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Pre-DUS screening of elite maize inbreds via SSR markers and ideotype selection

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[2]Department of Agronomy, Faculty of Agriculture, Universitas Brawijaya. Jl. Veteran, Malang 65145, East Java, Indonesia. Tel.: +62-341-575826, Fax.: +62-341-559701, ♥email: sugiharto.noor@gmail.com
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Abstract. *Mitreka RF, Atmojo ED, Asyari SND, Arifin AG, Saptadi D, Sugiharto AN. 2026. Pre-DUS screening of elite maize inbreds via SSR markers and ideotype selection. Biodiversitas 27 (4): d270433. https://doi.org/10.13057/biodiv/d270433*. Maize is a key cereal crop in Indonesia, but traditional pre-DUS evaluation relies heavily on morphological traits, which are often confounded by environmental influences. This limitation hinders precise genetic differentiation and efficient selection of elite inbred lines. This study aimed to support pre-DUS differentiation by integrating Simple Sequence Repeat markers with quantitative phenotypic traits and multivariate selection. Twelve elite maize inbred lines were evaluated under field conditions using a randomized complete block design; nine quantitative traits were recorded, and 10 SSR markers were used for genotyping. Our findings present three parallel outputs: phenotypic performance, molecular characterization, and multivariate selection outcomes. Phenotypically, integrated analysis revealed significant variation, with JUNRT1 and JUNRT3 demonstrating superior performance in key yield components (kernel weight, ear diameter, dehusked ear weight, and potential yield) alongside earlier flowering. Molecularly, SSR markers provided evidence consistent with genetic differentiation and predominantly single-fragment patterns in bulked SSR profiles for JUNRT1 and JUNRT3, complementing phenotypic data for pre-DUS characterization. Through multivariate analysis, Factor Analysis effectively reduced nine quantitative traits into three meaningful components: flowering time, yield components, and ear/kernel attributes. Subsequently, the Multi-Trait Genotype-Ideotype Distance Index simultaneously favored early maturity and high yield components, ranking JUNRT1 and JUNRT3 among the top lines. Collectively, this integrated morpho-molecular workflow provides screening-level evidence for pre-DUS characterization and prioritization of candidate parents for subsequent multi-location testing and potential hybrid development. It is crucial to reiterate that these outcomes are intended to support, not replace, formal DUS testing. However, single location-season phenotyping and bulked SSR profiles cannot confirm uniformity or stability under formal DUS requirements.

Keywords: Genetic resources, germplasm characterization, Indonesian maize inbreds, pre-DUS screening, SSR markers

INTRODUCTION

Maize serves as a fundamental agricultural commodity in Indonesia due to its critical role in food security. National demand for high-quality animal feed continues to drive the development of high-yielding hybrid varieties (Syahruddin et al. 2020). Developing elite inbred lines as prospective hybrid parents is therefore a primary breeding objective within the national program. Before these varieties are commercially distributed, they must satisfy rigorous criteria for Distinctness, Uniformity, and Stability (Yang et al. 2021a). The pre-DUS screening phase provides preliminary baseline data to support this regulatory requirement. This stage is essential for identifying promising genotypes before entering formal registration trials. However, the formal DUS process requires comprehensive multi-environment testing that is often resource-intensive. Early characterization of inbred lines facilitates the prioritization of candidate parents for field evaluations. Effective germplasm management requires a robust preliminary assessment framework to navigate regulatory hurdles (Yang et al. 2021a). Accurate identification reduces the risk of rejection during the final certification stages. Establishing a solid phenotypic baseline early ensures the long-term viability of commercial hybrids.

Conventional characterization for pre-DUS evaluation relies heavily on morphological descriptors. These phenotypic assessments are traditional tools used to distinguish between closely related genotypes (Chanda et al. 2014). Nevertheless, relying exclusively on morphology presents significant technical challenges in modern breeding. One primary constraint is the inherent sensitivity of morphological traits to Genotype-by-Environment interactions (Bouchetat et al. 2023). These interactions often mask the true genetic potential of a line and reduce the precision of variety discrimination. Furthermore, elite maize inbreds frequently exhibit narrow phenotypic variation. Limited variation among closely related genotypes often complicates variety discrimination (Yadav and Singh 2010). The labor-intensive nature of manual data collection also increases the risk of human error during large-scale evaluations. Consequently, morphology-only screening can lead to ambiguous results in early-stage selection. This ambiguity often complicates

the identification of unique genetic resources within elite germplasm pools (Yadav and Singh 2010). Precise discrimination is vital to ensure that new varieties meet the legal standards of uniqueness required for plant variety protection (Yang et al. 2021b). Utilizing more stable indicators is necessary to overcome these phenotypic limitations.

Molecular marker technologies have emerged as a powerful adjunct to traditional phenotypic descriptors. Simple Sequence Repeats are widely utilized due to their high polymorphism, reproducibility, and co-dominant nature (Akbari et al. 2025). These markers allow breeders to detect genetic differences that are not expressed at the morphological level. However, molecular data are intended to provide screening-level evidence and cannot replace formal regulatory requirements. The official DUS system still necessitates morphological descriptions to define the physical characteristics of a variety (Figàs et al. 2018). SSR markers were employed to provide high-resolution genetic profiling of elite inbred lines. This molecular evidence reinforces the biological aim of characterization by detecting underlying genetic variability. Molecular characterization is particularly valuable for validating the genetic purity of inbred lines (Cai et al. 2020). Genomic information provides a stable baseline that is unaffected by environmental fluctuations. Integrating molecular insights with phenotypic data creates a more comprehensive profile for candidate varieties. This multi-layered approach supports the decision-making process during the transition from breeding to formal registration.

Effective pre-DUS screening requires the simultaneous evaluation of multiple correlated traits to avoid selection bias. Single-trait selection often fails to account for the complex trade-offs between yield components. To address this complexity, multivariate frameworks like the Multi-Trait Genotype-Ideotype Distance Index with factor analysis provide a more holistic selection tool. This index facilitates the identification of genotypes that align closely with a predefined breeding ideotype (Olivoto and Nardino 2021). Factor analysis is also essential for reducing high-dimensional data into meaningful, non-correlated components (Olivoto and Nardino 2021). These statistical methods allow breeders to quantify trait correlations and avoid the pitfalls of subjective visual assessment. The practical decision point in this study involves screening elite inbreds for subsequent DUS and hybrid development. By combining phenotypic evaluation, SSR-based profiling, and MGIDI-based selection, this study aims to refine the characterization of Indonesian elite maize germplasm. The specific objectives were to quantify phenotypic variation, assess molecular diversity, and apply ideotype-based ranking to identify superior lines. This integrated workflow offers a robust mechanism for early-stage variety identification and prioritization. Implementing such a framework ensures a higher success rate in official trials.

MATERIALS AND METHODS

Study area

The research comprised two distinct stages: field and laboratory experiments. The research was conducted in Areng-areng Hamlet, Dadaprejo Village, Junrejo Sub-district, Batu City, East Java, Indonesia (7°54'21.4"S 112°33'53.3"E). Field trials were conducted during January-July 2025. Batu City is situated at an average elevation of 897 masl, characterized by an average monthly rainfall of 141 mm and an average temperature of 22°C (Handayani et al. 2023). The soil at the experimental site consists of a sandy clay texture, which provides balanced aeration for maize growth. Laboratory experiments were performed at the Biotechnology Laboratory, Universitas Brawijaya.

