_{\delta 12} = 2pq$ for A_1A_2, and $Z_{\delta 22} = -2p^2$ for A_2A_2, where p = frequency of A_1 allele and q = frequency of A_2 allele; and ε_{ij} is the effect of error of SNP genotype A_iA_j (i,j = 1,2). We used a GBLUP parametrization, and the additive and dominance relationship matrices among 15 maize inbred lines were estimated from SNP markers based on method proposed by VanRaden (2008) and Vitezica et al. (2013), respectively.

In the first stage, we fitted a model for each trait in each environment and season according to the following Equation: $y_{ijk} = u + g_i + r_j + b_k + \varepsilon_{ijk}$, where y_{ijk} is the phenotypic value of ith hybrid within kth block at jth replication; u is the constant; g_i is the fixed effect of ith hybrid; r_j is the fixed effect of jth replication; b_k is the random effect of kth block with $b_k \sim N(0, \sigma_b^2)$, and ε_{ijk} is the random effect of error with $\varepsilon_{ijk} \sim N(0, \sigma_\varepsilon^2)$. In the second stage, we fitted a joint diallel model for each trait across all environments using the best linear unbiased estimate (BLUE) of each hybrid estimated in the first stage and associated weights, according to the following Equation: $y_{ijl} = \mu + g_{ij} + s_{ij} + e_l + ge_{ijl} + se_{ijl} + \varepsilon_{ijl}$, where y_{ijl} is the phenotypic value of hybrid combination between the ith and jth parents within lth environment; μ is the general mean; g_{ij} is the random effect of GCA of ith or jth parent, with $g_{ij} \sim (0, \sigma_{GCA}^2)$; s_{ij} is the random effect of SCA of hybrid combination between the ith and jth parents, with $s_{ij} \sim (0, \sigma_{SCA}^2)$; e_l is the fixed effect of lth environment; ge_{ijl} is the random effect of GCA x environment interaction, with $ge_{ijl} \sim (0, \sigma_{GCAxE}^2)$; se_{ijl} is the random effect of SCA x environment interaction, with $se_{ijl} \sim (0, \sigma^2_{SCAxE})$; and ε_{ijl} is the random effect of error with $\varepsilon_{ijk} \sim N(0, \sigma^2)$. The joint diallel analyses were performed based on the mixed models approach using the R package “ASReml” (Butler et al. 2018). A likelihood ratio test was used to test random effects via the chi-square statistics (Neyman and Pearson 1928).

Based on the variance components of GCA (σ^2_{GCA}) and SCA (σ^2_{SCA}) across environments, we estimated the additive (σ^2_A) and dominance (σ^2_D) components of genetic variance across environments as follows (Hallauer et al. 2010): $\sigma^2_A = 2\sigma^2_{GCA}$ and $\sigma^2_D = \sigma^2_{SCA}$. Then, broad-sense heritability ($H^2_{\bar{X}}$) and narrow-sense ($h^2_{\bar{X}}$) were estimated as follows (Hallauer et al. 2010): $H^2_{\bar{X}} = \frac{(\sigma^2_A + \sigma^2_D)}{(\sigma^2_A + \sigma^2_D + \sigma^2)}$, and $h^2_{\bar{X}} = \frac{\sigma^2_A}{(\sigma^2_A + \sigma^2_D + \sigma^2)}$, where σ^2_A and σ^2_D are the additive and dominance genetic variance components of the genetic variance, respectively, and σ^2 is residual variance. The proportion of genotypic variance among single-cross hybrids (sch) due to σ^2_{GCA} was calculated as follows (Baker 1978): $\sigma^2_{sch/GCA} = \frac{2\sigma^2_{GCA}}{(2\sigma^2_{GCA} + \sigma^2_{SCA})}$. It indicates the predictability of the hybrids performance based only on the GCA effects. Also, we estimated Pearson’s correlation coefficients between tested traits using the estimates of adjusted means of experimental hybrids, and the estimates of GCA effects of elite inbred lines. Correlations coefficients were estimated using the cor.test function in the R package “stats” (R Core Team 2022).

2.2.5.3 Heterotic grouping

The 15 tropical maize inbred lines were assigned into heterotic groups based on genetic distance (GD) from SNP markers and estimates of SCA effects for GY across all environments. In the SNP-based GD method, the GD among inbred lines was estimated from 3,083 SNPs markers according to modified Roger’s genetic distance (MRD; Goodman and Stuber 1983) as follows: $MRD = \sqrt{\frac{\sum_{i=1}^{m}\sum_{j=1}^{a_i}(p_{ij} - q_{ij})^2}{2m}}$, where p_{ij} and q_{ij} are the allele frequencies of the jth allele at the ith locus in the inbred lines in each pair, a_i is the number of alleles at the ith marker, and m is the number of marker loci. The MRD was estimated using the R package “adegenet” (Jombart 2008). Then, we used the MRD matrix to cluster the 15 tropical maize inbred lines based on the Unweighted Pair Group Method using Arithmetic Averages (UPGMA; Sokal and Michener 1958). In the SCA method, the estimates of SCA effects for GY across all environments were considered as genetic distance among the inbred lines and used to cluster the 15 inbred lines based on UPGMA dendrogram (Sokal and Michener 1958). Both UPGMA dendrograms were generated using the R package “ape” (Paradis and Schliep 2019). In both dendrograms, the Mojena method (Mojena 1977) was used to allocate the inbred lines into heterotic groups. According to this method, the dendrogram must be cut as a function of the mean value of the genetic distance of fusion levels and the standard deviation of the distance values.

2.3 Results

2.3.1 Ranges, means, analysis of variance and correlations among traits

We observed wide ranges among the adjusted means of hybrids for most traits across all environments, mainly for GY, which exhibited a range of approximately 10,600 kg ha^{-1} (it ranged from 1,840 to 12,500 kg ha^{-1}; Table 2). DTP showed a range of around 14 days, whereas DTS ranged 17 days. PH ranged from 212.4 to 270.8 cm and EH ranged from 110 to 160.3 cm, whereas the ranges for BENN and AENN were very small – three and one nodes, respectively. SD exhibited a range of 6 mm, and LA varied by 470 cm^2. Regarding yield components, the ranges for EL and ED were 6 cm and 26 cm, respectively, NKR ranged approximately five kernel rows, and NKE varied by 360 kernels per ear. The ranges for GA and GP were 69.5 to 112.6 mm^2 and 33.3 to 42.9 mm, respectively, while GL ranged from 10.9 to 14.6 mm and GW ranged from 8.6 to 10.7 mm. Finally, TKW exhibited a range of approximately 170 g.

Joint analysis of variance across the four environments showed that experimental hybrid, check hybrid, and environment were highly significant ($P<0.001$) for all 18 tested traits by Wald test (Table 2). Also, experimental hybrid x environment interaction was highly significant ($P<0.001$) for almost all traits, except for AENN, EL and ED. The coefficient of variation (CV) ranged from 1.53% (DTP) to 13.5% (GY) and presented very low values (CV values < 7.0%) for most traits, mainly for plant architecture and grain morphology traits.

Most Pearson’s correlation coefficients using the estimates of adjusted means of experimental hybrids were non-significant ($P>0.10$) or presented low magnitude (Table 3, Fig. 1A). However, the strongest and positive correlations were observed between traits within the same group: DTP and DTS ($\hat{r} = 0.90$) for cycle traits, BENN and EH ($\hat{r} = 0.88$) for plant stature, GP and GA ($\hat{r} = 0.97$), GL and GA ($\hat{r} = 0.81$), GL and GP ($\hat{r} = 0.92$), GW and GA ($\hat{r} = 0.88$), and GW and GP ($\hat{r} = 0.75$) for grain morphology. Also, we found strong and positive correlations between traits from different groups: TKW and GA ($\hat{r} = 0.84$), TKW and GP ($\hat{r} = 0.76$), and TKW and GW ($\hat{r} = 0.79$); and a moderate correlation between two yield components: NKE and NKR ($\hat{r} = 0.71$). Finally, GY presented negative correlations with DTP ($\hat{r} = -0.29$)

and DTS (r̂ = -0.34), but it was positively correlated with LA (r̂ = 0.21), EL (r̂ = 0.42), ED (r̂ = 0.28), NKE (r̂ = 0.48), GA (r̂ = 0.33), GP (r̂ = 0.37), GL (r̂ = 0.44) and TKW (r̂ = 0.32).

Table 2 Estimates of variance components for blocks within replications and mean squares from the joint analysis, means, ranges (minimum and maximum) and coefficient of variation (CV%) for 18 agronomic traits measured in 105 experimental and five checks hybrids of tropical maize across four environments in Brazil

Source of variation	df	DTP[1/]	DTS	PH	EH	BENN	AENN
Block/Rep/Env		0.27**[2/]	0.23**	25.6**	13.4**	0.04**	0.04**
Rep/Env	8	1.13	0.25	72.6	118.3*	2.20**	1.89**
Hybrid	109	40.39**	53.94**	1,542.1**	1,082.9**	2.84**	0.81**
Experimental (Exp)	104	32.82**	43.49**	1,549.8**	1,054.2**	2.88**	0.81**
Check	4	95.00**	99.50**	1,097.0**	1,759.5**	1.73**	1.03**
Exp. x Check	1	1.00	26.00**	2,709.0**	1,251.0**	2.90**	0.10
Environment (Env)	3	2,314.66**	2,479.67**	5,717.3**	4,887.7**	4.47**	2.07**
Exp. *vs* Env	312	3.15**	2.13**	70.9**	69.5**	0.27**	0.19
Residual		1.10	1.11	51.5	51.9	0.21	0.17
CV(%)		1.53	1.55	2.95	5.44	6.03	6.63
Minimum		63.6	62.5	212.4	110.0	6.5	5.7
Mean		68.5	68.1	243.0	132.5	7.5	6.3
Maximum		78.0	80.0	270.8	160.3	9.3	7.0
	df	SD	LA	EL	ED	NKR	NKE
Block/Rep/Env		0.28**	475.6**	0.09	1.0	0.04*	117.9**
Rep/Env	8	5.13*	3,938.9	2.00	71.5*	0.50	2,575.0
Hybrid	109	11.79**	62,430.4**	13.83**	94.0**	11.29**	31,199.0**
Experimental (Exp)	104	11.94**	64,605.3**	13.88**	93.4**	11.57**	32,547.4**
Check	4	9.50**	26,759.0**	7.25*	79.8	6.75**	3,594.5
Exp. *vs* Check	1	0.00	129.0	27.00**	208.0*	1.00	776.0
Environment (Env)	3	125.00**	4,468,786.3**	339.00**	560.7**	7.67**	237,527.7**
Exp. x Env	312	3.02**	5708.1**	2.61	33.9	0.99**	2,740.5**
Residual		2.33	3832.0	2.51	34.3	0.74	1,997.3
CV(%)		6.90	6.44	9.92	12.29	5.71	8.13
Minimum		19.3	761.4	12.4	36.8	12.3	317.2
Mean		22.1	960.5	16.0	47.6	15.1	549.6
Maximum		25.2	1231.1	18.4	62.6	17.1	677.2
	df	GA	GP	GL	GW	TKW	GY
Block/Rep/Env		12.8**	1.12**	0.07**	0.03**	52.0**	398,512**
Rep/Env	8	196.8**	16.50**	1.39**	0.70**	542.5	889,413
Hybrid	109	629.5**	27.42**	4.23**	2.07**	1,2032.9**	15,611,009**
Experimental (Exp)	104	592.3**	25.06**	3.91**	2.11**	11,922.0**	15,373,077**
Check	4	185.5**	8.25**	1.68**	0.90**	2,214.8**	10,764,250**
Exp. *vs* Check	1	6,235.0**	348.00**	47.70**	3.10**	63,277.0**	49,693,000**
Environment (Env)	3	701.3**	87.33**	7.17**	3.03**	27,764.3**	108,176,667**
Exp. x Env	312	23.8**	1.47**	0.17**	0.07**	976.6**	2,324,601**
Residual		11.0	0.72	0.08	0.04	598.6	1,667,285
CV(%)		3.47	2.15	2.06	2.00	7.55	13.5
Minimum		69.5	33.3	10.9	8.6	242.0	1,840
Mean		95.6	39.6	13.4	9.6	324.0	9,550
Maximum		112.6	42.9	14.6	10.7	410.8	12,500

[1/] DTP: days to pollen (days); DTS: days to silking (days); PH: plant height (cm); EH: ear height (cm); BENN: bellow ear node number; AENN: above ear node number; SD: stalk diameter (mm); LA: leaf area (cm^2); EL: ear length (cm); ED: ear diameter (mm); NKR: number of kernel rows; NKE: number of kernels per ear; GA: grain area (mm^2); GP: grain perimeter (mm); GL: grain length (mm); GW: grain width (mm); TKW: one thousand kernel weight (g), and grain yield (GY, g ha^{-1}). [2/] **, * Significant at P=0.01 and P=0.05, respectively, by Wald test.

Fig. 1. Correlation network between pairs of adjusted means of experimental hybrids for 18 agronomic traits **(A)** and between estimates of general combining effects for 18 agronomic traits of 15 tropical maize inbred lines **(B)** across four environments in Brazil. Red and green lines represent negative and positive correlations, respectively. Line width is proportional to the strength of the correlation. DTP: days to pollen (days); DTS: days to silking (days); PH: plant height (cm); EH: ear height (cm); BEN: bellow ear node number; AEN: above ear node number; SD: stalk diameter (mm); LA: leaf area (cm^2); EL: ear length (cm); ED: ear diameter (mm); NKR: number of kernel rows; NKE: number of kernels per ear; GA: grain area (mm^2); GP: grain perimeter (mm); GL: grain length (mm); GW: grain width (mm); TKW: one thousand kernel weight (g), and grain yield (GY, kg ha^{-1}).

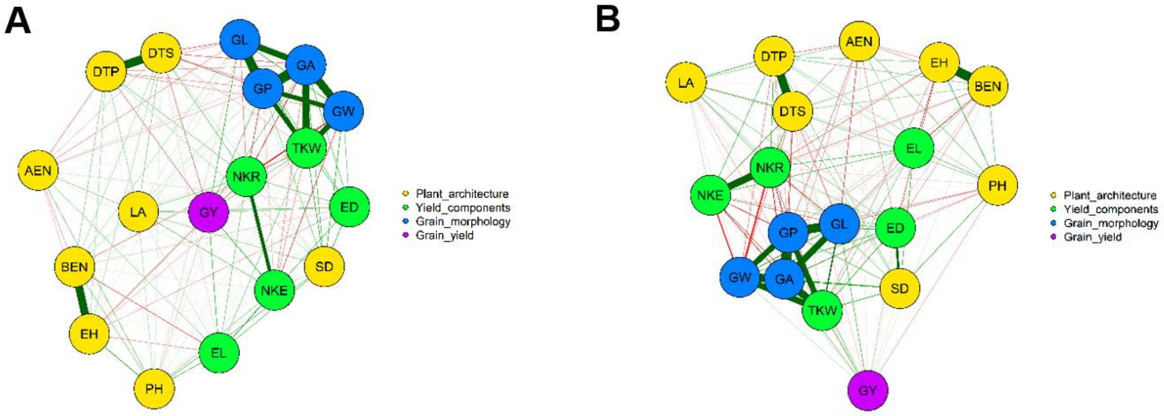

Table 3. Estimates of Pearson correlation coefficients between traits based on estimates of adjusted means of experimental hybrids (above diagonal) and general combining ability effects (below diagonal) of elite inbred lines of tropical maize across four environments in Brazil

Traits	DTP[1/]	DTS	PH	EH	BENN	AENN	SD	LA	EL	ED	NKR	NKE	GA	GP	GL	GW	TKW	GY
DTP		**0.90***[2/]	0.05	0.20*	0.25*	-0.27*	0.17	0.23*	-0.03	-0.04	0.28*	0.03	-0.21*	-0.24*	-0.20*	-0.22*	0.01	-0.29*
DTS	**0.90***		0.08	0.19*	0.20*	-0.13	0.14	0.26*	-0.10	-0.03	0.33*	0.01	-0.35*	-0.40*	-0.39*	-0.27*	-0.15	-0.34*
PH	0.20	0.27		0.52*	0.28*	0.31*	0.36*	0.16	0.42*	-0.06	-0.01	0.25*	-0.04	-0.07	-0.12	0.04	0.07	0.18
EH	0.28	0.30	0.47		**0.88***	-0.15	0.03	0.05	-0.23*	-0.20*	-0.24*	0.05	0.01	0.03	0.07	-0.01	-0.15	0.13
BENN	0.28	0.24	0.32	**0.94***		-0.21*	0.03	0.02	-0.38*	-0.14	-0.24*	-0.06	0.09	0.13	0.17	0.05	-0.12	0.06
AENN	-0.32	-0.13	0.30	-0.23	-0.24		0.04	0.27*	0.22*	-0.10	0.02	-0.05	-0.12	-0.17	-0.23*	0.01	0.04	0.01
SD	0.26	0.24	0.43	-0.02	-0.01	0.01		0.26*	0.35*	0.47*	0.10	0.15	0.37*	0.28*	0.09	0.47*	0.47*	0.04
LA	0.46	0.51	0.01	-0.02	0.03	0.28	0.23		0.12	0.28*	0.31*	0.32*	0.03	0.05	0.10	-0.04	0.10	0.21*
EL	0.20	0.12	0.38	-0.47	-0.50	0.27	0.44	-0.10		0.19	0.29*	0.53*	0.05	0.03	0.01	0.01	0.29*	0.42*
ED	0.09	0.07	-0.26	-0.36	-0.22	-0.22	**0.66***	0.28	0.13		0.32*	0.32*	0.39*	0.41*	0.35*	0.29*	0.35*	0.28*
NKR	0.44	0.48	-0.09	-0.32	-0.30	0.03	0.09	0.31	0.41	0.26		**0.71***	-0.51*	-0.44*	-0.29*	**-0.61***	-0.31*	0.12
NKE	0.42	0.39	0.04	-0.13	-0.09	-0.18	0.10	0.15	0.39	0.25	**0.85***		-0.25*	-0.15	0.02	-0.45*	-0.25*	0.48*
GA	-0.19	-0.35	-0.14	-0.04	0.10	-0.22	0.43	-0.07	-0.09	0.50	-0.60*	-0.54*		**0.97***	**0.81***	**0.88***	**0.84***	0.33*
GP	-0.19	-0.38	-0.22	-0.02	0.14	-0.31	0.31	-0.08	-0.17	0.49	-0.57*	-0.47	**0.97***		**0.92***	**0.75***	**0.76***	0.37*
GL	-0.10	-0.35	-0.32	0.02	0.20	-0.43	0.04	-0.05	-0.24	0.34	-0.47	-0.33	**0.82***	**0.93***		0.46*	0.58*	0.44*
GW	-0.28	-0.32	0.03	-0.02	0.05	-0.01	0.59*	-0.07	-0.04	0.48	**-0.63***	**-0.62***	**0.91***	**0.80***	0.53*		**0.79***	0.11
TKW	0.03	-0.15	-0.02	-0.24	-0.13	-0.04	0.58*	0.04	0.32	0.48	-0.34	-0.40	**0.87***	**0.79***	**0.62***	**0.82***		0.32*
GY	-0.04	-0.11	-0.19	-0.02	0.09	-0.06	-0.07	-0.17	0.11	0.15	0.03	0.11	0.30	0.31	0.39	0.13	0.35	

[1/] DTP: days to pollen (days); DTS: days to silking (days); PH: plant height (cm); EH: ear height (cm); BENN: bellow ear node number; AENN: above ear node number; SD: stalk diameter (mm); LA: leaf area (cm^2); EL: ear length (cm); ED: ear diameter (mm); NKR: number of kernel rows; NKE: number of kernels per ear; GA: grain area (mm^2); GP: grain perimeter (mm); GL: grain length (mm); GW: grain width (mm); TKW: one thousand kernel weight (g), and grain yield (GY, kg ha^{-1}). Pearson correlation coefficients lower than -0.60 or higher than 0.60 are highlighted in bold.[2/]* significant at P=0.05 by t test.

2.3.2 Variance components and heritability estimates

Variance components of GCA and SCA were significant ($P<0.05$) by likelihood ratio test for all traits across environments (Table 4). Regarding interactions, variance components of GCA x environment interaction were significant ($P<0.05$) for almost all traits, except for SD and ED, whereas variance component of SCA x environment interaction was significant ($P<0.05$) only for LA and GY. Estimates of additive genetic variance component ($\hat{\sigma}^2_{\mathrm{A}}$) were much greater than estimates of dominance genetic variance component ($\hat{\sigma}^2_{\mathrm{D}}$) for all plant architecture and grain morphology traits, and the yield components ED, NKR and TKW. Conversely, the $\hat{\sigma}^2_{\mathrm{D}}$ values were greater than $\hat{\sigma}^2_{\mathrm{A}}$ for the yield components NKE and EL, and GY by approximately 15%, 25%, and 180%, respectively.

Estimates of broad-sense heritability ($\hat{\mathrm{H}}^2_{\bar{\mathrm{X}}}$) ranged from 0.26 (ED) to 0.87 (GL and GW), and most traits presented high $\hat{\mathrm{H}}^2_{\bar{\mathrm{X}}}$ values (>0.70), mainly for cycle, plant stature and grain morphology traits. Concerning the estimates of narrow-sense heritability ($\hat{\mathrm{h}}^2_{\bar{\mathrm{X}}}$), they ranged from 0.16 (ED and GY) to 0.72 (GW); grain morphology, cycle and plant stature (PH and EH) traits along with BENN, LA, NRK and TKW had intermediate $\hat{\mathrm{h}}^2_{\bar{\mathrm{X}}}$ values (> 0.50), whereas plant architecture traits AEEN ($\hat{\mathrm{h}}^2_{\bar{\mathrm{X}}}$=0.24), SD ($\hat{\mathrm{h}}^2_{\bar{\mathrm{X}}}$=0.28) along with yield components EL ($\hat{\mathrm{h}}^2_{\bar{\mathrm{X}}}$=0.20), ED ($\hat{\mathrm{h}}^2_{\bar{\mathrm{X}}}$=0.16) and NKE ($\hat{\mathrm{h}}^2_{\bar{\mathrm{X}}}$=0.36), and also GY ($\hat{\mathrm{h}}^2_{\bar{\mathrm{X}}}$=0.16) showed low $\hat{\mathrm{h}}^2_{\bar{\mathrm{X}}}$ values (<0.40). The $\hat{\mathrm{h}}^2_{\bar{\mathrm{X}}}$ value for GY was 75% lower than its $\hat{\mathrm{H}}^2_{\bar{\mathrm{X}}}$ values - the highest difference between $\hat{\mathrm{H}}^2_{\bar{\mathrm{X}}}$ and $\hat{\mathrm{h}}^2_{\bar{\mathrm{X}}}$ values among tested traits. The proportion ($\hat{\sigma}^2_{\mathrm{sch/GCA}}$) of genotypic variance estimates of hybrids that is due to GCA ($\hat{\sigma}^2_{\mathrm{sch/GCA}}$) ranged from 0.26 (GY) to 0.93 (BENN), and almost all traits had $\hat{\sigma}^2_{\mathrm{sch/GCA}}$ values greater than 0.60, except EL (0.44), NKE (0.47) and GY (0.26). Finally, estimates of correlation between estimates of adjusted means of hybrids and SCA effects ranged from 0.28 (BENN) to 0.84 (GY), and the hybrids performance showed weak-to-moderate (<0.60) correlation with estimates of SCA effects for almost all traits, except for EL ($\hat{r}$=0.69), ED ($\hat{r}$=0.65), NKE ($\hat{r}$=0.73) and GY ($\hat{r}$=0.84).

Table 4. Estimates of variance components of general (σ^2_{GCA}) and specific (σ^2_{SCA}) combining ability, residual variance (σ^2), additive (σ^2_{A}) and dominance (σ^2_{D}) genetic variance components, narrow-sense heritability ($h^2_{\bar{X}}$) and broad-sense heritability ($H^2_{\bar{X}}$), proportion of genotypic variance estimates of hybrids that is due general combining ability ($\sigma^2_{sch/GCA}$) and estimates of correlation between adjusted means and SCA effects ($r_{\mathrm{adj.mean\ and\ SCA}}$) of hybrids for 18 agronomic traits measured in 105 single-crosses hybrids of tropical maize across four environments in Brazil

Parameters	DTP[1/]	DTS	PH	EH	BENN	AENN
$\hat{\sigma}^2_{\mathrm{GCA}}$	0.60**[2/]	0.77**	33.60**	23.30**	0.06**	0.01**
$\hat{\sigma}^2_{\mathrm{SCA}}$	0.54**	0.79**	25.05**	11.10**	0.01**	0.01**
$\hat{\sigma}^2_{\mathrm{GCAxE}}$	0.09**	0.10**	2.09**	2.16**	4.17x10^{-3}**	1.66x10^{-3}**
$\hat{\sigma}^2_{\mathrm{SCAxE}}$	0.08	0.11	0.35	~ 0	~ 0	~ 0
$\hat{\sigma}^2$	0.40	0.38	19.23	17.91	0.08	0.06
$\hat{\sigma}^2_{\mathrm{A}}$	1.21	1.54	67.20	46.60	0.12	0.02
$\hat{\sigma}^2_{\mathrm{D}}$	0.54	0.79	25.05	11.10	0.01	0.01
$\hat{H}^2_{\bar{X}}$	0.81	0.86	0.83	0.76	0.60	0.37
$\hat{h}^2_{\bar{X}}$	0.56	0.57	0.60	0.62	0.56	0.24
$\hat{\sigma}^2_{sch/GCA}$	0.69	0.66	0.73	0.81	0.93	0.66
$\hat{r}_{\mathrm{adj.mean\ and\ SCA}}$	0.46**[3/]	0.46**	0.51**	0.38**	0.28**	0.55**
	SD	LA	EL	ED	NKR	NKE
$\hat{\sigma}^2_{\mathrm{GCA}}$	0.22**	1,411.76**	0.15**	1.03**	0.20**	492.52**
$\hat{\sigma}^2_{\mathrm{SCA}}$	0.12**	840.15**	0.39**	1.20*	0.15**	1,118.80**
$\hat{\sigma}^2_{\mathrm{GCAxE}}$	0.01	116.83**	0.02*	0.04	0.02**	36.08**
$\hat{\sigma}^2_{\mathrm{SCAxE}}$	0.01	288.10*	~ 0	3.06	0.03	~ 0
$\hat{\sigma}^2$	1.00	1,231.14	0.85	9.33	0.25	844.99
$\hat{\sigma}^2_{\mathrm{A}}$	0.44	2,823.51	0.31	2.06	0.41	985.05
$\hat{\sigma}^2_{\mathrm{D}}$	0.12	840.15	0.39	1.20	0.15	1,118.80
$\hat{H}^2_{\bar{X}}$	0.36	0.75	0.45	0.26	0.69	0.71
$\hat{h}^2_{\bar{X}}$	0.28	0.58	0.20	0.16	0.51	0.33
$\hat{\sigma}^2_{sch/GCA}$	0.78	0.77	0.44	0.63	0.73	0.47
$\hat{r}_{\mathrm{adj.mean\ and\ SCA}}$	0.50**	0.49**	0.69**	0.65**	0.45**	0.73**
	GA	GP	GL	GW	TKW	GY
$\hat{\sigma}^2_{\mathrm{GCA}}$	10.77**	0.42**	0.07**	0.05**	249.67**	126,945.2**
$\hat{\sigma}^2_{\mathrm{SCA}}$	7.27**	0.33**	0.08**	0.02**	160.73**	704,283.6**
$\hat{\sigma}^2_{\mathrm{GCAxE}}$	0.99**	0.06**	0.01**	2.89x10^{-3}**	26.24**	37,591.4**
$\hat{\sigma}^2_{\mathrm{SCAxE}}$	0.33	0.04	~ 0	~ 0	30.42	140,466.9*
$\hat{\sigma}^2$	5.07	0.34	0.03	0.02	226.31	589,908.5
$\hat{\sigma}^2_{\mathrm{A}}$	21.54	0.84	0.15	0.09	499.35	253,890.5
$\hat{\sigma}^2_{\mathrm{D}}$	7.27	0.33	0.08	0.02	160.73	704,283.5
$\hat{H}^2_{\bar{X}}$	0.85	0.78	0.87	0.87	0.74	0.62
$\hat{h}^2_{\bar{X}}$	0.64	0.56	0.58	0.72	0.56	0.16
$\hat{\sigma}^2_{sch/GCA}$	0.75	0.72	0.66	0.83	0.76	0.26
$\hat{r}_{\mathrm{adj.mean\ and\ SCA}}$	0.34**	0.39**	0.46**	0.30**	0.42**	0.84**

[1/] DTP: days to pollen (days); DTS: days to silking (days); PH: plant height (cm); EH: ear height (cm); BENN: bellow ear node number; AENN: above ear node number; SD: stalk diameter (mm); LA: leaf area (cm^2); EL: ear length (cm); ED: ear diameter (mm); NKR: number of kernel rows; NKE: number of kernels per ear; GA: grain area (mm^2); GP: grain perimeter (mm); GL: grain length (mm); GW: grain width (mm); TKW: one thousand kernel weight (g), and grain yield (GY, kg ha^{-1}). [2/] **, * significant at P=0.01 and P=0.05, respectively, by the likelihood ratio test. [3/] ** significant at P=0.01 by t test.