Genetic materials

The research subjects consisted of twelve elite maize inbred lines (JTKT1, JTKT2, JTKT3, JUNRT1, JUNRT2, JUNRT3, DR1, DR2, DR3, JUNRA1, JUNRA2, and JUNRA3). These lines were sourced from the CV. Blue Akari collection and were developed as candidate parental lines for hybrid maize breeding. The set represents the fifth selfing generation (S_5) within an ongoing selection program aimed at improving parental uniformity and stabilizing key agronomic traits. The line codes reflect internal breeding populations and selection streams maintained by CV. Blue Akari, and all entries were handled and stored as inbred materials to support downstream pre-DUS characterization and parental evaluation.

Experimental design and field management

This study was conducted as a field experiment using a randomized complete block design with three replications, with treatments randomized independently within each block. A total of 36 plots were established, with seeds sown at 60 × 30 cm spacing and 60 plants per plot. To minimize edge effects, border rows and end plants were excluded from sampling. Morphological observations were recorded from 10 plants per plot, following the UPOV TG/2/7 Rev. technical guidelines. Standard local agronomic practices, including fertilization (60 kg N ha^{-1}, 45 kg P_2O_5 ha^{-1}, and 45 kg K_2O ha^{-1}), manual weed management, and pest control, were uniformly applied across all plots. Pest control included chlorantraniliprole-based insecticide (30 g a.i. ha^{-1}) against *Spodoptera frugiperda* (J.E.Smith, 1797) at V6 and VT stages, and propiconazole-based fungicide (125 g a.i. ha^{-1}) for rust disease at VT and R1 stages.

Working procedures for molecular screening

Total genomic DNA from mixed samples in each maize inbred line was isolated from 100 mg of two-week-old fresh leaf tissue using a Plant Genomic DNA Mini Kit (Geneaid Biotech Ltd., Taiwan) following the manufacturer's protocol. Samples were initially frozen in liquid nitrogen and ground into a fine powder. Key steps included RNase treatment, column-based DNA binding and washing, and elution in 100 µL buffer; the purified DNA was collected and stored at -20°C for subsequent analyses. A total of 40 SSR primer pairs (Sigma-Aldrich, USA) were initially screened; 21 primers were polymorphic, and 10 SSR markers were selected for all molecular analyses presented here based on high informativeness (PIC>0.5) and their reproducibility with a clear scoring (Table 1). Each sample

reaction consisted of a 25 µL volume containing 3 µL of DNA template and 22 µL of master mix, which comprised dNTPs, MgCl, Tris-HCl buffer, KCl, Taq DNA polymerase, specific forward and reverse primers, and nuclease-free water. PCR was performed under the following cycling conditions: initial denaturation at 95°C for 3 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 50-54°C for 30 s, and extension at 72°C for 30 s; followed by a final extension at 72°C for 7 min. Amplified PCR products were held at 4°C until electrophoresis.

Amplified DNA fragments were resolved using agarose gel electrophoresis. A 1.5% agarose gel was prepared by dissolving 0.6 g of agarose in 40 mL of 1× TAE buffer, heating until melted, and cooling before casting. Following solidification, DNA samples, mixed with 1 µL of 6× loading dye, were loaded into the wells with a 50-500 bp DNA ladder as a molecular marker. Electrophoresis was performed in 1× TAE buffer at 90 V for 60 min. Gels were stained with ethidium bromide for 10 min, destained in distilled water for 10 min, and visualized under UV illumination using a UV transilluminator. This approach was utilized as the selected SSR markers were expected to exhibit allele size differences exceeding 10 bp. While this method is robust for baseline profiling, its resolution limits for very small differences; thus, for formal DUS registration, advanced platforms like capillary electrophoresis are recommended to complement these results.

Data collection

Phenotypic data were recorded from 10 plants per plot, selected by restricted random sampling from non-border rows and columns to minimize edge effects. Importantly, trait observations from these individual plants were averaged at the plot level prior to statistical analysis. Nine quantitative traits were measured: 50% Anthesis (AA), 50% Silking (SA), Ear Length (EL), Ear Diameter (ED), Number of kernel Rows (NR), 100-Kernel Weight (KW), dehusked Ear Weight (EW), Kernel Percentage (KP), and Potential yield (PK). AA and SA were recorded as days from sowing to when 50% of plants in a plot reached anthesis (pollen shed) and silking (visible silk emergence), respectively. Ear-related traits were measured at physiological maturity and standardized to 11% grain moisture. The explicit formula used for PK estimation is as follows: PK (t ha^{-1}) = [(grain weight per plot area × 10 × (100-moisture content)) / ((100-adjusted moisture content) × plot area)] where grain weight is in kg, moisture content is in percentage, and plot area is in square meters.

Genomic DNA was extracted from 14-day-old bulked leaf tissue comprising 10 randomly sampled plants per inbred line and amplified using the 10 selected SSR primers (Table 1) to generate locus-specific fragments. Amplicons were separated by gel electrophoresis, photographed, and allele sizes were estimated (bp) from gel images based on pixel-intensity distributions using Gel Analyzer calibrated with a 50-500 bp DNA ladder. Given that the expected allele size differences exceeded 10 bp, this digital profiling provided sufficient resolution for initial screening by fitting univariate functions to the intensity distributions, effectively resolving bands that might appear buried under a single peak. Alleles were recorded as fragment sizes (bp) for each locus (two alleles when heterozygous) to construct an allele-size genotype matrix for each inbred line, which served as input for diversity estimates, genetic distance calculation, and clustering analyses. The use of bulked DNA samples facilitates efficient high-throughput screening and provides a cost-effective initial assessment of genetic profiles at the line level. However, it implicitly assumes homogeneity within the pooled sample, which may limit the direct detection of low-frequency heterozygosity and within-line variability that could impact strict uniformity assessments for DUS purposes, necessitating single-plant genotyping for confirmation of observed multi-allelic patterns.

Operational definitions of Pre-DUS components

Operationally, evaluations were framed as pre-DUS screening indicators rather than formal certification tests: distinctness was inferred from separable phenotypic patterns and multivariate separation supported by distance-based clustering from phenotypic and SSR data; uniformity was evaluated from low within-plot variability among the 10 sampled plants and supported by predominantly single-fragment in bulked SSR profiles across assayed loci; and stability was not formally assessed across environments or seasons and cannot be inferred from the current single-location data; thus, phenotypic consistency and molecular profiles observed here serve only as baseline evidence for future multi-environment testing.

Table 1. Primer list for SSR marker

Primer code	Forward	Reverse	Amplicon (bp)
Primer_RF-09	ACAGCCACCACCACTCCATC	AGATCTTCGAGAGCGTGGAGAAC	108
Primer_RF-11	CACCGGAACACCTTCTTACAGTTT	CGAAACCTTCTCGTGATGAGC	156
Primer_RF-16	TCTAGCTTGTGGTGGTGGTTGA	ACATGAGCACAAAGACTGACGC	152
Primer_RF-21	TGAGCCGAAGCTAACCTCTC	GATGCAAAGGAGGTTCAGGA	208
Primer_RF-22	GTAAAGTACGATGCGCCTCCC	CGGGGTAGAGGAGAGTTGTG	145
Primer_RF-24	CATGGGACAGCAAGAGACACAG	ACCTTCATCACCTGCAACTACGAC	118
Primer_RF-25	TTGCCGATAAGAACCAAACA	ACGACCGGTGTGGTTACATT	118
Primer_RF-31	AGGGTTTTGCTCTTGGAATCTCTC	GAGGAAGGAGGAGGTCGTATCGT	80
Primer_RF-34	AGAGAATCCCCAAGCAAACAAAC	CTTCATCGGAGCCATGGTGT	90
Primer_RF-38	AAGCTAATTAAGGCCGGTCATCCC	TCCGTGTACTCGGCGGACTC	148

Statistical analysis

Evaluation of phenotypic performance and trait distribution was conducted through univariate and descriptive statistical methods. Analysis of Variance was performed under the RCBD model, wherein genotypes were treated as fixed effects and blocks as random effects, with the plot mean serving as the experimental unit for all statistical analyses. Following confirmation of normality and homogeneity of variance assumptions using the Shapiro-Wilk (stats::shapiro.test) and Levene's tests (car::leveneTest), no data transformations were required as the raw data satisfied these statistical assumptions. Significant genotypic effects were further examined using the Scott-Knott test (agricolae::SK, $\alpha = 0.05$), which was specifically chosen to provide clear and non-overlapping differentiation between genotypes, facilitating a more robust variety characterization. Descriptive statistics, including standard deviations, were presented to evaluate the variation in quantitative traits. All statistical analyses were performed using RStudio version 2023.09.1 + 394.

To capture the complex interrelationships among multiple traits and classify genotypes based on their overall performance, various multivariate analytical tools were employed. Hierarchical Cluster Analysis was used for genotype classification using Pearson distance with the complete linkage method (pheatmap), while Genotype × Trait biplot analysis was used to assess partial genotype distinctness (Yan and Rajcan 2002) using (metan::gtb). All phenotypic data for the GT biplot were standardized as z-scores to ensure each trait contributed equally to the results regardless of its original numerical scale. Finally, the Multi-Trait Genotype Ideotype Distance Index (metan::mgidi) determined genotypes aligning with the breeding ideotype using a 15% selection intensity (Olivoto and Nordino 2021). The ideotype was strategically defined to prioritize earlier maturity by decreasing phenological traits, while simultaneously increasing all yield components to maximize productivity. Broad-sense heritability, selection differentials, and selection gain reported in the MGIDI output were computed from variance components. All multivariate statistical analyses were performed using RStudio version 2023.09.1 + 394.

Complementing the phenotypic evaluation, molecular genetic analysis was performed to assess locus-specific variation and determine genetic relationships among the inbred lines. Genetic profiles were analyzed based on locus-specific amplification using 10 selected SSR primers to ensure variation originated from the same genomic regions. SSR alleles were scored as fragment sizes (bp) from agarose gel images based on pixel-intensity distributions using Gel Analyzer, calibrated against a 50-500 bp DNA ladder. Alleles were recorded as bp values for each locus (two alleles when heterozygous) to construct an allele-size genotype matrix for each inbred line. Genetic distance between lines was estimated according to Nei's 1978 standard genetic distance formula (Nei 1978). Statistical summaries of polymorphism information content, allele number, heterozygosity, and gene diversity index were calculated using PowerMarker V3.25 software. Since genomic DNA was extracted from bulked leaf tissue, the observed heterozygosity is interpreted as an indicator of within-line genetic heterogeneity or residual heterozygosity. Furthermore, 10 selected SSR markers were included in the genetic distance and clustering analyses, and no missing allele calls were observed in the final allele-size matrix. Cluster analysis was performed using RStudio, with bootstrapping (1000 replicates) to assess tree support and relationships among the maize inbred lines.

RESULTS AND DISCUSSION

Morphological characterization on quantitative performance of inbred lines

The pronounced phenotypic variation observed among the twelve elite maize inbred lines for all nine quantitative traits is a fundamental prerequisite for effective selection and genetic advancement in maize breeding programs. The Scott-Knott test effectively delineated these variations, grouping genotypes into distinct performance levels (Table 2).

Table 2. Comparison of Mean Quantitative Traits of Twelve Maize Inbred Lines

Genotipe	AA	SA	NR	EL	ED	KW	EW	KP	PK
JTKT1	50.07 ^{d}	51.87 ^{d}	13.53 ^{b}	15.54 ^{a}	4.34 ^{a}	25.08 ^{a}	151.57 ^{a}	86.49 ^{a}	6.20 ^{b}
JTKT2	49.60 ^{d}	51.47 ^{d}	13.47 ^{b}	15.90 ^{a}	3.98 ^{a}	19.98 ^{c}	117.37 ^{b}	84.07 ^{b}	4.80 ^{c}
JTKT3	50.33 ^{d}	52.17 ^{d}	15.20 ^{a}	15.90 ^{a}	4.18 ^{a}	22.68 ^{b}	149.37 ^{a}	82.78 ^{b}	6.11 ^{b}
JUNRT1	57.20 ^{c}	57.57 ^{c}	13.07 ^{c}	14.03 ^{b}	4.05 ^{a}	27.40 ^{a}	180.57 ^{a}	88.43 ^{a}	7.39 ^{a}
JUNRT2	58.37 ^{b}	59.27 ^{b}	12.67 ^{c}	15.58 ^{a}	3.83 ^{a}	25.10 ^{a}	180.07 ^{a}	87.65 ^{a}	7.37 ^{a}
JUNRT3	57.57 ^{c}	58.30 ^{c}	13.73 ^{b}	13.67 ^{b}	4.15 ^{a}	25.83 ^{a}	169.37 ^{a}	88.14 ^{a}	6.93 ^{a}
DR1	61.73 ^{a}	64.50 ^{a}	12.40 ^{c}	13.00 ^{b}	2.73 ^{b}	27.70 ^{a}	102.47 ^{b}	88.18 ^{a}	4.19 ^{c}
DR2	61.17 ^{a}	63.77 ^{a}	12.67 ^{c}	12.19 ^{c}	3.06 ^{b}	26.84 ^{a}	137.80 ^{b}	89.55 ^{a}	4.28 ^{c}
DR3	61.13 ^{a}	63.83 ^{a}	12.40 ^{c}	11.11 ^{c}	3.12 ^{b}	27.07 ^{a}	153.10 ^{a}	88.83 ^{a}	4.90 ^{c}
JUNRA1	61.30 ^{a}	64.07 ^{a}	13.07 ^{c}	15.96 ^{a}	4.12 ^{a}	24.03 ^{b}	134.07 ^{b}	84.68 ^{b}	5.49 ^{c}
JUNRA2	61.33 ^{a}	64.10 ^{a}	14.27 ^{b}	16.30 ^{a}	4.21 ^{a}	23.94 ^{b}	141.77 ^{b}	82.95 ^{b}	5.80 ^{b}
JUNRA3	60.90 ^{a}	63.37 ^{a}	14.07 ^{b}	15.97 ^{a}	4.30 ^{a}	27.85 ^{a}	161.10 ^{a}	85.69 ^{b}	6.59 ^{b}
p-value	**	**	**	**	**	**	**	**	**