2.3.3 GCA effects

Although most Pearson's correlation coefficients based on GCA effects between traits were either non-significant (*P>0.05*) or showed low magnitude, we observed moderate-to-strong positive (>0.60) for some pairs of traits (Table 3, Fig. 1B). Likewise the correlations of adjusted means, we found strong and positive correlations between traits within the same group: DTP and DTS ($\hat{r}$ = 0.90) for cycle traits, EH and BENN ($\hat{r}$ = 0.94) for plant stature, NKR and NKE ($\hat{r}$ = 0.85) for yield components, GA and GP ($\hat{r}$ = 0.97), GP and GL ($\hat{r}$ = 0.93), GA and GW ($\hat{r}$ = 0.91), GA and GL ($\hat{r}$ = 0.82), GP and GW ($\hat{r}$ = 0.80) for grain morphology. Additionally, we observed strong correlations between traits from different groups: GA and TKW ($\hat{r}$ = 0.87), GW and TKW ($\hat{r}$ = 0.82), and GP and TKW ($\hat{r}$ = 0.79). Moderate and positive correlations were observed between SD and ED ($\hat{r}$ = 0.66), GL and TKW ($\hat{r}$ = 0.62), SD and GW ($\hat{r}$ = 0.59), and SD and TKW ($\hat{r}$ = 0.58), from different groups; and also grain morphology since GL presented moderate correlation with GW ($\hat{r}$ = 0.53). Negative and moderate correlations were observed between traits of yield components and morphology grain: GW and NKR ($\hat{r}$ = -0.63), NKE and GW ($\hat{r}$ = -0.62), NKR and GA ($\hat{r}$ = -0.60), NKR and GP ($\hat{r}$ = -0.57), and NKE and GA ($\hat{r}$ = -0.54). Finally, the GY exhibited no correlation with any tested traits.

The tropical maize inbred lines VML004, VML016, VML062, VML081, VML083, VML131 and VML157 had positive GCA effects for GY (Table 5). Also, almost all of them presented negative GCA effects for at least one cycle trait and one plant stature trait. The VML131 had the highest GCA effects for GY (1,186.89 kg ha^{-1}), and negative GCA effects for DTP (-0.54 day), DTS (-0.74 day), PH (-6.83 cm), EH (-4.08 cm); it also had positive GCA effects for TKW (41.11 g) and almost all grain morphology traits. The inbred line VML062 also showed good performance for GY (120.53 kg ha^{-1}), and negative effects for DTP (-0.87 day), DTS (-0.36 day), PH (-5.76 cm) and EH (-7.47 cm). Similarly, VML016 presented a positive GCA estimate for GY (84.67 kg ha^{-1}) associated with negative GCA effect for EH (-12.13 cm). VML083 also contributes to increasing GY (256.41 kg ha^{-1}), while reducing the PH (-8.83 cm). The inbred line VML081 stood out for its positive estimate for GY (44.79 kg ha^{-1}) associated with desirable estimates for DTP (-2.54 days) and DTS (-2.13 days). Although VML004 exhibited a high GCA effect for GY (535.02 kg ha^{-1}), it presented undesirable GCA effects for EH (12.43 cm). Likewise, VML157 presented high estimate of GCA effect for GY (225.77 kg ha^{-1}), but showed high frequency of favorable alleles for increasing cycle (GCA effects of 2.04 and 2.87 days for DTP and DTS, respectively) and PH (4.47 cm). Therefore, VML016, VML062, VML081, VML083 and VML131 are promising tropical maize inbred lines for improving GY and reducing cycle and plant stature in our maize breeding program targeting the development of breeding populations, new inbred lines and, consequently, highest-yielding hybrids.

Table 5. Estimates of general combining ability effects of 15 tropical maize inbred lines for 18 agronomic traits across four environments in Brazil

Inbred Lines	DTP$^{1/}$	DTS	PH	EH	BENN	AENN	SD	LA	EL
VML131	-0.54	-0.74	-6.83	-4.08	-0.22	-0.02	0.21	7.22	0.07
VML004	0.37	-0.13	4.75	12.43	0.83	-0.12	0.05	-45.16	-0.11
VML083	0.21	-0.83	-8.83	-0.66	0.12	-0.03	-0.70	-17.13	-0.13
VML157	2.04	2.87	4.47	0.87	0.18	0.12	0.98	103.45	0.18
VML062	-0.87	-0.36	-5.76	-7.47	-0.32	0.01	-0.56	-32.27	0.28
VML016	-0.17	-0.94	4.24	-12.13	-0.60	0.12	0.20	-39.26	1.25
VML081	-2.54	-2.13	0.03	2.84	0.10	0.22	-1.23	-16.40	-0.98
VML134	1.48	1.88	11.97	8.79	0.17	-0.09	-0.31	2.89	0.26
VML115	-0.64	-1.39	-4.82	-1.08	-0.07	-0.31	0.51	-60.35	-0.27
VML066	-0.57	0.00	1.06	0.85	-0.03	-0.12	0.50	-47.12	-0.19
VML154	1.60	1.79	5.58	3.77	0.09	-0.08	-0.13	0.39	0.06
VML105	0.32	0.94	-13.59	-4.28	-0.26	0.01	-0.68	1.35	-0.29
VML176	-1.26	-1.00	11.55	-2.62	-0.25	0.33	0.95	6.41	0.79
VML140	0.37	-0.10	-7.80	-6.03	-0.24	-0.07	-0.05	101.96	-0.30
VML055	0.18	0.15	4.00	8.81	0.51	0.03	0.24	34.02	-0.65
	ED	NKR	NKE	GA	GP	GL	GW	TKW	GY
VML131	1.20	-0.48	-17.70	8.69	1.48	0.51	0.49	41.11	1,186.89
VML004	0.09	-0.11	22.46	1.64	0.52	0.33	-0.01	-5.70	535.02
VML083	-1.51	0.27	11.16	-2.14	-0.32	0.03	-0.29	-5.25	256.41
VML157	2.41	1.15	25.68	-0.21	-0.08	-0.10	0.00	11.50	225.77
VML062	-0.08	0.47	17.35	-3.19	-0.27	0.04	-0.26	-10.69	120.53
VML016	0.21	-0.11	-2.52	3.19	0.59	0.19	0.10	27.53	84.67
VML081	-2.04	-0.70	-33.43	-3.43	-0.45	-0.08	-0.18	-31.59	44.79
VML134	-1.97	0.37	24.96	-6.88	-1.26	-0.30	-0.49	-23.96	-107.96
VML115	1.70	-0.26	-18.28	7.55	1.67	0.57	0.37	22.78	-192.89
VML066	1.34	-0.16	16.68	-0.44	-0.29	-0.35	0.16	-15.60	-218.38
VML154	-1.89	0.06	-2.62	-6.13	-1.32	-0.46	-0.28	-12.82	-250.65
VML105	-0.13	0.48	-6.61	-4.32	-1.04	-0.41	-0.19	-16.31	-264.18
VML176	-0.23	0.21	-1.99	-2.48	-1.03	-0.69	0.19	3.45	-437.20
VML140	1.57	0.50	29.79	-1.15	0.08	0.21	-0.21	-12.59	-489.43
VML055	-0.68	-1.68	-64.95	9.29	1.72	0.52	0.60	28.13	-493.37

$^{1/}$DTP: days to pollen (days); DTS: days to silking (days); PH: plant height (cm); EH: ear height (cm); BENN: bellow ear node number; AENN: above ear node number; SD: stalk diameter (mm); LA: leaf area (cm^2); EL: ear length (cm); ED: ear diameter (mm); NKR: number of kernel rows; NKE: number of kernels per ear; GA: grain area (mm^2); GP: grain perimeter (mm); GL: grain length (mm); GW: grain width (mm); TKW: one thousand kernel weight (g), and grain yield (GY, kg ha^{-1}).

2.3.4 Grouping of inbred lines

The estimates of SCA effects for GY ranged from -5,214.67 kg ha^{-1} (VML134 and VML154) to 1,881.53 kg ha^{-1} (VML081 and VML134) with a range of 7,096 kg ha^{-1} (Table 6). Based on Mojena's method (1977), the UPGMA dendrogram grouped the 15 tropical maize inbred lines into four groups (Fig. 2). Group I consisted of inbred lines VML016, VML066, VML081 and VML176, group II consisted of lines VML062, VML131 and VML140, group III consisted of lines VML004, VML055 and VML115 and group IV consisted of lines VML083, VML105, VML134, VML154 and VML157. The inbred lines VML134 and VML154 were allocated into cluster IV and were the most similar inbred lines, whereas the VML081 and VML134 were allocated into groups I and IV, respectively, and also were the most divergent lines based on SCA effects.

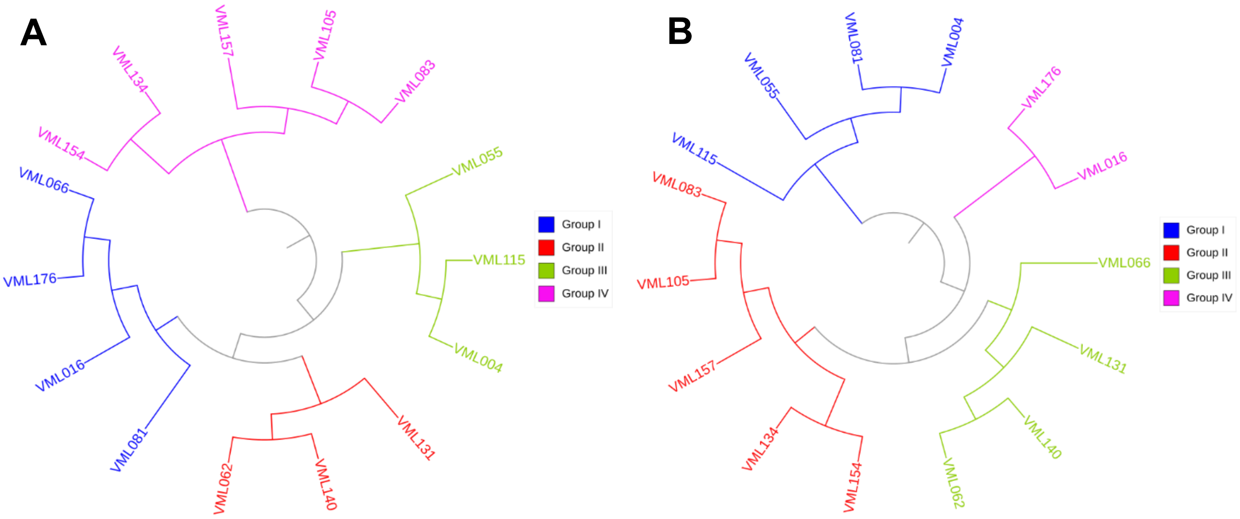

Fig. 2. Dendrograms from unweighted pair-group method of arithmetic clustering for 15 tropical maize inbred lines using estimates of specific combining ability effects for grain yield across four environments in Brazil **(A)** and using Roger's modified genetic distances calculated from 3,083 SNP markers **(B)**.

Regarding the SNP-based DG method, the mean of Roger's modified genetic distance among the 15 inbred lines was 1.1734, and it ranged from 0.3304 (VML134 and VML154) to 1.2464 (VML055 and VML083; Table 6). The UPGMA dendrogram grouped the 15 inbred lines into four groups based on Mojena's method (1977; Fig. 2). Group I consisted of inbred lines VML004, VML055, VML081 and VML115, group II consisted of lines VML083, VML105, VML134, VML154 and VML157, group III of lines VML062, VML066, VML131 and VML140, and group IV consisted of just two lines, VML016 and VML176.

Table 6. Estimates of specific combining ability effects for grain yield (above diagonal) and modified Roger's genetic distances (bellow diagonal) among 15 tropical maize inbred lines across four environments in Brazil

Lines	VML004	VML016	VML055	VML062	VML066	VML081	VML083	VML105	VML115	VML131	VML134	VML140	VML154	VML157	VML176
VML004		-117.85	-234.33	211.73	558.09	-609.61	49.96	491.06	-941.10	58.10	**1,518.94**	25.83	**1,215.67**	925.89	-330.69
VML016	1.2028		-391.07	727.39	-74.74	27.56	245.45	727.05	327.21	161.88	498.03	-162.61	563.26	**1,119.02**	-1,138.47
VML055	1.1915	**1.2435**		209.08	206.15	148.27	481.34	1,037.38	-497.58	-226.14	673.97	136.06	661.31	-292.22	619.62
VML062	1.1652	1.1882	1.2049		5.15	-364.36	311.84	304.63	32.58	-361.14	**1,255.86**	-1,434.95	1,026.83	771.42	347.34
VML066	**1.2295**[1/]	1.1819	1.2093	1.1778		-812.55	172.87	599.34	344.52	-394.79	956.41	221.48	886.24	618.44	-1,369.27
VML081	1.0885	1.2172	1.1776	1.1846	1.2104		-57.83	490.50	362.16	290.68	**1,881.53**	-338.53	**1,720.53**	-149.98	-63.16
VML083	1.2133	1.2133	**1.2464**	1.2128	1.1872	1.2169		-1,481.10	-294.46	256.25	755.42	**1,674.56**	940.97	-149.24	389.10
VML105	1.2151	1.1821	1.2158	1.1648	1.2132	1.1902	0.9764		428.05	152.11	-384.23	396.32	-279.25	-796.83	**1,451.71**
VML115	1.1934	1.2083	1.2125	1.2059	1.1818	1.2049	1.1854	1.1972		939.88	175.05	759.99	506.86	249.04	593.67
VML131	1.2084	1.2187	**1.2220**	1.1405	1.1472	1.2032	**1.2209**	1.1723	**1.2220**		**1,401.22**	-766.05	**1,259.85**	292.77	-2.46
VML134	1.2001	1.1916	1.2038	1.1757	1.2131	1.1822	1.1205	0.9965	1.2196	**1.2255**		1,016.17	-5,214.67	-291.08	567.44
VML140	1.1543	1.1908	1.2111	0.9128	1.1885	1.2006	1.2040	1.1444	1.1813	1.1495	1.1652		801.82	-369.10	535.01
VML154	1.2010	1.1936	1.2048	1.1722	1.2129	1.1751	1.1133	1.0055	1.2184	1.2114	0.3304	1.1594		-238.35	721.50
VML157	1.2059	1.1600	1.2172	1.1906	1.1733	1.1629	1.0943	1.0106	1.2096	1.1775	1.1188	1.1724	1.1104		840.71
VML176	1.1882	1.1852	1.2144	1.1897	1.2095	1.2016	1.2187	1.2112	**1.2376**	**1.2308**	1.1971	1.2032	1.1942	**1.2285**	

[1/] The ten highest estimates of SCA effects and modified Roger's genetic distances are highlighted in bold.

We observed a strong and positive correlation between genetic distances based on SNP markers and SCA effects for GY ($\hat{r} = 0.74$). Consequently, both grouping approaches were consistent in the allocation of inbred lines into "divergent groups"; only the inbred lines VML066 and VML081 were not allocated in the same group by both methods. The lines VML083, VML105, VML134, VML154 and VML157, and VML016 and VML176 were grouped together by the two methods. Likewise, the sets of three lines VML062, VML131 and VML140; and VML004, VML055 and VML115 were also placed into the same groups by both methods.