Note: AA: 50% Anthesis (day), SA: 50% Silking (day), NR: Number of kernel Rows, EL: Ear Length (cm), ED: Ear Diameter (cm), KW: 100-Kernel Weight (g), EW: Dehusked Ear Weight (g), KP: Kernel Percentage (%), PK: Potential yield (t ha^{-1}). Values followed by the same letter in the same column are not significantly different based on the Scott-Knott test at a 5% significance level. **: p-value ANOVA<0.01, *: 0.01<p-value ANOVA<0.05, ns: p-value ANOVA>0.05

Table 3. Comparison of standard deviations across all observed characters

Genotipe	AA	SA	NR	EL	ED	KW	EW	KP	PK
JTKT1	1.29	1.73	1.43	1.32	0.32	4.03	31.89	4.42	1.13
JTKT2	0.99	1.43	1.36	1.51	0.28	2.87	24.45	3.65	0.74
JTKT3	1.37	1.71	1.76	1.92	0.32	4.49	43.80	9.24	0.66
JUNRT1	1.33	1.54	1.24	1.51	0.26	4.08	35.69	3.37	0.48
JUNRT2	1.25	2.22	1.30	1.54	0.18	2.84	30.94	2.94	0.86
JUNRT3	1.28	1.19	1.44	1.99	0.34	4.55	45.36	4.18	0.93
DR1	1.90	2.39	2.09	1.54	0.41	3.99	40.77	13.76	0.63
DR2	1.53	1.99	1.58	3.06	0.67	4.27	33.93	6.87	1.32
DR3	1.41	1.67	1.50	1.60	0.82	4.95	59.18	10.56	2.08
JUNRA1	1.46	1.41	1.24	1.39	0.27	4.09	28.00	3.69	0.42
JUNRA2	0.94	1.04	1.61	1.42	0.32	5.29	43.62	7.34	1.41
JUNRA3	0.98	1.33	1.59	1.34	0.26	5.55	34.79	3.97	0.75

Note: AA: 50% Anthesis (day), SA: 50% Silking (day), NR: Number of kernel Rows, EL: Ear Length (cm), ED: Ear Diameter (cm), KW: 100-Kernel Weight (g), EW: Dehusked Ear Weight (g), KP: Kernel Percentage (%), PK: Potential yield (t ha^{-1})

A notable pattern was that longer growth duration tended to coincide with higher potential yield, with intermediate-late maturing lines in the JUNRT family consistently showing superior productivity. This tendency has been reported in maize, where extended growth duration can support assimilate allocation and grain filling (Zhao et al. 2022). In breeding programs targeting heterogeneous environments, early maturity remains valuable for escaping terminal drought or heat stress in short or unpredictable seasons, although it may reduce attainable yield under optimal conditions (Prasanna et al. 2021). However, the maturity-yield tendency was not uniform across genotypes. The latest-maturing DR lines (DR1-DR3) displayed comparatively low yields. suggesting that extended duration alone may not ensure efficient grain filling and could reflect less favorable source-sink coordination during key developmental stages. This finding is consistent with literature indicating that prolonged vegetative periods do not inherently guarantee increased grain yield if biomass partitioning to reproductive structures is constrained or inefficient (Devasree et al. 2020; Abo-Hamed et al. 2025). Conversely, the earliest-maturing JTKT lines generally produced lower yields than intermediate-late materials.

The superior performance of lines such as JUNRT1, JUNRT2, and JUNRT3 in terms of kernel weight and dehusked ear weight indicates enhanced grain filling efficiency and robust sink capacity. These findings are consistent with extensive literature highlighting strong positive correlations between key ear morphology traits, including ear length, ear diameter, and kernel weight, underscoring their pivotal role as direct yield components (Kyi et al. 2021; Baduwal et al. 2022). Notably, the number of kernel rows, while intuitively linked to yield, did not consistently correlate with higher total kernel weight or ear weight in this study, as exemplified by JTKT lines. This is consistent with findings that there remains scope for increasing kernel size in maize, despite the recognized trade-off between kernel number and kernel weight, which poses a challenge for future yield improvement (Fernández et al. 2022).

Further analysis through standard deviations across traits offered insights into the descriptive variability within the evaluated genotypes (Table 3). The standard deviation values are treated as indicators of descriptive consistency rather than definitive regulatory benchmarks, as no formal pre-DUS uniformity threshold was applied in this study. Genotypes consistently exhibiting low standard deviations across multiple traits typically manifest a more homogeneous and stable expression of agronomic characteristics. This consistent phenotypic expression constitutes a highly desirable attribute for the development of pure lines and the selection of consistent hybrid parents (Kovinčić et al. 2023). For example, the JUNRT family exhibited relatively low standard deviations across several key traits, suggesting a higher degree of phenotypic consistency within these lines, which is advantageous for pre-DUS assessment. Conversely, larger standard deviations may indicate greater inherent genetic variability or heterozygosity, which, if not merely attributable to measurement error, can represent a valuable reservoir of genetic diversity for targeted selection and trait improvement in breeding programs aiming for broader adaptation (Swarup et al. 2020).

Hierarchical cluster analysis (Figure 1) separated the twelve inbred lines into two major groups based on multi-trait performance. Cluster 1 was characterized by moderate to low potential yields, despite substantial variation in maturity (from early JTKT to later JUNRA). Although some entries in this cluster expressed favorable ear morphology, overall productivity remained lower. This indicates that specific phenological or morphological attributes did not consistently translate into superior yield performance in this study, a phenomenon also observed in other contexts where yield is influenced by complex genetic and environmental interactions (Genievskaya et al. 2025). Cluster 2 was generally late-maturing, with the JUNRT lines within this group demonstrating the highest potential yields alongside superior dehusked ear weight and kernel percentages. This performance is consistent with the physiological role of extended growth duration in facilitating enhanced resource capture and the partitioning of assimilates to the grain for maximizing yield potential (Zhao et al. 2022; Liu et al. 2023). In contrast, the DR lines combined late maturity with markedly lower yields, emphasizing that late maturity alone is not a sufficient predictor of high yield, but must be accompanied by efficient physiological processes and favorable genetic architecture, as reflected by the JUNRT genotypes.

Trait-based clustering delineated two distinct groups. Cluster 1 encompassed traits directly related to grain yield, specifically ear diameter, ear length, number of kernel rows, dehusked ear weight, and potential yield. This indicates that selection targeting ear structure and ear biomass is likely to provide the most direct gains in yield within this panel, consistent with research demonstrating that maize yield breakthroughs are fundamentally driven by the accumulation and efficient allocation of dry matter to the ear (Liu et al. 2023). In practical breeding terms, these traits can be prioritized as primary selection criteria because they capture sink-related variation that is closely aligned with yield formation. Conversely, Cluster 2 grouped several key traits such as 50% anthesis, 50% silking, 100-kernel weight, and kernel percentage, suggesting that

phenology and kernel attributes should be considered complementary targets to maintain adaptation and grain characteristics while advancing yield-driven selection.

PC1 and PC2 explained 51.9% and 24.7% of the total variation, respectively (cumulative 76.6%) (Figure 2.A). As an exploratory tool, the GT biplot confirms multi-trait association patterns within this specific dataset. In the PC1-PC2 biplot space, the proximity of vectors for dehusked ear weight, ear length, ear diameter, and kernel percentage suggests positive associations among these yield components, consistent with earlier reports linking grain yield with ear-related traits (Kandel et al. 2018). This pattern supports the practical use of ear morphology traits for indirect selection, as improvement in one component may coincide with gains in related traits (Carnimeo et al. 2020). By contrast, the opposing orientation of kernel weight and kernel percentage relative to some ear-dimension traits indicates an inverse tendency in this two-dimensional projection; this is treated as a working hypothesis consistent with resource-allocation constraints that can limit the simultaneous improvement of grain size and yield components (Chen et al. 2016; Simmons et al. 2020).