2.3.5 Hybrid performance

Two out of the 15 highest-yielding tropical maize hybrids evaluated in our study outperformed the best check hybrid, BM709PRO2 (Table 7). The hybrid VML131/VML134 (12,545 kg ha^{-1}) out-yielded the best commercial check, BM709PRO2 (11,794 kg ha^{-1}) by approximately 6%, whereas the hybrid VML004/VML134 (11,989 kg ha^{-1}) had similar yield performance to the best check. Furthermore, thirteen experimental hybrids had yield means higher than the second highest-yielding check, DKB390PRO3 (10,887 kg ha^{-1}). Overall, the field performance of the 15 highest-yielding hybrids was comparable to the checks regarding plant stature, yield components and grain morphology, except for the hybrid VML004/VML134. Although VML004/VML134 showed high grain yield, it presented undesirable performance for PH (265.76 cm) and EH (155.20 cm). The hybrid VML081/VML131 were the earliest (around 65 days for days to flowering), whereas hybrid VML083/VML140 shortest (226.74 cm and 130.37 cm for PH and EH, respectively) among the highest-yielding hybrids. Additionally, the hybrids VML115/VML131, VML131/VML157, VML131/VML154, VML083/VML131 and VML016/VML131 presented field performance comparable to the checks, highlighting their agronomic potential. In agreement with estimates of GCA effects, eight out of 15 highest-yielding hybrids had the inbred line VML131 as parental, which had the highest estimates of GCA effects for GY.

Table 7. Adjusted means for 18 agronomic traits of the 15 (~14%) highest-yielding tropical maize hybrids across four environments in Brazil

Hybrid	DTP[1/]	DTS	PH	EH	BENN	AENN	SD	LA	EL
VML131/VML134	67.81	67.84	245.53	134.37	7.31	6.13	21.91	1,001.28	17.45
VML004/VML134	69.59	69.14	265.76	155.20	8.41	6.04	21.74	979.03	16.63
BM709PRO2	68.61	69.91	249.41	140.98	7.83	6.56	22.33	980.70	17.46
VML081/VML134	66.67	67.01	262.20	151.62	8.09	6.14	21.83	985.67	15.89
VML115/VML131	68.59	67.11	229.79	131.07	7.32	5.78	22.24	920.07	16.67
VML004/VML157	71.62	71.24	254.65	148.26	8.60	6.28	23.53	1,008.57	15.70
VML004/VML131	69.32	68.19	245.85	143.14	8.12	6.17	21.92	914.11	16.59
VML131/VML157	69.51	69.70	243.19	132.08	7.55	6.32	23.56	1,045.38	15.81
VML131/VML154	68.41	67.52	242.30	129.76	7.31	6.17	22.38	988.34	16.36
VML083/VML131	67.84	66.37	230.81	129.76	7.43	6.20	22.51	959.15	16.62
VML083/VML140	69.09	66.92	226.74	130.37	7.42	6.09	20.84	1,058.58	15.97
VML081/VML131	65.48	65.65	237.58	133.58	7.47	6.58	20.32	958.95	15.13
VML016/VML157	70.08	70.24	261.43	126.01	7.08	6.54	23.35	990.97	17.87
VML016/VML131	67.70	66.63	236.82	117.30	6.85	6.21	21.79	914.83	16.99
DKB390PRO3	71.15	66.83	236.23	141.03	7.62	5.74	21.74	1,008.68	15.77

Hybrid	ED	NKR	NKE	GA	GP	GL	GW	TKW	GY
VML131/VML134	48.29	15.36	638.4	96.51	39.83	13.73	9.44	329.2	12,545
VML004/VML134	45.50	15.06	618.3	92.45	39.06	13.66	9.12	296.6	11,989
BM709PRO2	47.82	14.48	519.1	106.28	42.37	14.41	9.86	364.8	11,794
VML081/VML134	42.97	14.63	559.6	86.24	37.93	13.05	8.96	279.9	11,584
VML115/VML131	49.29	13.54	494.4	111.59	42.20	14.12	10.67	410.8	11,567
VML004/VML157	51.11	16.20	595.9	99.16	40.31	13.67	9.71	342.6	11,343
VML004/VML131	49.46	14.51	545.1	107.90	41.85	14.29	10.21	368.9	11,297
VML131/VML157	50.67	15.16	537.1	102.21	40.50	13.58	10.02	380.3	11,266
VML131/VML154	48.03	15.02	540.6	98.10	40.04	13.54	9.74	358.5	11,234
VML083/VML131	47.82	15.37	576.1	99.35	40.41	13.86	9.62	342.9	11,101
VML083/VML140	47.11	15.61	584.6	94.52	39.89	13.81	9.17	331.3	11,045
VML081/VML131	47.29	13.79	504.7	98.64	40.09	13.55	9.90	320.2	10,991
VML016/VML157	50.12	16.74	604.1	97.37	40.01	13.59	9.54	370.1	10,978
VML016/VML131	47.99	14.89	529.0	106.04	41.14	14.05	10.04	370.7	10,894
DKB390PRO3	52.84	16.51	567.1	101.88	40.48	13.56	9.87	348.5	10,887

[1/] DTP: days to pollen (days); DTS: days to silking (days); PH: plant height (cm); EH: ear height (cm); BENN: bellow ear node number; AENN: above ear node number; SD: stalk diameter (mm); LA: leaf area (cm^2); EL: ear length (cm); ED: ear diameter (mm); NKR: number of kernel rows; NKE: number of kernels per ear; GA: grain area (mm^2); GP: grain perimeter (mm); GL: grain length (mm); GW: grain width (mm); TKW: one thousand kernel weight (g), and grain yield (GY, kg ha^{-1}).

2.4 Discussion

The hybrids exhibited a broad range of variation for GY and all other measured traits. The large genotypic variation among the hybrids for plant architecture, yield components, grain morphology, and grain yield associated with low coefficients of variation across environments will allow good genetic progress through selection in this set of hybrids (Hallauer et al. 2010; Swarup et al. 2021). The presence of genotype by environment interaction (GEI) for almost all traits, except for AENN, EL and ED, suggested that the hybrids differed in their response pattern across the tested environments. In tropical environments, strong GEI effects are expected due to the frequent occurrence of abiotic stress, such as drought, heat and low N availability (Edmeades et al., 2017; Nurmberg et al., 2022; Von Pinho et al., 2022). The limited predictability of environmental conditions across growing seasons highlights the importance of evaluating hybrids over multiple years to identify cultivars with stable performance, and consequently, commercial potential (Oyekunle et al. 2017). Thus, the evaluation of maize hybrids across diverse environments and seasons (years) is essential for accurately identifying high-yielding and stable hybrids (Badu-Apraku et al. 2016; Sserumaga et al. 2016; Zuffo et al. 2021; Uberti et al. 2023; Bocianowski et al. 2024). Designing an efficient breeding strategy to improve any trait of interest requires a comprehensive knowledge of genetic parameters such genetic variances components, heritability, and genetic trait associations (Hallauer et al. 2010; De La Fuente et al. 2013; Cobb et al. 2019). Regarding the trait associations, most Pearson's correlation coefficients using the estimates of adjusted means of experimental hybrids were non-significant or of low magnitude, indicating none or weak associations between many traits. Nevertheless, we observed strong and positive correlations between DTP and DTS, BENN and EH, GP and GA, GL and GA, GL and GP, GW and GA, GW and GP, TKW and GA, TKW and GP, TKW and GW, indicating that the simultaneous selection for these traits is feasible, as the correlations are in the favorable direction. GY exhibited negative correlations with DTP and DTS, but positive correlations with LA, EL, ED, NKE, GA, GP, GL and TKW. Therefore, truncated selection targeting GY is favorable for the improvement of other traits, as it leads to a reduction of cycle traits and an increase of grain yield components, like EL, ED, NKE and TKW.

In any breeding program, understanding the inheritance of a trait is a pre-requisite to define the best breeding strategies and, consequently, increase the genetic gain. A great challenge for breeders is that some important agronomic traits are controlled by multiple genes and strongly influenced by the environment factors (Hallauer et al. 2010; Carvalho et al. 2018; Hossain et al. 2020). Regarding the genetic parameters, the presence of variance components of GCA and SCA effects for all traits in our study indicates that both additive and non-additive gene actions were important in the inheritance of these traits. Although our set of inbred lines previously selected based on per se and testcross performance for most agronomic traits, we observed differences in the frequency of favorable alleles associated with GCA and also for non-additive gene actions (SCA effects) for almost all traits. Therefore, even with high-performing germplasm, the genetic variability can be exploited through additive and non-additive breeding strategies. The $\hat{\sigma}^2_{\mathrm{A}}$ predominated over $\hat{\sigma}^2_{\mathrm{D}}$ for all plant architecture and grain morphology traits, which also showed moderate narrow-sense heritability estimates, and thus the favorable alleles of those traits can be fixed in the early generations in a breeding cycle, such as intra-recurrent selection (Hallauer et al. 2010; Dermail et al. 2023). On the other hand, for the yield components NKE and EL, and also GY, $\hat{\sigma}^2_{\mathrm{D}}$ were greater than $\hat{\sigma}^2_{\mathrm{A}}$, hybridization strategies should be adopted to explore heterosis and selection must be based on hybrid performance (Aslam and Zafar 2020; Xiao et al. 2021). The $\hat{\sigma}^2_{\mathrm{sch/GCA}}$ indicates the predictability of hybrids performance based exclusively on GCA effects. If this ratio is close to one, it suggests that the hybrids performance can be accurately predicted based on the average of parental GCA effects (Baker 1978). Overall, most plant architecture and grain morphology traits can be predicted using GCA effects and their selection can be done based on per se performance of the inbred lines, whereas we found low $\hat{\sigma}^2_{\mathrm{sch/GCA}}$ estimates for GY, which is associated with the highest $\hat{r}_{\mathrm{adj.mean\ and\ SCA}}$ estimate, confirming the importance of SCA effects in predicting hybrid performance. In addition to GY, the yield components NKE and EL also presented high $\hat{r}_{\mathrm{adj.mean\ and\ SCA}}$ estimates. Therefore, our results indicate the predominance of non-additive gene effects influencing hybrids performance for GY, NKE and EL. Similarly to us, the preponderance of additive gene action over non-additive gene action for cycle and plant stature traits have been reported in tropical maize (Badu-Apraku et al. 2015; Zhou et al. 2018). Inconsistency between our findings and those of previous studies may be explained by the use of different germplasm, which could result in varying patterns of gene action (Akinwale et al. 2014; Annor and Badu-Apraku 2016; Abu et al. 2020; Ribeiro et al. 2020).

The value of an inbred line in a breeding program is defined by its GCA effect since it reflects the number of favorable genes in the parent (Viana et al. 2013; Sprague and Tatum 1942). Therefore, breeding programs generally aim to develop hybrid combinations with high SCA estimates, involving at least one parent with a good GCA effect to ensure the complementary additive gene contribution (Mengistu et al. 2020; Seye et al. 2020; Zhou 2021). The inbred lines VML004, VML016, VML062, VML081, VML083, VML131 and VML157 exhibited positive GCA effects for GY, indicating the presence of favorable alleles associated with this trait. Therefore, these inbred lines have the potential to contribute to developing high-yielding progenies. Although the correlations among the GCA effects were low, some inbred lines also have high estimates for cycle traits and plant stature, such as VML004 and VML157. The selection of these inbred lines as parents in a breeding program must be done with caution since the increase in yield will be associated with a longer cycle, greater plant height, and high ear placement. On the other hand, VML016, VML062, VML081, VML083 and VML131 presented negative GCA effects for at least one cycle trait and one plant stature trait. Inbred lines exhibiting negative GCA effects for cycle and plant stature traits, combined with positive GCA effects for GY, hold the potential for developing cultivars with increased grain yield while maintaining early maturity and short stature (Annor and Badu-Apraku 2016; Gichuru et al. 2016; Yong et al. 2020).

A clear understanding of heterotic patterns among inbred lines is the key to success in a hybrid breeding program, mainly for maize since hybrid is the most varieties planted in the world (Hallauer et al. 2010; Aslam and Zafar 2020; Akinwale 2021). The inbred lines developed by the UFV program have a complex genetic background since most of them were derived from commercial hybrids and tropical populations of diverse genetic origins (Faria et al. 2022). Consequently, our maize inbred lines cannot be accurately allocated to the major tropical heterotic groups, such as Tuxpeño and non-Tuxpeño (Trevisan 2018). Nevertheless, heterotic groups can be identified using SCA estimates and/or molecular markers. The SCA effects for GY have been successfully used for heterotic grouping in tropical maize, as this method efficiently detects hybrid vigor through field evaluations (Badu-Apraku et al. 2013; Akinwale et al. 2014; Badu-Apraku et al. 2015; Gichuru et al. 2016; Amegbor et al. 2017; Bhatla et al. 2025). However, the SCA estimates are influenced by environmental conditions and are time-consuming, which can be limiting in studies with a large number of inbred lines (Bhatla et al. 2025). On the other hand, molecular markers are not influenced by the environment, and they provide faster results than diallel schemes (Ahmad et al. 2017). Therefore, molecular markers should be applied as complementary tools in plant breeding to provide a comprehensive understanding of the genetic diversity in the breeding germplasm (Amiteye 2021). We identified four groups based on SCA estimates for GY and SNP markers, with a strong concordance regarding the inbred lines of each group. Our results highlight the efficiency of using molecular markers to assign inbred lines to heterotic groups in a faster, less labor-intensive, and more cost-effective way (Badu-Apraku et al. 2013; Akinwale et al. 2014; Gichuru et al. 2016); Aslam and Zafar 2020; Akinwale 2021). Also, the correlation between both approaches reinforces the potential of molecular markers to classify inbred lines not yet evaluated in hybrid combinations, supporting the findings of Badu-Apraku et al. (2013). The inbred lines from each group could be recombined separately to develop breeding populations. Also, VML016, VML062, VML081, VML083 and VML131 that are promising inbred lines for improving GY and reducing cycle and plant stature can be used as source germplasm for new breeding populations. These populations can be utilized to develop new inbred lines through pedigree breeding or double haploid method (Hallauer et al. 2010). Finally, the inbred lines or progenies derived from these populations within a group should be crossed and tested with the most promising lines from the opposite group.

A comprehensive understanding of the combining ability associated with field performance is essential for maize breeders to develop modern hybrids that exhibit high yield potential and tolerance to stress conditions (Luz et al. 2024). The presence of GEI for most traits evaluated in our study implies the need for testing hybrids across different environments, mainly under stress conditions and diverse management practices. According to Sandhu and Dhillon (2021), the advancement of tropical maize breeding is related to tolerance to high plant density, therefore it is also important that tropical maize hybrids are evaluated for this trait. Moreover, the increase in global temperatures has contributed to world reduction in maize production, highlighting the need for hybrids adapted to this challenging condition, particularly when combined with water stress (El-Sappah et al. 2022). In our study, the experimental hybrid VML131/VML134 had the best field performance among the hybrids evaluated. Also, the hybrids VML081/VML131, VML083/VML140, VML115/VML131, and VML131/VML157 exhibited agronomic performance comparable to those of the commercial checks, especially the hybrid VML081/VML131 for cycle traits, and VML083/VML140 for plant stature. These outstanding experimental hybrids must be further evaluated in on-farm trials across more diverse environments and management conditions for potential release and commercialization in Southeastern Brazil.