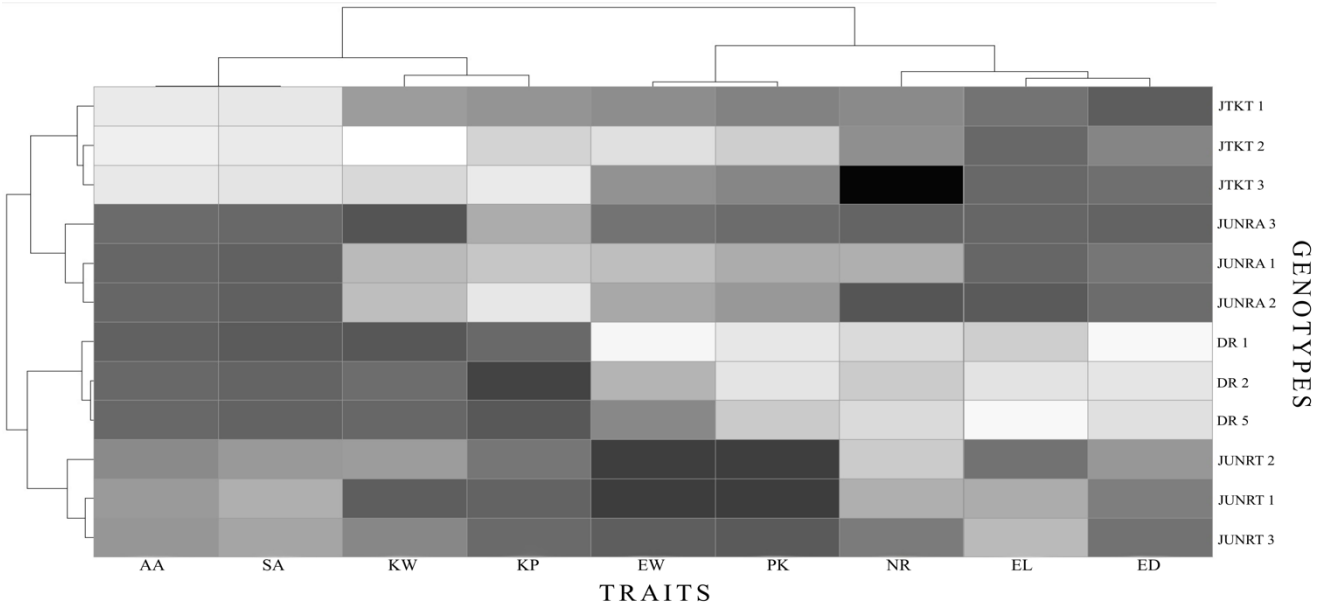

Figure 1. Hierarchical Clustering Analysis (HCA). AA: 50% Anthesis, SA: 50% Silking, NR: Number of kernel Rows, EL: Ear Length, ED: Ear Diameter, KW: 100-Kernel Weight, EW: Dehusked Ear Weight, KP: Kernel Percentage, PK: Potential yield. Darker colors represent a higher mean value of a trait in the genotype group

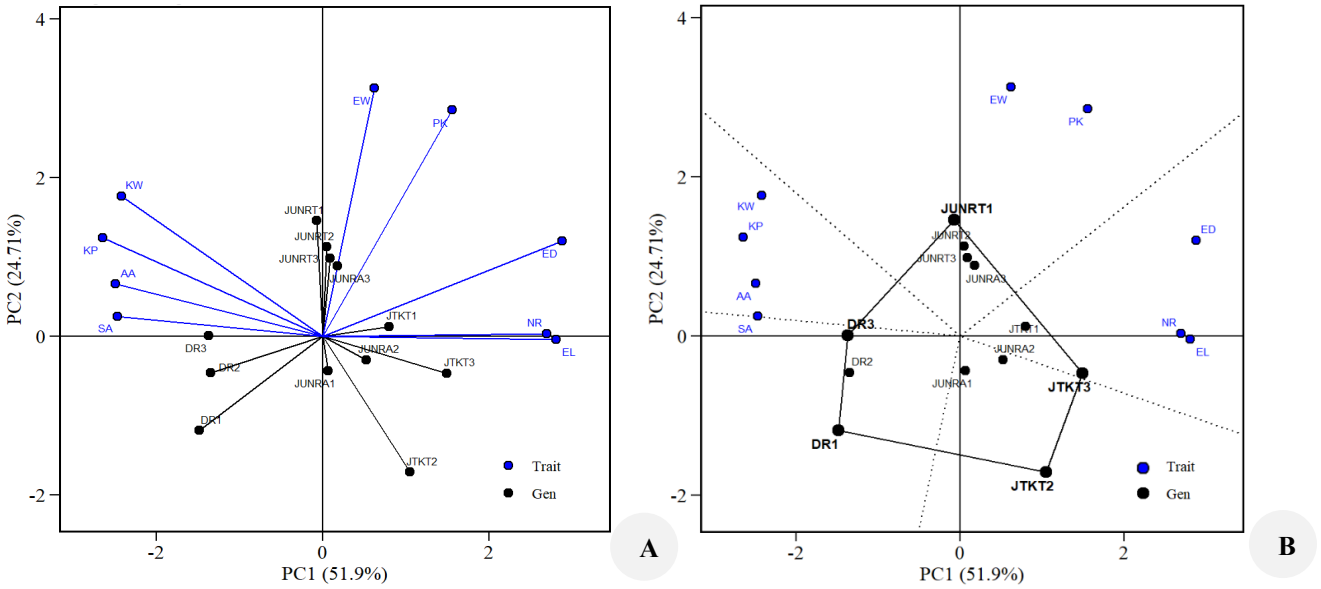

Figure 2. Genotype × Trait (GT) biplot analysis: A. Relationships among traits, B. Which-won-where polygon view. PC1: Principal Component 1, PC2: Principal Component 2, AA: 50% Anthesis, SA: 50% Silking, NR: Number of kernel Rows, EL: Ear Length, ED: Ear Diameter, KW: 100-Kernel Weight, EW: Dehusked Ear Weight, KP: Kernel Percentage, PK: Potential yield

Table 4. Explained descriptive statistics of all SSR primers

Primer code	MAF	NA	GD	HS	PIC
Primer_RF-09	0.4167	5	0.7361	0.0000	0.6990
Primer_RF-11	0.1667	10	0.8715	0.3333	0.8579
Primer_RF-16	0.2917	6	0.7986	0.4167	0.7694
Primer_RF-21	0.2083	7	0.8472	0.3333	0.8281
Primer_RF-22	0.5417	5	0.6493	0.2500	0.6135
Primer_RF-24	0.3750	6	0.7535	0.0833	0.7172
Primer_RF-25	0.5000	7	0.6736	0.5000	0.6335
Primer_RF-31	0.3333	8	0.8194	0.0000	0.8003
Primer_RF-34	0.3750	5	0.7292	0.6667	0.6838
Primer_RF-38	0.2500	9	0.8507	0.4167	0.8339

Note: MAF: Major Allele Frequency, NA: Allele Number, GD: Gene Diversity, HS: Observed Heterozygosity, PIC: Polymorphic Information Content

The polygon view (Figure 2.B) highlights vertex genotypes with sector-specific strengths. JTKT3 was a vertex genotype for the sector containing ear length, ear diameter, and number of kernel rows, whereas JUNRT1 was a vertex for dehusked ear weight and potential yield, indicating superior yield-related performance. DR3 formed a vertex associated with later flowering and relatively high kernel percentage and kernel weight, but its dehusked ear weight and potential yield were lower, helping explain why it was not prioritized in multi-trait ranking compared with the JUNRT lines. This visualization is widely used to identify trait-specific winners in GT biplot analyses (Singamsetti et al. 2024). Genotypes such as JUNRT2 and JUNRT3, characterized by high yield potential, exhibit a balanced contribution of all yield-related traits, even though this genotype is not a vertex for each trait.