2.5 Conclusion

We concluded that additive and non-additive gene actions are important in controlling the inheritance of plant architecture traits, yield components, grain morphology, and grain yield. For plant architecture and grain morphology traits, the predominance of $\hat{\sigma}^2_{\mathrm{A}}$ over $\hat{\sigma}^2_{\mathrm{D}}$, indicates that breeding schemes that capitalize on the additive effects of the parents, such as recurrent selection, must be used in their genetic improvement. On the other hand, for the yield components NKE and EL, and GY, which $\hat{\sigma}^2_{\mathrm{D}}$ were greater than $\hat{\sigma}^2_{\mathrm{A}}$, and we must adopt hybridization strategies to exploit heterosis. Five inbred lines, VML016, VML062, VML081, VML083 and VML131, presented negative GCA effects for at least one cycle trait and one plant stature trait. Therefore, these inbred lines represent valuable germplasm sources for developing breeding populations, new hybrids, and open-pollinated varieties targeted to Brazilian maize production systems. We identified four groups based on SCA estimates for GY and SNP markers, with a strong concordance regarding the inbred lines of each group. These findings will be useful for exploring the heterosis among lines, guiding crosses, selecting testers, developing highly heterotic and yielding hybrids for Brazilian tropical environments as well as for establishing heterotic groups in the UFV breeding program. Finally, the experimental hybrids VML081/VML131, VML083/VML140, VML115/VML131, VML131/VML134, and VML131/VML157 were the highest-yielding hybrids across environments and should be extensively evaluated in on-farm trials to confirm the potential release and commercialization in Southeastern Brazil.

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3. Chapter two

Genotypic variation and association mapping for plant stature and anthesis-silking interval in set of temperate-adapted maize lines developed by DH and SSD methods

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Abstract

BS39 is a broad-based population of tropical maize adapted to temperate environments that can provide useful and unique alleles to U.S. Corn Belt breeding programs. Doubled-haploid (DH) has been used as an efficient alternative method to speed up the development of maize lines. Our objectives were to compare genetic parameters for plant height (PH), ear height (EH) and anthesis-silking interval (ASI) among the BS39-derived lines using Single Seed Descent (SSD) and DH methods, identify SNP markers associated with these traits, and investigate the relationship among BS39-derived lines and their testcross performance for PH and EH. We developed four sets of 96 lines derived from BS39 and from a cross between BS39 and A427, a spontaneous haploid genome doubling (SHGD) donor, by DH and SSD methods, named BS39_DH, BS39_SSD, BS39xA427_DH and BS39xA427_SSD. DH lines were genotyped using genotype-by-sequencing, whereas SSD lines were genotyped using Diversity Array Technology Sequencing. We observed extensive genotypic variation for PH, EH and ASI in all sets of lines. Lines developed by SSD method had larger genotypic variation and higher predicted genetic gains for all traits at all selection intensity than those developed using the DH method. However, the production of DH lines associated with SHGD appears be a feasible strategy to reduce the costs and time required to complete the breeding cycle and enhance genetic gains in maize breeding programs. PH and EH presented moderate-to-strong positive correlations between BS39-derived lines and their testcross performance. We found nine

significant SNPs for PH and EH in DH lines, and most of them are involved in development process in plants.

Keywords *Zea mays* L. - Doubled haploid - Single-seed descent - Spontaneous haploid genome doubling - Genome-wide association study - Maize germplasm.

3.1 Introduction

Genetic diversity is essential for a more sustainable agricultural food production and indispensable for plant breeders to develop new cultivars (Mayer et al. 2017; Carena 2021; Swarup et al. 2021). The genetic base of modern maize cultivars has narrowed due to selection, leading to a higher susceptibility to biotic and abiotic stresses, and reduced adaptability and stability (Andorf et al. 2019). It remains necessary to characterize and monitor useful genetic diversity in plant breeding programs, through the understanding of genetics and physiology that allow the identification of specific genes or chromosomal regions associated with phenotypic effects (Smith et al. 2022). Tropical maize germplasm has an enormous potential to contribute with useful and unique alleles to the U.S. Corn Belt breeding programs and, consequently, expand their genetic base (Hallauer and Carena 2014; Hallauer and Carena 2016). An example is the BS39 maize population which has contributed with novel alleles for economically important traits for temperate maize breeding programs (Teixeira et al. 2015; Verzegnazzi et al. 2021; Santos et al. 2022). The temperate-adapted BS39 population is an open-pollinated accession, composed of five exotic accessions of Tusón germplasm, representing South American regions. The BS39 was developed by the maize breeding program at Iowa State University and it includes 100% tropical germplasm photoperiod adapted to temperate environments (Teixeira et al. 2015; Hallauer and Carena 2016).

Maize inbred line development relied upon at least six to eight generations of recurrent selfing in the past (Hallauer and Carena 2009; Chaikam et. al 2019). Doubled haploid (DH) technology has emerged as an efficient alternative to speed up the development of maize lines. The use of DH lines saves time in a breeding program, allows better use of genetic variation, and also increases response to selection by increasing heritability for both per se and testcross evaluation (Bordes et al. 2007; Sleper and Bernardo 2016; Maqbool et al. 2020). Moreover, DH technology effectively uncovers genetic load and purges deleterious alleles expressed at haploid level in landraces populations (Strigens et al. 2013; Brauner et al. 2019; Maqbool et al. 2020). However, one of the major bottlenecks in DH production is the artificial genome doubling step that uses chemicals, such as toxic colchicine (Melchinger et al. 2013; Chaikam et al. 2019).

Artificial genome doubling (AGD) adds extra steps to DH development, such as sowing of putative haploids in the greenhouse, colchicine application and transplanting. Using genotypes with spontaneous haploid genome doubling (SHGD) ability is useful to facilitate DH line development (Wu et al. 2014; Boerman et al. 2020). In the population studied by Santos et al. (2022), SHGD was compared with AGD and resulted in twice as many DH lines. However, genomic regions associated with SHGD may cause linkage drag in the new DH lines. Consequently, effects of SHGD on performance of DH lines must be more studied, mainly under field conditions.

One effective way to compare whether potential genetic variability is affected by the DH process is to compare the DH lines with inbred lines developed by the single-seed descent (SSD) breeding method (Bordes et al. 2007). Verzegnazzi et al. (2021) found a modest increase in genetic diversity across the genome with the SSD compared to the DH method through assessment of 663 inbred lines derived from BS39 maize population or the cross between BS39 and A427 (SHGD donor) by both methods. At the testcross level, Santos et al. (2022) showed that both SSD and DH methods are efficient to develop high-performing lines with the same materials used by Verzegnazzi et al. (2021). Similar results were observed by Bordes et al. (2007). While testcross trials demonstrated efficiency of the DH method in maintaining trait variability, no respective information is available for the per se level.

Traits important for parent line performance need to be selected at per se level, whereas the performance of inbred lines per se is an ineffective predictor of hybrid performance for traits showing dominance effects (Hallauer and Carena 2009; Andorf et al. 2019). Maize breeding pipelines usually require per se evaluations of lines to select for highly heritable traits, such as plant height, ear height, and flowering time (Peiffer et al. 2014; Li et al. 2016; Wallace et al. 2016; Vanous et al. 2018). Therefore, the evaluation per se of inbred lines is essential for selection of parents with desirable agronomic traits aiming at the development of superior hybrids. Per se performance can only access part of the testcross performance since the latter carries half of the genomic information of the tester (Hallauer et al. 2010).

Considering the foregoing, the main proposal of our study was to characterize the genetic variation for plant stature and anthesis-silking interval in a large set of maize lines derived directly from the BS39 population and the crosses between BS39 and the inbred line A427 (SHGD donor) developed using SSD and DH methods. Thus, our objectives were to i) compare genotypic variance, heritability and predicted genetic gains for plant height, ear height and anthesis-silking interval among maize lines (per se) derived from the BS39 population using SSD and DH methods; ii) identify SNP markers and candidate genes associated with plant height, ear height and anthesis-silking interval; and iii) investigate the relationship among BS39-derived lines and their testcrosses performance for plant height and ear height.

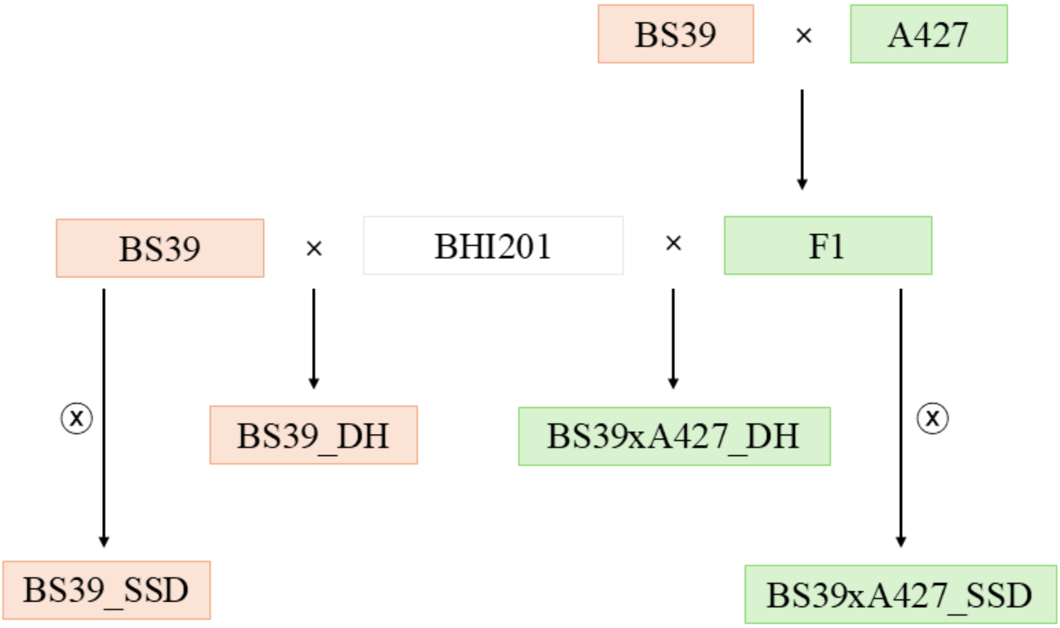
3.2 Materials and Methods

3.2.1 Plant materials

The BS39 is an open-pollinated population, composed of five exotic accessions of Tusón germplasm: PI 449556 and PI 583912 from Brazil, NSL 283507 from Cuba, PI 487940 from Ecuador, and PI 498583 from Guatemala. This population was developed by the maize breeding program at Iowa State University through 11 cycles of stratified mass selection for earlier flowering, using silk expression as the primary selection criterion (Hallauer and Carena 2016). Four sets each of 96 lines derived from the BS39 maize population were used in our study: BS39_DH, BS39_SSD, BS39xA427_DH and BS39xA427_SSD, total of 384 maize lines (Santos et al. 2022; Verzegnazzi et al. 2021). Briefly, the sets of lines named BS39_DH and BS39xA427_DH were developed using DH method, whereas BS39_SSD and BS39xA427_SSD were developed using SSD method. Moreover, the sets of lines BS39_DH and BS39_SSD were derived directly from a sample of the BS39 population and their lines have 100% of BS39 genetic background. The sets BS39xA427_DH and BS39xA427_SSD were derived from crosses between a sample of the BS39 and the inbred line A427, and they have genetic background from both BS39 and A427 germplasm. The A427 is a non-stiff stalk (NSS) public line developed at University of Minnesota and was used as source of SHGD donor in our study. A427 is known as a source of a major SHGD QTL (Boerman et al. 2020; Ren et al. 2020; Trampe et al. 2020) that was shown to be stable across environments (De la Fuente et al. 2020). Thus, we were primarily interested in the comparison between lines derived from the same genetic background: BS39 and BS39xA427 sets.

In relation to development of DH lines, initially, a large set of individual plants from each BS39 and BS39xA427 population were crossed with maternal haploid inducer BHI201 (http://isurftech.technologypublisher.com/technology/19126), at the Iowa State University Agriculture Engineering and Agronomy Farm (ISU-AEA) during 2015 summer (Fig. 1). Then, at physiological maturity, 520 and 750 ears were harvested from BS39 and BS39xA427 population, respectively, dried and individually shelled. After that, putative haploids were identified and classified manually in kernels from both BS39 and BS39xA427 population based on embryo and aleurone coloration (*R1-nj*; Nanda and Chase 1966). The BS39 and BS39xA427 sets of putative haploids followed different ways. During 2016 summer, haploids from BS39 population were sown in the greenhouse for treatment with colchicine to promote haploid genome doubling according to protocol described by Eder and Chalyk (2002). Then, haploids plants at V3-4 leaf collar stage (Abendroth et al. 2011) were transplanted to the field at ISU-AEA, and putative haploids plants shedding pollen were self-pollinated. At physiological maturity, the DH lines were harvested and shelled individually, and these lines were named BS39_DH lines (Verzegnazzi et al. 2021). Conversely, putative haploids from BS39xA427 population were not treated with colchicine. They were identified and directly sown in the field at ISU-AEA during the summer of 2016. Then, putative DH plants that spontaneously shed pollen were self-pollinated. During the summer of 2017, ~20 seeds from each DH line were increased in the field at ISU-AEA. Here, DH lines presenting substantial phenotypic variation were discarded. Also, for homogenous DH lines, all plants were self-pollinated, and the seed bulked. DH lines derived from this process were named BS39xA427_DH lines (Verzegnazzi et al. 2021).

Fig 1. Breeding scheme used to derive DH and SSD inbred lines from BS39 and from the cross between BS39 and A427.



In the development of SSD lines, a large set of different plants from each BS39 and BS39xA427 population were self-pollinated to produce S_1 progenies during 2015 winter, at Tuniche Seed Services in Graneros, Chile (Fig. 1). At physiological maturity, 600 ears were harvested and two kernels from each ear were taken to generate a balanced bulk of 1200 S_2

seeds from each population, BS39 and BS39xA427. Then, both bulks were sown in 2016 summer at ISU-AEA, and 700 self-pollinated ears from each population were harvest and shelled individually. One kernel from each ear was taken to generate a balanced bulk of 700 S_3 seeds from each population that were sown in 2016 winter in Chile. At physiological maturity, 300 self-pollinated ears from each population were harvested, shelled individually, and ten kernels from each ear out of 300 S_4 ears were sown in an individual row in 2017 summer at ISU-AEA. Then, the first plant of each row was self-pollinated, and 120 rows were selected at physiological maturity from each population. Individual S_5 ears were harvested, shelled individually, and sown in 2017 winter in Chile to generate S_6 progenies from BS39 and BS39xA427 populations. Finally, they were named BS39_SSD and BS39xA427_SSD lines with 96 SSD lines each population (Verzegnazzi et al. 2021).