Molecular characterization of inbred lines

The molecular characterization, particularly through the application of SSR, was strategically employed to complement and validate phenotypic assessments (Table 4). Meanwhile, molecular characterization additionally serves as a valuable tool for establishing a comprehensive database to safeguard the genetic profiles of inbred lines. The final selection criteria for these 10 markers were based on their high PIC, which indicates a marker's ability to distinguish.

The major allele frequency ranged from 0.1667 to 0.5000, indicating that no single allele predominated at any locus and that allele frequencies were relatively balanced across markers. However, allele number estimates derived from agarose gel-based SSR profiling should be interpreted cautiously due to resolution limitations in distinguishing closely sized alleles (Masha et al. 2022). This allele-frequency profile is advantageous for applications such as population structure assessment and marker-assisted breeding, where maintaining diversity supports robust and adaptive selection. In addition, loci with higher allele numbers generally provide stronger discriminatory power among genotypes, strengthening the resolution of pre-DUS characterization and cultivar differentiation, which is relevant for variety registration and intellectual property protection (Yang et al. 2021b).

The high gene diversity observed (ranging from 0.6493 to 0.8715) reflects substantial allelic variation and suggests a broad genetic base among the evaluated lines. Comparable levels were reported by Saidi et al. (2022), who documented an average expected heterozygosity of 0.64 for SSR markers, supporting their effectiveness for capturing allelic richness and genetic structure. The observed heterozygosity values (ranging from 0.0000 to 0.6667) indicate low to moderate heterozygosity. However, utilizing bulked DNA provides a comprehensive representation of the aggregate allelic architecture within each genotype, whereby the observed heterozygosity characterizes the collective genetic profile rather than individual-level variation. Polymorphism Information Content (PIC), ranging from 0.6135 to 0.8579, further classified the primers as highly informative, consistent with PIC being driven by both allele number and allele-frequency distribution; thus, the selected SSRs are suitable for DNA fingerprinting and genetic relationship analyses (Kumar et al. 2024). This is in line with broader evidence that high-PIC markers are preferred for genotype differentiation in applied breeding contexts (Jahnke et al. 2022).

SSR marker analysis revealed the most abundant single band for both JUNRT1 and JUNRT3 genotypes across all tested SSR markers. This monomorphic banding pattern suggests sequence similarity on the target chromosome and its homolog, SSR marker analysis revealed the most abundant single band for both JUNRT1 and JUNRT3 genotypes across all tested SSR markers. This monomorphic banding pattern suggests sequence similarity on the target chromosome and its homolog, indicating genetic homogeneity in the assayed regions. Importantly, these molecular findings provide indirect evidence of genetic distinctness and homozygosity, supporting the superior phenotypic performance observed in quantitative characters for these genotypes, particularly in ear diameter, 100-kernel weight, ear weight, kernel percentage, and potential yield. The consistent presence of a single band in JUNRT 1 and JUNRT3 suggests genetic stability in the tested regions, which indirectly supports their advantageous phenotypic traits and potential DUS compliance. This coherence between molecular marker data and phenotypic evaluation implies that these genotypes possess desirable genetic attributes that could be highly beneficial for breeding programs focused on improving crop yield and quality (Gedil and Menkir 2019). The indirect evidence observed further supports the utility of these identified genotypes for exploitation in maize improvement strategies.

Meanwhile, JUNRT2 also exhibits similar advantageous phenotypic traits; however, it appears to possess more multi-allelic banding patterns in bulked DNA than JUNRT1 and JUNRT3, which necessitates further investigation (Figure 3). The presence of a multi-allelic band at this specific primer suggests a genetic heterogeneity, including the possible presence of heterozygous alleles that could influence the expression of certain traits. Recent studies have highlighted that such genetic variations, particularly in heterozygous states, can significantly impact trait expression and contribute to phenotypic diversity (de Pasqual et al. 2022). This implies that while JUNRT2 displays comparable phenotypic advantages in ear diameter, 100-

kernel weight, ear weight, kernel percentage, and potential yield, its underlying genetic diversity, indicated by heterogeneity, might offer additional breeding opportunities for enhancing these traits. Further studies are essential to fully elucidate the relationship between the observed multi-allelic band and its impact on these quantitative traits. Furthermore, subsequent validation using single-plant SSR or SNP genotyping is recommended to confirm the genetic purity of inbred lines.

UPGMA identified JTKT1 as a genetically distinct genotype (Figure 4); however, its lower phenotypic performance relative to the JUNRT lines and the presence of multi-fragment patterns at several SSR loci (Primer_RF-11, Primer_RF-25, Primer_RF-34, and Primer_RF-38) suggest a lower degree of allelic consistency (homogeneity) within the bulked sample. By contrast, JUNRT1 and JUNRT3 clustered closely and showed strong phenotypic similarity in yield-related traits (e.g., ear diameter, dehusked ear weight, kernel percentage, and potential yield), together with predominantly single-fragment (single-allele) SSR profiles across the assayed loci. This pattern is consistent with high allele fixation typical of advanced inbred lines and is desirable for hybrid breeding because homogenous parental lines support stable hybrid performance and consistent trait expression (Labroo et al. 2021). Accordingly, JUNRT1 and JUNRT3 were prioritized based on three criteria: (i) superior phenotypic performance in yield-related traits, (ii) predominantly single-fragment SSR profiles across tested loci, and (iii) genetic distinctness from other lines.

JUNRT2, although belonging to the same family, formed a nearby but separable sub-branch in the dendrogram, indicating minor genetic differentiation. Its SSR profile showed multi-fragment patterns at four loci, which may reflect within-line heterogeneity, including the possible presence of heterozygous alleles. Heterogeneity within lines is undesirable when assembling hybrid parents because residual within-line segregation and/or genetic impurity can increase off-types and reduce the uniformity and agronomic performance of the resulting F_1 seed lots (Kovinčić et al. 2023). Accordingly, parental inbred lines used for hybrid breeding are commonly expected to be highly homozygous, and lines showing elevated residual heterozygosity are typically recommended for additional purification (Josia et al. 2021). In the context of bulked SSR profiling, multi-allelic band patterns should therefore be interpreted as preliminary, line-level signals that warrant confirmatory single-plant genotyping before making strong inferences about segregation risk in hybrid seed production.