3.2.2 Experimental design

The 384 lines derived from BS39 population were evaluated across three locations in Iowa during the 2018 growing season. In all locations, the 384 BS39-derived lines were laid out in augmented block design (Federer 1956). We used 12 inbred lines as check treatments to correct the experimental error in the experiments. Ten out of them are from the Germplasm Enhancement of Maize Project: GEMN0048, GEMN0097, GEMN0098, GEMN0157, GEMN0177, GEMN0178, GEMN0187, GEMN0192, GEMN0225 and GEMN0238, and two are the ex-PVP inbred lines, GF1 and LH195. Each plot was a single 5.49-m long, with rows spaced 0.76 m apart. In all experiments, the plant population was ~76,500 plants ha−1. Trial management was the same for all experiments and they were carried out according to recommended agronomic practices for maize production in Central Iowa.

3.2.3 Trait measurements

We evaluated three traits: anthesis-silking interval (ASI), plant height (PH) and ear height (EH). Anthesis-silking interval was measured in days as the difference between female and male flowering (ASI=days to 50% silking – days to 50% anthesis). The PH and EH were measured in centimeters as the distance from the ground level to the collar of the uppermost leaf and to the collar of the upper ear leaf, respectively.

3.2.4 Testcross assays

Testcrosses performance based on crossing the 384 BS39-derived lines used in this study and Ex-PVP inbred line LH195, a stiff-stalk synthetic line, was previously assessed by

Santos et al. (2022). Briefly, 96 inbred lines from each set of BS39 population (BS39_DH, BS39xA427_DH, BS39_SSD, BS39xA427_SSD) were crossed with the Ex-PVP inbred line LH195, in winter 2017. Female rows were hand-emasculated, and wind pollinated by the tester to produce testcross seeds for the field trials. Then, the 96 testcross hybrids obtained from each set of BS39-derived lines along with six checks hybrids were evaluated for PH and EH across four locations in Iowa during the 2018 growing season.

3.2.5 Genotypic data

The 192 lines of sets BS39_DH and BS39xA427_DH were genotyped using genotyping-by-sequencing (GBS; Elshire et al. 2011). Genotypes were called with the TASSEL 5 - GBS production pipeline using the ZeaGBSv2.7 (AGPv2) as reference genome (Glaubitz et al. 2014). In total, 955,690 SNP markers distributed across the entire genome were generated by GBS. The 192 inbred lines of sets BS39_SSD and BS39xA427_SSD were genotyped using Diversity Arrays Technology (DArTSeq; Jaccoud et al. 2001), and SNP calling was done with the DArTsoft analytical pipeline using B73 AGPv4 as a reference genome. A total of 32,930 SNPs markers were generated by DArTSeq. In both sets of SNPs markers, SNPs with minor allele frequency lower than 0.05, call rate lower than 0.5 and monomorphic markers were removed before imputing using Beagle 5.0 (Browning and Yu 2009). After filtering and imputing, the final number of markers used for genome-wide association analysis (GWAS) was 228,562 SNPs and 16,244 SNPs distributed across all chromosomes in the DH lines and SSD lines, respectively.

3.2.6 Phenotypic data analyses

A mixed model implemented in the R package ‘sommer’ (Covarrubias-Pazaran 2016) was used to estimate variance components as well as to predict genotypic values of each inbred line across locations. Environment (locations) and checks were included in the model as fixed effects, whereas inbred line and block were considered random effects. The interactions between fixed and random effects were included in the model as random effects. The phenotypic values were modeled according to following equation: $\mathrm{y} = \mathrm{X\beta} + \mathrm{Zg} + \mathrm{Wge} + \mathrm{e}$, where y is the vector of phenotypic values of observations; β is the vector of fixed effects of locations, checks and set of lines; g is the vector of random effects of line, with $\mathrm{g} \sim \mathrm{N}(0, \mathrm{G}\sigma_{\mathrm{G}}^2)$, where G is a n x n matrix of relative kinship coefficients that define the degree of genetic covariance between a pair of lines and σ_{G}^2 is the genotypic variance due to lines; ge is the vector of random effects of line-by-location interaction, with $ge \sim N(0, I\sigma^2_{GE})$ and I is an identity matrix and σ^2_{GE} is the variance due to genotype-by-location interaction; e is the vector of random effects of error, with $e \sim N(0, I\sigma^2)$ and σ^2 is the error variance; X, Z and W are the incidence matrices associated with the effects β, g and ge, respectively. The G matrix was estimated based on VanRaden (2008) approach using the R package "AGHmatrix" (Amadeu et al. 2016).

Variance components were estimated using the restricted estimation of maximum likelihood (REML) approach (Patterson and Thompson 1971), and genotypic values of inbred lines were predicted using the best linear unbiased predictors (BLUPs; Piepho et al. 2008). A likelihood ratio test (LRT) deviance analysis was used to test random effects via the chi-square statistic (Neyman and Pearson 1928). Ranges and mean values were based on BLUP. Broad-sense heritabilities ($\hat{h}^2$) were estimated for each trait based on the generalized heritability estimator (Cullis et al. 2006; Piepho and Möhring 2007): $\hat{h}^2 = 1 - \frac{\bar{v}_{BLUP}}{2\hat{\sigma}^2_G}$, where $\bar{v}_{BLUP}$ is the mean variance of a difference of two BLUPs and $\hat{\sigma}^2_G$ is the genotypic variance estimate due to maize lines. Additionally, Pearson's correlation coefficients for PH and EH were estimated between the genotypic values of the lines per se and their testcrosses using the R package "Hmisc" (Harrell Jr and Dupont 2023).

We also predicted the genetic gain (ΔG) from selection in each set of maize lines using the following formula (Falconer and Mackay 1996; Hallauer et al. 2010): $\Delta G_\alpha = k_\alpha \hat{h} \hat{\sigma}_G$, where k_α is the selection differential in standard deviation units for selection intensities (α) equals to 5% (k=2.06), 10% (k=1.76), and 40% (k = 0.97); $\hat{h}$ is the square root of heritability estimate; and $\hat{\sigma}_G$ is the standard deviation of genotypic variation estimate. Then, we used the usefulness criterion (U) to compare the breeding potential of each set of maize lines as (Schnell and Utz 1975; Bernardo 2020): $U_\alpha = \mu - \Delta G$, where μ is the overall mean of maize lines for a trait in each set.

3.2.7 GWAS analysis

Firstly, we treated the genetic component as fixed effect in a model similar to that described above to estimate the Best Linear Unbiased Estimates (BLUEs) for BS39 lines. Then, the BLUEs of ASI, PH and EH were used for performing GWAS in SSD and DH lines separately using 16,244 SNPs and 228,562 SNPs markers in the SSD and DH lines, respectively. GWAS was performed using the FarmCPU (Fixed and random model Circulating Probability Unification) model implemented in R package "GAPIT" (Lipka et al. 2012). The

FarmCPU model controls false positives and prevents over-fitting by applying algorithms that resolve confounding problems among testing markers and covariates (Liu et al. 2016). We included two principal components and the kinship matrix in the model to control spurious associations due to population structure and relatedness, respectively (Yu et al. 2006). To determine the significance threshold for multiple testing and to reduce Type I error, the False Discovery Rate (FDR) control was applied (Benjamini and Hochberg 1995). Thus, the significance threshold was set as $P = 1x10^{-7}$. The MaizeGDB database was used to find linked candidate genes for each SNP based on the maize B73 RefGen_V3 genome, and to find matches with B73 RefGen_V5.

3.3 Results

3.3.1 Means, variance components and heritabilities

We observed substantial differences among the means of four sets of lines derived from the BS39 maize population, mainly for PH and EH (Table 1). Maize lines developed using the SSD method showed larger means of PH and EH than lines developed using a DH method. In relation to ASI, there were neither source germplasm (BS39 or BS39xA427) nor method effects, and lines of the BS39_DH set had the highest mean ASI (1.76), whereas BS39_SSD lines showed the lowest mean (0.90). When we compared the DH and SSD methods within each source population, we found that means of PH and EH were greater in the BS39_SSD lines than in the BS39_DH set, while for ASI we observed the opposite. In the BS39xA427 sets, the means of SSD lines were greater than of DH lines for all traits.

Genotypic variance components ($\hat{\sigma}_G^2$) were significant ($P = 0.05$) using the likelihood ratio test for all traits and sets of lines (Table 1). Variance components for line-by-environment interactions were not significant ($P > 0.10$), except for PH in the BS39_SSD set. The magnitude of $\hat{\sigma}_G^2$ values was affected by source populations and methods used in the development of maize lines. Maize lines developed using the SSD method had larger genotypic variation than those developed using the DH method for all traits from both source populations, BS39 and BS39xA427. Also, the BS39xA427_SSD lines exhibited the largest $\hat{\sigma}_G^2$ values for PH and EH among the four sets of BS39 lines, whereas the BS39_SSD set showed the largest $\hat{\sigma}_G^2$ value for ASI followed by BS39xA427_SSD lines.

Table 1. Means, variance component estimates due to lines (σ^2_G) and genotype-by-environment interactions (σ^2_{GE}), and estimates of residual variance (σ^2) with standard error in brackets, and broad-sense heritability (h^2) for plant height (PH), ear height (EH) and anthesis-silking interval (ASI) measured in four sets of lines derived from BS39 maize population across three environments

Traits	Sets	Mean	$\hat{\sigma}^2_G$	$\hat{\sigma}^2_{GE}$	$\hat{\sigma}^2$	$\hat{h}^2$
PH (cm)	BS39_DH	163.33	245.67*/(40.02)	1.46 (13.84)	128.16 (18.64)	0.65
	BS39_SSD	188.04	328.79*(60.30)	140.66*(23.84)	48.01 (14.44)	0.64
	BS39xA427_DH	179.35	194.43*(31.24)	<0.001 (8.69)	89.63 (12.22)	0.68
	BS39xA427_SSD	189.23	458.30*(76.36)	22.15 (23.97)	103.05 (23.93)	0.79
EH (cm)	BS39_DH	74.40	126.10*(22.22)	<0.001 (13.18)	134.95 (18.76)	0.48
	BS39_SSD	85.44	160.81*(30.93)	32.63 (23.16)	81.99 (21.83)	0.58
	BS39xA427_DH	79.24	86.08*(15.69)	<0.001 (8.98)	92.61 (12.62)	0.48
	BS39xA427_SSD	87.82	256.82*(44.01)	<0.001 (8.34)	86.08 (11.71)	0.75
ASI (days)	BS39_DH	1.76	0.82*(0.17)	0.60 (0.40)	1.37 (0.37)	0.29
	BS39_SSD	0.90	1.80*(0.37)	0.77 (0.29)	0.84 (0.24)	0.53
	BS39xA427_DH	1.16	1.17*(0.21)	<0.001 (0.12)	1.28 (0.17)	0.48
	BS39xA427_SSD	1.32	1.51*(0.33)	<0.001(0.15)	1.50(0.20)	0.50

*/Significant at P=0.05 by the likelihood ratio test.

The estimates of broad-sense heritability were intermediate to high (>0.45) for PH and EH, and low to intermediate for ASI ($\hat{h}^2$values ranged from 0.29 to 0.53). In general, the $\hat{h}^2$ values of BS39_SSD and BS39_DH were very close, as well as the values of BS39xA427_SSD and BS39xA427_DH. The correlations between line per se and testcrosses performance were significant and positive (>0.52) for both PH and EH and for both BS39 and BS39xA427 (Table 2).

Table 2. Pairwise Pearson correlation coefficients of BLUPs between BS39 and BS39xA427 from testcross and per se evaluation trials for plant height (PH) and ear height (EH)

	BS39_PH per se	BS39_EH per se	BS39xA427_ PH per se	BS39xA427_EH per se
BS39_PH testcross	0.76**	0.61**	-	-
BS39_EH testcross	0.62**	0.74**	-	-
BS39xA427_ PH testcross	-	-	0.76**	0.52**
BS39xA427_EH testcross	-	-	0.69**	0.72**

**Significant at P=0.01 by t test.

3.3.2 Predicted genetic gains (ΔG) and usefulness criteria (U)

The ΔG estimates for PH and EH were intermediate-to-high, depending on selection intensity (Table 3). The ΔG values for ASI were small and ranged from 0.47% (at α=40% in the BS39_DH set) to 2.01% (at α = 5% in the BS39_SSD set). The ΔG values for all traits were greater in SSD versus DH sets of maize lines, for both BS39 and BS39xA427, at all selection intensities. Thus, the ΔG values were approximately 15%, 24% and 100% greater in the

BS39_SSD set than in the BS39_DH set for PH, EH, and ASI, respectively. In the BS39xA427 set, the ΔG values were 65%, 116% and 16% greater for SSD than DH lines for PH, EH, and ASI, respectively, irrespective of selection intensity. The set of lines derived from the BS39xA427 population using the SSD method, presented the highest ΔG values for PH and EH, whereas the lowest ΔG values for these traits were also observed for the lines from BS39xA427, but using the DH method.

Table 3. Predicted genetic gain from selection (ΔG) and usefulness criteria (U) at selection intensity α for plant height (PH), ear height (EH) and anthesis-silking interval (ASI) for four sets of lines derived from BS39 maize population across three environments

Trait	Sets	α= 5%		α= 10%		α= 40%	
		ΔG	U[a/]	ΔG	U	ΔG	U
PH (cm)	BS39_DH	26.03	137.30	22.24	141.09	12.26	151.07
	BS39_SSD	29.88	158.16	25.53	162.51	14.07	173.97
	BS39xA427_DH	23.69	155.66	20.24	159.11	11.15	168.20
	BS39xA427_SSD	39.20	150.03	33.49	155.74	18.46	170.77
EH (cm)	BS39_DH	16.03	58.37	13.69	60.71	7.55	66.85
	BS39_SSD	19.89	65.55	17.00	68.44	9.37	76.07
	BS39xA427_DH	13.24	66.00	11.31	67.93	6.24	73.00
	BS39xA427_SSD	28.59	59.23	24.43	63.39	13.46	74.36
ASI (days)	BS39_DH	1.01	0.76	0.86	0.90	0.47	1.29
	BS39_SSD	2.01	-1.11	1.72	-0.82	0.95	-0.05
	BS39xA427_DH	1.54	-0.38	1.32	-0.16	0.73	0.43
	BS39xA427_SSD	1.79	-0.47	1.53	-0.21	0.84	0.48

[a/] U= μ - ΔG, where μ and ΔG are the mean and predicted genetic gain, respectively, of maize lines for a trait in each set, respectively.

Concerning to usefulness criteria, lower U values for PH and EH are desirable to reduce maize plant height, whereas lower values for ASI are important to increase kernel set due to better synchronization of flowering in maize. The U ranged from 137.3 cm and 58.4 cm (at α = 5% in the BS39_DH) to 174.0 cm and 76.1 cm (at α = 40% for BS39_SSD) for PH and EH, respectively (Table 3). For ASI, the BS39_DH showed the highest U values at all selection intensities, while the BS39_SSD showed the lowest U values and all those were negative. At all selection intensities, the lowest U values for PH and EH were found for BS39_DH lines. In contrast, for PH the highest U values were observed in the BS39_SSD set, whereas for EH the highest U values were observed in the BS39xA427_DH set at α = 5% and in the BS39_SSD set at α = 10% and 40%. Regarding the impact of lines development methods on U values, we observed that, in the BS39 sets, the U values for PH and EH were 13% (approximately 20 cm) and 11.5% (approximately 8 cm), respectively, greater in the lines developed using SSD (BS39_SSD) than in the lines from BS39_DH set, irrespectively of selection intensity.