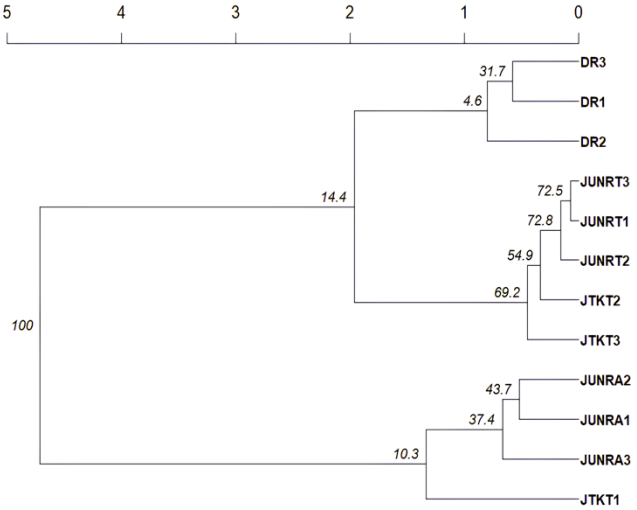

Figure 4. Unweighted Pair Group Method with Arithmetic mean (UPGMA) tree between observed maize inbred lines

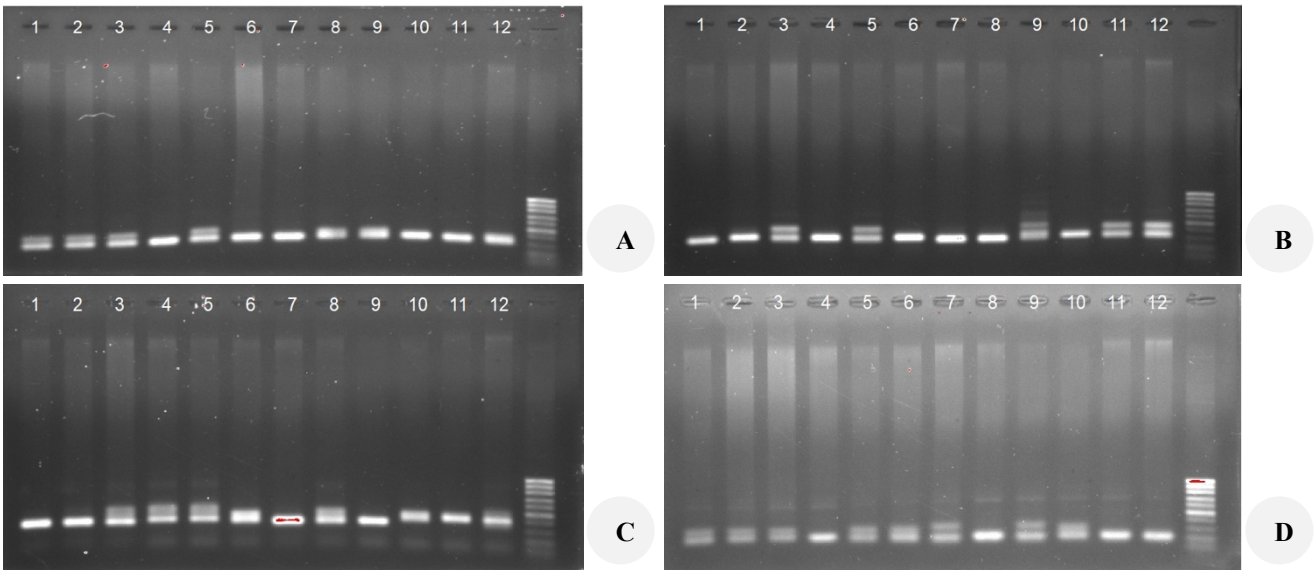

Figure 3. Heterogeneity in JUNRT2 lines was observed using four primers, with their amplified fragments resolved via 1.5% agarose gel electrophoresis. A. Primer_RF-11, B. Primer_RF-16, C. Primer_RF-21, D. Primer_RF-34. 1: JTKT1, 2: JTKT2, 3: JTKT3, 4: JUNRT1, 5: JUNRT2, 6: JUNRT3, 7: DR1, 8: DR2, 9: DR3, 10: JUNRA1, 11: JUNRA2, 12: JUNRA3, 13: DNA-Ledder. The inherent resolution of gel electrophoresis may limit the visual discernment of very subtle allelic size differences

Identification of elite inbred lines with superior performance

The identification of elite inbred lines with superior performance is a crucial outcome for advancing maize breeding programs. While Hierarchical Cluster Analysis (HCA) provided initial insights into phenotypic groupings and the Genotype × Trait (GT) biplot visually represented complex trait-genotype relationships, these methods primarily serve descriptive purposes, offering a foundational understanding of the maize inbred lines. To move beyond descriptive analyses and enable more robust, objective, and efficient selection, Factor Analysis (FA) and the Multi-Trait Genotype-Ideotype Distance Index (MGIDI) become essential. FA reduces trait dimensionality into biologically meaningful mega-traits, simplifying complex genetic architecture without losing critical information. Subsequently, MGIDI integrates these refined trait structures to objectively rank genotypes against a predefined ideotype, enabling precise and robust selection. This multi-layered approach ensures comprehensive and precise evaluation for elite maize inbred line selection.

The Factor Analysis was validated by a mean communality of 0.91, confirming that the extracted mega-traits provide a robust and highly representative model for the nine quantitative traits (Table 5). Factor 1 primarily reflects ear structure and kernel efficiency, showing strong positive associations with kernel percentage and 100-kernel weight, while being inversely related to ear length, ear diameter, and number of kernel rows, capturing the efficiency of converting ear structure into harvestable kernel mass. Factor 2 clearly represents overall yield potential, strongly characterized by dehusked ear weight and potential yield, aggregating the direct components of harvestable biomass. Factor 3 unequivocally represents plant maturity and developmental timing, with equally high positive loadings for 50% anthesis and 50% silking, isolating the phenological aspects critical for adaptation. These three biologically anchored factors were subsequently used as the condensed trait structure feeding into the MGIDI framework, allowing simultaneous ranking of genotypes toward an ideotype combining favorable ear structure, high yield capacity, and desirable flowering time. This dimensional reduction not only simplifies genotype evaluation but also enhances the precision of subsequent multivariate selection indices, such as MGIDI, by focusing on the most relevant and interpretable axes of variation (Olivoto et al. 2022).

The selection process, guided by the MGIDI, successfully achieved selection gain for the traits comprising Factor Analysis, with potential yield and dehusked ear weight exhibiting the highest gains (Table 6). Concurrently, the index effectively shifted flowering phenology towards earlier maturity, demonstrating negative selection gain percentages for 50% silking and 50% anthesis, which aligns with the desired selection direction. The simultaneous achievement of gain in FA2 and the desired reduction in maturity time traits of FA3 confirms the index's efficacy in managing the complexities of multivariate selection. However, within FA1, a negative selection gain was observed for ear length, which counteracted the intended direction of increase. Conversely, ear diameter and the number of kernel rows within the same factor showed a positive gain. Positive genetic gain signifies that a breeding program has successfully altered the genetic makeup of a population in a desired direction for a particular trait or set of traits (Seck et al. 2023).