Conversely, in the BS39xA427 sets, the U values were greater for PH (approximately 3%) in the lines developed using a DH method (BS39xA427_DH) at $\alpha = 5\%$ and 10%, while at $\alpha = 40\%$, the BS39xA427_SSD set was slightly greater than BS39xA427_DH (approximately 1.5%). We found similar results for EH since the BS39xA427_DH set was greater than BS39xA427_SSD at $\alpha = 5\%$ and 10% (approximately 10% and 6.7%, respectively), but at $\alpha = 40\%$, the BS39xA427_SSD set also was slightly greater than BS39xA427_DH (approximately 1.8%).

3.3.3 GWAS for traits characterized at per se level for the BS39 line panel

We found three and six SNPs significantly ($P < 1x10^{-7}$) associated with PH and EH, respectively, and no SNP was found associated with ASI ($P > 1x10^{-7}$) in the DH line set (Fig. 2). For PH, significant SNPs were found on chromosome (chr.) 3 (Table 4). For EH, we found two SNPs on chr. 3, and one on chr. 1, 2, 4 and 5. The nine significant SNPs found in the DH line set were associated with six candidate genes (Table 5). The SNP S3_148977424 was located within the gene Zm00001eb140620 and codes for non-specific phospholipase C gene family. The SNP S4_28330337 is linked to gene Zm00001eb17118 that codes for tetratricopeptide repeats (TPRs) containing proteins, and S3_31555212 is linked to gene Zm00001eb126840, which encodes basic helix–loop–helix (BHLH) domain-containing proteins. We did not find any significant SNP ($P > 1x10^{-7}$) associated with any tested trait in the SSD line set (Fig. 3).

Fig. 1 Manhattan and quantile-quantile (Q-Q) plots for GWAS of plant height (a), ear height (b) and anthesis-silking interval (c) using the FarmCPU model on set of DH lines derived from BS39 and BS39xA427 populations.

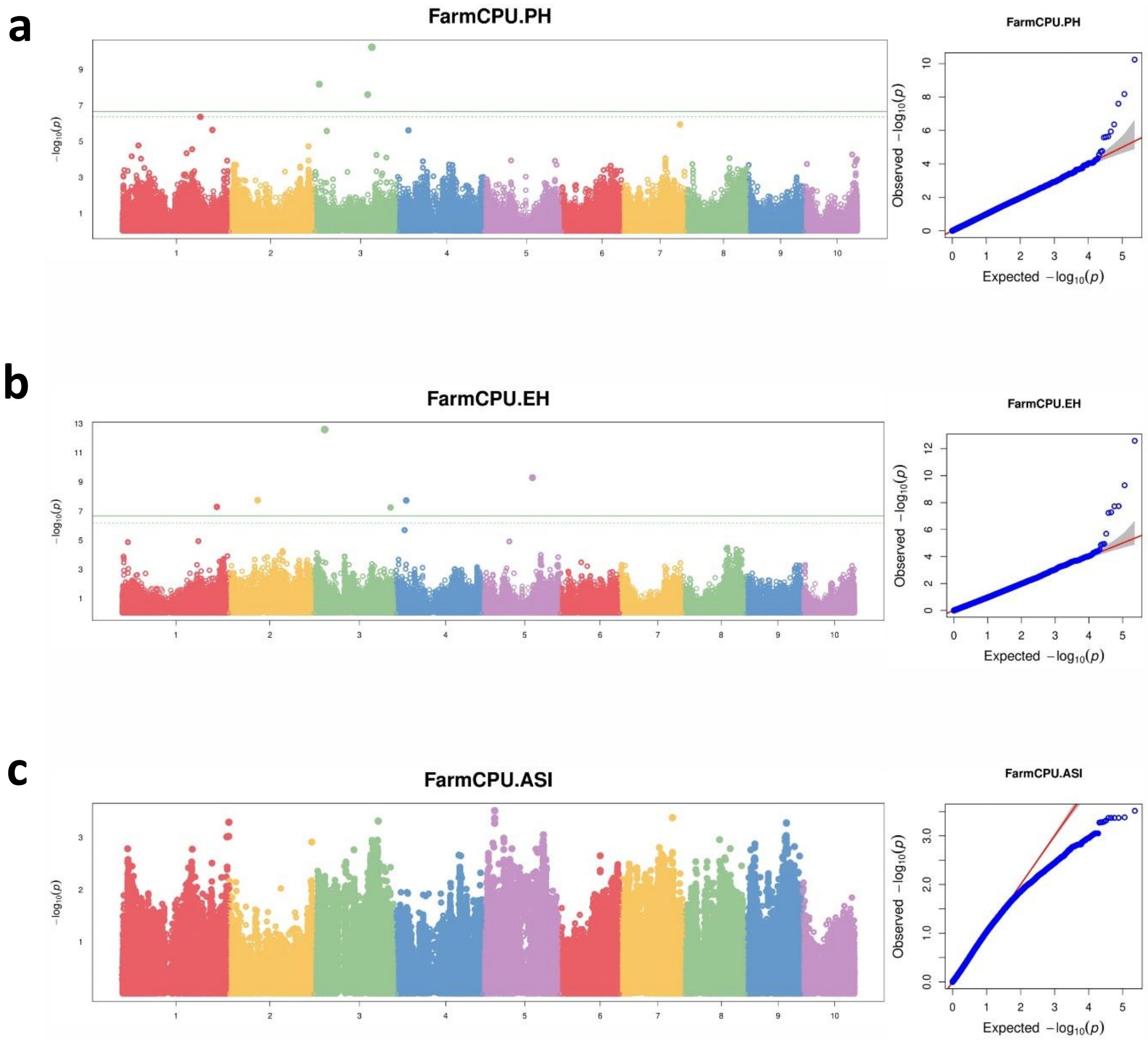


Table 4. Significant SNPs associated with plant height (PH, cm) and ear height (EH, cm) identified by GWAS using the FarmCPU model on set of DH lines derived from BS39 and BS39xA427 populations.

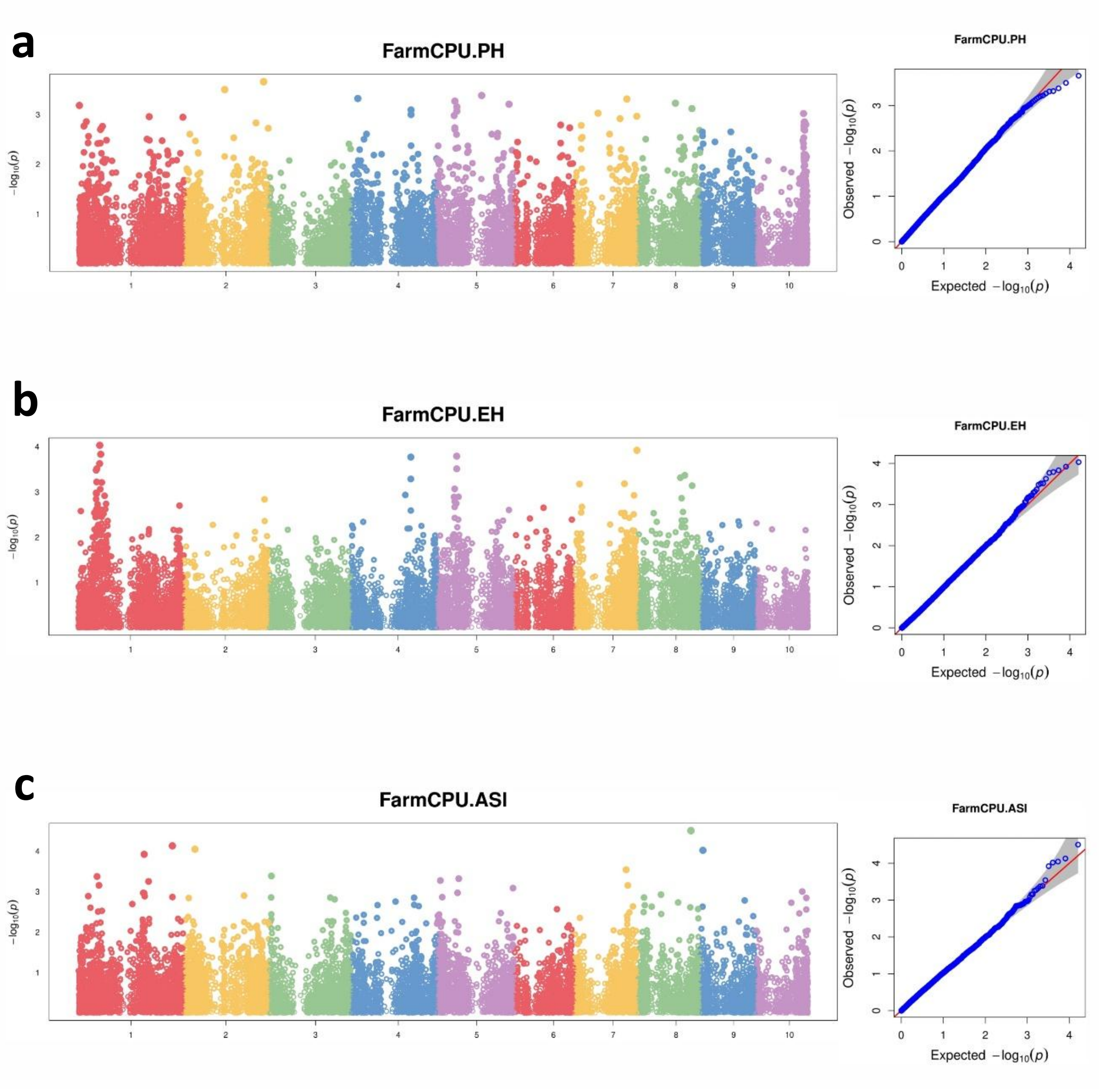
Trait	SNP	Chr	Position	P-Value	SNP effect
PH	S3_13485056	3	13,485,056	6.68E-09	6.4644
	S3_148977424	3	148,977,424	2.51E-08	5.8812
	S3_160667501	3	160,667,501	5.85E-11	7.8481
EH	S1_267198903	1	267,198,903	5.20E-08	4.7557
	S2_80446866	2	80,446,866	1.83E-08	4.0057
	S3_31555212	3	31,555,212	2.60E-13	12.9613
	S3_216472260	3	216,472,260	5.78E-08	4.7547
	S4_28330337	4	28,330,337	1.88E-08	7.3435
	S5_141252132	5	141,252,132	5.16E-10	5.7497

Table 5. Significant SNP markers and candidate genes associated with plant height (PH) and ear height (EH) identified by GWAS using the FarmCPU model on set of DH lines derived from BS39 and BS39xA427 populations

Trait	SNP	B73 Gene ID[a/]	Zm Gene ID[b/]	Function Annotation[c/]
PH	3	GRMZM2G479112	Zm00001eb140620	Non-specific phospholipase C2
PH	3	GRMZM2G092112	Zm00001eb142950	Beclin-1-like protein
EH	1	GRMZM2G467893	Zm00001eb053090	Nodulin-like domain-containing protein
EH	3	GRMZM2G163975	Zm00001eb126840	BHLH domain-containing protein
EH	4	GRMZM2G146862	Zm00001eb171180	TPR-containing protein
EH	5	GRMZM2G069332	Zm00001eb237860	Expansin domain protein

[a/] Based on B73 RefGen_v3; [b/] Based on B73 RefGen_v5; [c/] Obtained from MaizeGDB and Gramene.

Fig. 2 Manhattan and quantile-quantile (Q-Q) plots for GWAS of plant height (a), ear height (b) and anthesis-silking interval (c) using the FarmCPU model on set of SSD lines derived from BS39 and BS39xA427 populations.



3.4 Discussion

3.4.1 Genetic parameters of BS39 lines developed by SSD and DH methods

Extensive genotypic variation was observed for PH, EH and ASI in each set of maize lines derived from the BS39 population, and associated with intermediate-to-high $\hat{h}^2$ values for almost all traits and sets. Selection for PH, EH and ASI should thus allow good genetic progress in the genetic improvement of the BS39 maize population using any or all sets of lines (Hallauer et al. 2010; Hallauer and Carena 2016). The lower genotypic variation among the maize lines developed using DH method in both sets, BS39 and BS39xA427, compared with those developed using the SSD method for all traits, agreed with results reported by Verzegnazzi et al. (2021). These authors characterized the same four sets of BS39 lines using molecular markers and observed that the SSD method was more suitable to retain genetic diversity compared to the DH method. Consistent with these expectations, they found that LD decayed slower for DH lines over larger distances than for SSD lines, in both BS39 and BS39xA427 sets. In a recent study, Zeitler et al. (2020) reported a dramatic loss of genetic diversity among DH lines derived from European maize landraces at both the haplotype and individual SNP level. The narrower genetic diversity among the maize DH lines associated with the presence of slower LD decay compared to SSD lines may be due to selection done during DH process to discard plants with extreme phenotypes and DH plants with poor or none pollen production, seedling loss during chromosome doubling procedures and establishment of DH seedling in the field, fixation of particular haplotypes and also less recombination undergone by DH lines compared with SSD lines (Murigneux et al. 1993; Jannink and Abadie 1999; Frisch and Melchinger 2007; Smith et al. 2008; Lübberstedt and Frei 2012; Zeitler et al. 2020). Finally, it could also be due to a more stringent purge of genetic load present in exotic germplasm (Wilde et al. 2010; Prasanna et al. 2012; Prigge et al. 2012; Strigens et al. 2013; Böhm et al. 2017; Brauner et al. 2019; Chaikam et al. 2019).

Regarding the introgression of SHGD genes into the BS39 population, the alleles coming from inbred line A427 affected both PH and EH traits during the DH production, since these sets of lines showed lower genotypic means and genetic variances compared with SSD lines for plant stature. According to Verzegnazzi et al. (2021), the presence of SHGD alleles in the BS39 population interfered with the development of DH lines reducing their allelic diversity, mainly due to selection of particular A427-derived SHGD alleles. Thus, LD decay distance (~150 kb) in the set of DH lines from BS39xA427 was greater than the LD decay distance (~94 kb) in the set of DH lines from BS39 per se. Conversely, the introgression of temperate inbred line A427 into BS39 tropical population, with absence of selection for SGHD alleles during SSD lines development, broadens the genetic base of the source population and reduced its genetic load. During the six self-pollinations required to produce inbred lines by SSD method from BS39xA427, the SSD lines undergone multiple recombination events that were important to break up linkage blocks and A427 haplotypes present in BS39xA472 inbred progenies (Frisch and Melchinger 2007). This is consistent with much faster LD decay (~51 kb) in the BS39xA427_SSD set than LD decay distance (~150 kb) in the BS39xA427_DH set (Verzegnazzi et al. 2021). After that, favorable alleles of A427 were incorporated in the BS39 lines replacing those recessive alleles that have large detrimental effects on inbred lines and cause severe inbreeding depression and poor vigor in them. Consequently, SSD lines from BS39xA427 had larger genotypic variation and higher genotypic means for PH and EH than DH lines from BS39xA427. Additionally, the SSD lines derived from BS39 and BS39xA427 exhibited greater genotypic variation for ASI, whereas the BS39_DH set showed the highest mean value for this trait.

Even though maize lines developed using DH methods appear to have smaller genetic divergence and genetic variation for agronomic traits than SSD lines, diverse and high-yielding hybrids have been successfully developed from DH lines derived from elite germplasm (Beyene et al. 2011; 2013; 2017; Prigge et al. 2012; Sserumaga et al. 2016; Maqbool et al. 2020) and maize landraces (Wilde et al. 2010; Brauner et al. 2019; Hölker et al. 2019; Maqbool et al. 2020). Bordes et al. (2007) compared maize testcross performance of DH with SSD lines derived from the same broad-based population and found that genetic variance due to testcrosses of DH lines was greater than the genetic variance among testcrosses from SSD lines for grain yield and plant stature traits. Recently, Santos et al. (2022) evaluated maize testcrosses based on crosses of an elite inbred line with the four sets of BS39 lines used in our study. Genotypic variance among testcrosses of DH lines were greater than of SSD lines for grain yield, plant and ear height, in both BS39 and BS39xA427 sets. Also, some testcrosses of DH lines from the BS39 population had comparable agronomic performance with maize hybrids developed from ex-PVP inbred lines. These genotypic differences could be attributed to residual heterozygosity in SSD lines providing weaker allelic complementation among SSD lines and tester and, consequently, decreasing the genetic variance among SSD testcrosses compared with DH lines. Consequently, DH method has largely replaced recurrent selfing in the development of new lines of maize in private and public breeding programs (Prasanna et al. 2012; Andorf et al. 2019; Chaikam et al. 2019) and has also been proposed for unlocking the untapped genetic variability of maize landraces and open-pollinated varieties (Strigens et al. 2013; Böhm et al. 2017; Melchinger et al. 2017; Brauner et al. 2019; Maqbool et al. 2020). However, the effect of method of development of lines on genetic variance among lines per se and among their testcrosses must be more studied for other agronomic traits and, mainly, using elite breeding populations as source germplasm.

In outcrossing populations, such as the BS39 population, the DH method appears to be more stringent for eliminating recessive detrimental alleles in the parental gene pool than traditional inbreeding methods based on successive generations of self-pollination (Geiger and Gordillo 2009; Strigens et al. 2013; Böhm et al. 2017; Chaikam et al. 2019). Therefore, to prevent the losses of lines due to genetic load and genetic diversity during the maize line development using DH method, we suggest that maize breeders increase the size of source populations and use large numbers of individuals from each population, mainly from maize landraces. Furthermore, to reduce the effects of unfavorable alleles from A427 and the risk of fixing its genomics regions, we suggested that the development of DH lines using SHGD be done from backcross populations with 75% breeding population and 25% SHGD donors instead of F_1 plants used in our study. As the inheritance of SHGD is mainly mediated by a single large-effect QTL (Ren et al. 2017; Trampe et al. 2020; Verzegnazzi et al. 2021), and after the identification of additional major genes, favorable alleles may be transferred to breeding populations using marker-assisted backcrossing and marker-assisted gene pyramiding (Boerman et al. 2020; Trampe et al. 2020).

3.4.2 Predicted genetic gain (ΔG) and usefulness criteria (U) of lines derived from BS39

As the ΔG is function of the genotypic variance, heritability and selection intensity (Falconer and Mackay 1996; Hallauer et al. 2010; Bernardo 2020), the sets of BS39 lines that had the greatest estimates of both genotypic variance and heritability parameters, at the same value of selection intensity, presented the highest ΔG values in our study. Thus, in both the BS39 and BS39xA427 sets, maize lines developed using the SSD method had the highest ΔG values for all traits and at all selection intensities. As mentioned above, maize lines developed by the SSD method were more suitable to capture genetic variability in both BS39 and BS39xA427 sets than DH lines for all tested traits. This implies that selection for plant stature and ASI based on SSD lines performance will lead to a greater genetic gain in the improvement of the BS39 population than selection based on DH lines. However, although the ΔG values observed in both DH sets (BS39_DH, BS39xA427_DH) were lower than those of SSD lines, ΔG values in both sets of DH lines were intermediate to high. Consequently, good genetic progress can still be made in the BS39 population by selecting DH lines for plant stature and ASI. Moreover, rates of genetic gain per year using DH lines might be higher than using SSD lines since the DH lines can be developed in two generations compared to six to eight generations for SSD lines. Thus, the breeding strategy of using DH lines can reduce the time required to complete the breeding cycle and consequently can enhance genetic gains in maize breeding programs (Andorf et al. 2019; Chaikan et al. 2019; Maqbool et al. 2020). Finally, the production costs per DH line can be greatly reduced with the use of SHGD without a negative impact on the genetic gain of the BS39 population.

Usefulness criteria account for differences in mean, genetic background and selection response, and thus it is appropriate to compare the types of source germplasm (Schnell and Utz 1975; Melchinger et al. 1998; Bernardo 2020). The reduction in plant stature and ASI is crucial for the development of modern hybrids that are more tolerant to low nitrogen, drought, stalk lodging and high plant population (Trachsel et al. 2016; Adu et al. 2021; Sandhu and Dhillon 2021; Silva et al. 2022), the U values were obtained by the difference between the population means and ΔG. Consequently, lower U values for PH, EH and ASI are desirable in maize breeding, while for grain yield higher U values are desirable targeting the genetic improvement of maize yielding performance. The BS39_DH set showed the lowest U values for PH and EH at all selection intensities, and they should be used for the improvement of BS39 population per se for plant stature. Regarding BS39xA427, the BS39xA427_SSD set had the lowest U values for PH and EH at stronger selection. However, at weaker selection intensity, the development of maize lines using DH method and SHGD donor appears the best breeding strategies to improve BS39 population for plant stature and ASI. As BS39xA427_DH set showed lower genotypic variance estimates than other tested sets, we suggest to: i) monitor the genetic variance of BS39 during next breeding cycles, ii) use weaker selection intensity in its improvement, and also iii) increase the numbers of haploids obtained from them during next breeding cycles. These breeding strategies will help to avoid the exhaustion of genetic variability in the improved versions of BS39 population.

Santos et al. (2022) assessed the ΔG and U for agronomic traits in testcrosses hybrids derived from crosses among the four sets of BS39 lines used in our study and an elite inbred line. In contrast to our findings, they found that, in both the BS39 and BS39xA427 sets, DH lines showed greater ΔG values than SSD lines for PH and EH, and also grain yield, at all selection intensities. These differences may be result of residual heterozygosity still present in

SSD lines since they were developed by six generations of self-pollination. Also, agronomic traits such as maturity, plant and ear height, tassel size, diseases and lodging were taken into consideration during self-pollination (Verzegnazzi 2019). Regarding the U values, they found that DH lines from BS39xA427 set had the lowest U values for PH and EH among tested sets. Also, for grain yield, which we are looking for higher U values, they found similar U values among almost all maize lines sets, except in the BS39xA427_SSD that presented the lowest U value for grain yield. This result is in agreement with our findings indicating that the use of the DH method associated with SHGD appears to be a viable breeding strategy to develop homozygous lines from BS39 and other exotic maize populations.

3.4.3 Correlations between testcross and per se performance of BS39 lines

In all maize breeding program, the evaluation and selection of new maize inbred lines based on testcross performance depends on the magnitude of correlation between performance of lines per se and their respective testcrosses (Hallauer and Carena 2009; Andorf et al. 2019). Thus, if testcross and line per se performance were weakly correlated for one or more traits, the selection of the maize breeding lines will be done based on their testcross performance (Betrán et al. 2003; Hallauer et al. 2010; Kebede et al. 2013). In our study, PH and EH of lines per se performance from both BS39 and BS39xA427 sets were strongly positively ($\hat{r} > 0.70$) correlated with testcross performance of PH and EH, respectively. These results imply that per se performance of BS39 lines appears to be an effective predictor of their hybrid performance. Consequently, the selection of BS39 lines for plant stature can be done based on their per se performance instead of their testcrosses eliminating the need for making crosses and carrying out an extensive amount of trials. In agreement with our results, previous studies have reported positive and moderate to high associations between line per se and their testcrosses performance for plant stature traits in maize (Mihaljevic et al. 2005; Meseka et al. 2006; Zhang et al. 2011; Môro et al. 2017). This is consistent with the genetic control of PH and EH in maize since several studies have reported that both PH and EH are mainly governed by multiple small additive genes with some large effect loci likely fixed during maize domestication (Mihaljevic et al. 2005; Peiffer et al. 2014; Li et al. 2016; Ding et al. 2017; Vanous et al. 2018; Fei et al. 2022). Additionally, Môro et al. (2017) investigated the use of genomic selection in lines to predict their respective testcrosses for seven agronomic traits in tropical maize, and concluded that is only possible to use genomic selection in the maize lines to select testcrosses for less complex traits with prevailing additive effects such as cycle and plant stature traits.

3.4.4 SNPs associated with candidate genes in BS39

GWAS enables to identify beneficial alleles in non-elite germplasm, which is the primary source of novel alleles (Vanous et al. 2018; Santos et al. 2022; Uberti et al. 2025). SNPs significantly associated with PH and EH for the DH line set were located in homologues of genes responsible for important metabolic pathways for plant growth and development. Vanous et al. (2018) also found candidate genes related to height traits in maize DH lines. Candidate gene Zm00001eb140620 that codes for a non-specific phospholipase C operates as key component of the regulatory system for cellular growth and development in living organisms (Pokotylo et al. 2013). The non-specific phospholipase C in *Arabidopsis thaliana* was identified and characterized firstly by Nakamura et al. (2005). Moreover, non-specific phospholipase C revealed multiple physiological functions under stress conditions (Nakamura and Ngo 2020; Fan et al. 2023). The gene Zm00001eb142950 related to beclin proteins plays a crucial role in molecular mechanisms of autophagy (Xu and Qin 2019). Autophagy induced under environmental stress such as hypoxia is an adaptive response that helps cells survive under these conditions, and beclin 1 is a positive regulator with a critical role in the biogenesis of autophagosomes (Tran et al. 2021). Candidate gene Zm00001eb126840, belongs to the BHLH domain-containing, plays a central role in a wide range of metabolic, physiological and developmental processes in plants (Sun et al. 2015). The BHLH protein family is one of the most important families of transcription factors and has been related to flowering time (Huang et al. 2024), fruit development (Sun et al. 2015), iron deficiency responses (Wang et al. 2013), and salt (Zi et al. 2024) and drought stress tolerance (Li et al. 2021). Candidate gene Zm00001eb171180 is involved in tetratricopeptide repeats (TPR) biogenesis, which regulates diverse biological processes including protein folding and import, organelle targeting, vesicle fusion, plant development and drought stress response (Wei and Han 2017; Graham et al. 2019). Wang et al. (2024) showed that the TPR gene family in Cucurbitaceae is involved in the regulation of salt, chilling and temperature-high-humidity stress tolerance. Similarly, Wei and Han (2017) demonstrated the role of TPR proteins during plant development, physiological processes and drought stress in *Arabidopsis*, rice and maize. In addition to the candidate genes mentioned, other genes close to them could also be identified due to slower LD decay present in the set of DH lines. The use of the BS39 population as a source of novel alleles for elite germplasm can be confirmed by our results. Future research is needed to validate the candidate genes found in our study and examine their possible causal relationships with plant stature traits.

If confirmed, they can be used for speeding up and increasing the genetic gain of these traits in maize breeding programs through marker-assisted selection (MAS; Hasan et al. 2021).

The absence of significant SNPs associated with any tested trait in the SSD lines set can be attributed to the fast LD decay associated with a lower marker density in the SSD lines (16,244 SNPs). Verzegnazzi et al. (2021) reported LD decay values of 94 kb for BS39_DH and 150 kb for BS39xA427_DH. In contrast, the LD decay observed in the sets of BS39_SSD and BS39xA427_SSD lines were 4 kb and 51 kb, respectively. Thus, as the power of GWAS depends on LD between a functional locus and molecular markers, populations with very fast LD decay require high marker density to successfully uncover the genetic architecture of complex traits such as plant stature in maize (Flint-Garcia et al. 2003; Zhu et al. 2008; Cortes et al. 2021). Moreover, the population size likely influenced our results since smaller populations reduce the power to detect significant SNPs by GWAS analysis (Ball 2013; Gibson 2018; Tao et al. 2020). Initially, our strategy was to perform GWAS on all four sets of lines. However, due to the low number of common SNP markers between the SSD and DH sets, a high level of imputation would have been required. To address this limitation, we performed separate GWAS analyses in the SSD set and DH set, which reduced the population size and, consequently, the power to detect significant SNPs compared to a combined analysis. Finally, the effects of SNPs for PH, EH and ASI may be small in the SSD lines limiting detection in smaller GWAS populations (Gibson 2018).

3.5 Conclusion

In conclusion, the DH method appears to be more stringent than SSD in eliminating recessive detrimental alleles from the parental gene pool. To prevent the loss of genetic diversity during the maize line development using DH method, we suggest that maize breeders increase the size of source populations and use large numbers of individuals, mainly from maize landraces. The use of the DH method associated with SHGD is a viable breeding strategy to develop homozygous lines from BS39 and other exotic maize populations. To reduce the fixation of undesirable genomic regions from A427, we suggest developing DH lines from backcross populations with 75% of the breeding population and 25% of SHGD donors. The selection of BS39 lines for plant stature can be done based on their per se performance instead of their testcrosses, eliminating the need for making crosses and carrying out an extensive amount of trials. Our findings support the potential of the BS39 population as a source of novel alleles for elite germplasm. Future research should focus on validating the candidate genes identified in our study and examining their possible causal relationships with plant stature traits to enable marker-assisted selection.

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4. General conclusions

We concluded that both additive and non-additive gene actions are important in controlling the inheritance of key agronomic traits in maize. Five inbred lines from the Programa Milho® are promising genetic resources for the development of breeding populations, new inbred lines and hybrids, and also open-pollinated varieties of tropical maize. Our 15 tropical maize inbred lines were allocated into four heterotic groups based on SNP markers and SCA estimates, which will be useful to optimize human and financial resources in the UFV maize breeding program. Five experimental hybrids stood out for their high-yield performance, and they must be evaluated across more diverse environments and management conditions to validate their potential commercialization in Brazil. In relation to association mapping for plant stature in panel consisting of DH and SSD lines, we observed that SSD method is more efficient than the DH method in generating genetic diversity and increasing the genetic gain for plant architecture traits; no significant SNPs were detected in the SSD set, but in the DH lines nine markers were associated with PH and EH, including candidate genes involved in metabolic pathways related to plant growth.