The negative selection gain for ear length highlights the trade-offs that certain traits must be sacrificed to achieve greater trait improvement. Such trade-offs are a common phenomenon in quantitative genetics, where intensive selection for kernel efficiency or total mass may inadvertently compromise the size of supporting structures (Dwivedi et al. 2021). This observed trade-off for ear length can be physiologically attributed to resource allocation priorities within the plant. Maize plants possess a finite pool of assimilates (Borrás and Vitantonio-Mazzini 2017). Intensive selection pressure for high-order yield components, such as kernel efficiency and the number of kernel rows, often prioritizes the flow of these assimilates towards kernel development and filling, thereby potentially reducing the resources available for ear structural elongation.

To mitigate this trade-off, breeders can consider several strategies. One approach involves actively crossing selected lines with germplasm known to possess superior ear length. This approach aims to introduce favorable alleles for this trait into the breeding population, as identifying superior inbred lines through crossing and combining ability analysis is a fundamental method for maize genetic improvement (Gamea et al. 2018). Subsequent selection would then focus on identifying recombinant genotypes that combine improved ear length with desired gains in other yield components, effectively breaking the negative genetic link between these traits. The selected inbreds can be utilized as testers through a selection program focused on specific combining ability to enhance the ear length character in the final hybrid crosses. Furthermore, advanced genomic approaches could be employed to identify specific genetic loci controlling both ear length and yield components within this panel, potentially facilitating the precise manipulation of resource partitioning for simultaneous trait improvement.

Table 5. Explained factorial loadings and communalities estimated in the factor analysis

Characters	FA1	FA2	FA3	Communality	Uniquenesses
50% anthesis	-0.25	-0.05	0.96	0.98	0.02
50% silking	-0.20	-0.17	0.96	0.99	0.01
Number of kernel rows	-0.79	0.20	0.25	0.73	0.27
Ear length	-0.89	-0.17	0.18	0.86	0.14
Ear diameter	-0.74	0.56	0.26	0.92	0.08
100-kernel weight	0.60	0.32	-0.61	0.82	0.18
Dehusked ear weight	0.08	-0.97	0.00	0.94	0.06
Kernel percentage	0.95	-0.19	-0.18	0.97	0.03
Potential yield	-0.23	-0.95	0.09	0.96	0.04
Communality mean				0.91	

Note: FA1: Factor 1; FA2: Factor 2; FA3: Factor 3

Table 6. Predicted selection differentials, selection gains, and goals for traits in the MGIDI analysis

Characters	Factor	Xo	Xs	SD	SDperc	H	SG	SGperc	Sense	Goal
50% anthesis	FA3	57.56	57.39	-0.17	-0.30	0.99	-0.17	-0.30	Decrease	100
50% silking	FA3	59.52	57.94	-1.58	-2.66	0.99	-1.57	-2.65	Decrease	100
Number of kernel rows	FA1	13.38	13.40	0.02	0.14	0.81	0.02	0.11	Increase	100
Ear length	FA1	14.60	13.94	-0.66	-4.54	0.89	-0.59	-4.03	Increase	0
Ear diameter	FA1	3.84	4.06	0.22	5.78	0.86	0.19	4.97	Increase	100
100-kernel weight	FA3	25.29	26.32	1.03	4.07	0.78	0.80	3.16	Increase	100
Dehusked ear weight	FA2	148.22	168.12	19.90	13.40	0.74	14.80	9.98	Increase	100
Kernel percentage	FA1	86.45	87.76	1.31	1.52	0.72	0.94	1.09	Increase	100
Potential yield	FA2	5.84	6.99	1.15	19.80	0.87	1.01	17.2	Increase	100

Note: Xo: Original population mean, Xs: Mean of selected lines, SD: Selection Differential, SDperc: Selection Differential in percentage, H: Broad-sense heritability, SG: Selection Gains, SGperc: Selection Gains in percentage. Goals represent trait improvement success, (100) target fully achieved, and (0) no progress toward the ideotype

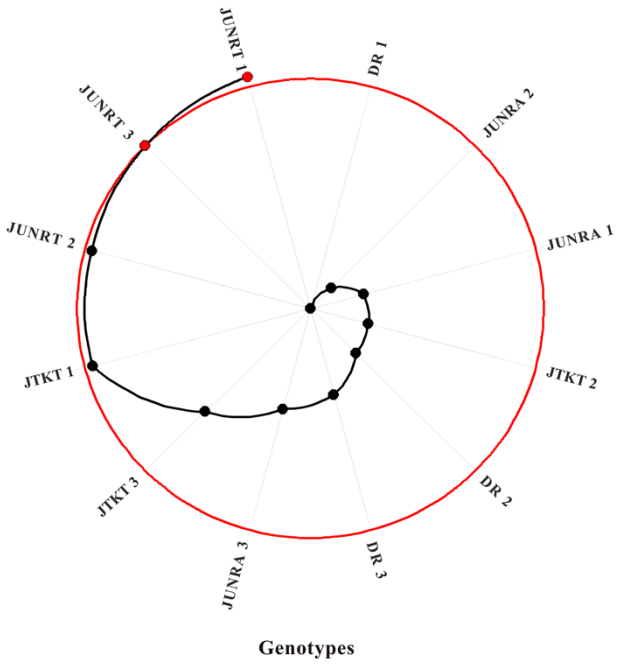

Figure 5. Maize inbred lines ranking in ascending order for the MGIDI. The selected lines are shown as red dots (dots that are on the circle line and outside the circle line), while the unselected lines are in black dots

The MGIDI analysis integrated multiple morphological traits into a single selection criterion, enabling multi-trait ranking and identification of lines with balanced performance (Figure 5). JUNRT1 and JUNRT3 ranked highest, combining earlier flowering with superior potential yield, and are therefore identified as strong candidates for pre-DUS prioritization in hybrid-parent evaluation. This combination is particularly relevant for tropical maize systems, where earlier flowering can reduce exposure to terminal drought and heat stress and thereby improve yield stability under variable conditions (Ehemba et al. 2019). Nevertheless, these rankings were derived from a small panel (12 lines) evaluated at a single location; their broader relevance and potential genotype × environment responses cannot be inferred reliably. Accordingly, future work should expand the germplasm set and implement multi-location trials to characterize G × E effects. The action can validate stability and adaptability prior to wider deployment (Nagesh et al. 2025). For true DUS stability, this validation should be conducted across multiple locations. In subsequent work, a full DUS descriptor set, including qualitative traits, should also be scored. A logical next experiment is a multisite trial of selected genotypes versus comparator varieties, accompanied by full DUS descriptor scoring and genotyping using a denser SSR/SNP panel.

In conclusion, this study successfully integrated morphological and molecular data to characterize twelve elite maize inbred lines and support a pre-DUS screening. This integrated approach strengthened characterization by complementing morphology with SSR fingerprints, but it does not remove environmental influences on phenotypic expression. JUNRT1 and JUNRT3 were identified as top-ranked lines, warranting their prioritization for pre-DUS screening as candidate parents. Their ranking was primarily associated with higher potential yield together with relatively earlier flowering, and their SSR profiles showed predominantly single-allele patterns across the assayed loci, providing molecular evidence consistent with high homozygosity. However, this study's primary limitation stems from its single-location and single-season evaluation. Therefore, multi-location and multi-season validation is mandatory for these lines to ensure their uniformity and stability. This essential next step must include applying a complete DUS descriptor set and strengthening molecular resolution with a denser SSR/SNP panel.

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