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Classification of new germplasm into existing heterotic groups of pearl millet [*Pennisetum glaucum* (L.) R. Br.]

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Abstract

The study assigned new germplasm, which includes populations and inbreds, to established heterotic groups using various approaches to broaden the existing genetic base while maintaining the heterotic pattern in pearl millet [*Pennisetum glaucum* (L.) R. Br.]. It utilized 13 pearl millet populations of African and Asian origins and 24 new inbred parents from ICRISAT's breeding program. Testers, both inbred and composite, were employed to categorize these materials into heterotic groups. Different sets of line × tester crosses were generated and evaluated during the rainy season at multiple locations in India. The pearl millet populations were assigned to heterotic group B (seed parental groups) (HGB1 and HGB2) and heterotic group R (pollinator parental groups) (HGR1 and HGR2) based on general combining ability (GCA) and specific combining ability effects. Composite testers were found to be more effective for the heterotic grouping of pearl millet populations. New inbred lines were classified into HGB and HGR based on GCA and hybrid performance using opposite heterotic group testers and also using genetic similarity obtained from genotype-by-sequencing data.The new germplasm classified into heterotic groups will help enhance the genetic gain for the long-term success of pearl millet hybrid breeding programs.

1 | INTRODUCTION

Pearl millet is the sixth most cultivated cereal, with a global area of 34 million ha and a production of 31 million metric tons (FAO, 2020). Africa and Asia produce the majority of the world's pearl millet; however, it has gradually spread to other nontraditional regions for feed and forage, such as the United States, Canada, Mexico, Brazil, West Asia, North Africa, and Central Asia (de Assis et al., 2018; Patil et al., 2020; Yadav & Rai, 2013). Pearl millet is valued for its nutrient-rich grain for human consumption as well as its green fodder and dry stover for livestock (Andrews & Kumar, 1992), and it provides a source of income and nutritional security for more than 90 million people in South Asia and sub-Saharan Africa (Serba et al.., 2020).

Indian pearl millet breeding businesses have capitalized on heterosis by developing hybrid cultivars. The commercial viability of hybrid pearl millet has been made possible due to the cross-pollination breeding system, increased heterosis, and the presence of stable cytoplasmic-nuclear male sterility (CMS) along with its fertility restorers. The

Abbreviations: CMS, cytoplasmic-nuclear male sterility; CT, composite tester; GBS, genotyping-by-sequencing; GCA, general combining ability; HGB, heterotic group B (seed parental groups); HGR, heterotic group R (pollinator parental groups); IT, inbred tester; OPV, open pollinated variety; SCA, specific combining ability; SNP, single nucleotide polymorphism.

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large-scale use of single A1 CMS sources (Burton, 1965) during the 1960s raised concerns about susceptibility to diseases and pests, leading to the identification of additional CMS sources, including A2, A3 (Athwal, 1961, 1966), A4 (Hanna, 1989), and A5 sources (Rai, 1995). In the years between 1984 and 2000, a considerable number of genetically diverse CMS lines were produced and utilized in hybrid breeding; subsequently, genetic improvement prioritized hybrid genetic diversification and adaptability to niche cultivation areas (2001–2018). Single-cross hybrids are widely grown in more than 70% of the total pearl millet production area (Yadav & Rai, 2013), which has increased annual productivity from 305 kg ha^{-1} in the 1950s to 1391 kg ha^{-1} in the present (ICAR–All India Coordinated Research Project on Pearl Millet, 2022).

Breeding programs in India's public and private sectors have collaborated closely with the ICRISAT-Asia pearl millet breeding program to enhance the genetic diversity of hybrid parents by utilizing significant breeding material of African and Asian origin (Gupta et al., 2020). A trait and adaptation-based breeding approach is being followed to develop a phenotypically diverse range of hybrid parental lines to meet the needs of various agroecologies. However, mere trait and adaptation-based breeding does not guarantee the predictive ability of hybrid parental lines, that is, the proportion of newly developed hybrid parental lines that are heterotic in terms of commercial significance. To continuously improve the genetic gain achieved by pearl millet hybrids, new strategies must be developed for increasing the higher magnitude of heterosis and ensuring the gain in the hybrid breeding program.

Genetic diversity and information on heterotic groups are valuable in the development of inbred lines. They assist breeders in using their germplasm more efficiently and consistently by selecting complementary lines to maximize the outcomes of a hybrid breeding program. Heterotic groups within a crop can be established based on pedigree information, morphological distinctions, germplasm origins, and combining abilities. Broad-based heterotic groups have been identified in various other crops. For example, maize (*Zea mays* L.) has groups like Reid Yellow Dent and Lancaster Sure Crop (Dudley et al., 1991), as well as Flint and Dent groups (Dhillon et al., 1993). Rice (*Oryza sativa* L.) has maintainer (B) and restorer (R) line-based heterotic groups (Wang et al., 2015; Xie et al., 2014), and in rye (Secale cereale L.), there are Petkus and Carsten groups (Hepting, 1978), among others. The presence of heterotic groups suggests that populations from different backgrounds may possess distinct allelic diversity, which could result from founder effects, genetic drift, or the accumulation of unique varieties through mutations or selection.

Studies on molecular diversity in pearl millet have categorized such lines into genetically distinct groups, confirming the existence of two broad groups in hybrid parents: one for seed parents and the other for restorer parents (Gupta et al., 2015, 2020; Nepolean et al., 2012; Ramya et al., 2018; S. Singh et al., 2018). These groups function as two broad-based heterotic groups, with heterosis between B × R found to be more significant than that between B × B or R × R heterosis (Singh & Gupta, 2019). The historical approach for breeding hybrid parents in pearl millet breeding programs may have contributed to the formation of these two broad heterotic pools. The majority of the seed parental lines (B-lines) have been developed at ICRISAT (International Crops Research Institute for the Semi-Arid Tropics) using germplasm from the "Togo origin," a region in western Africa comprising Burkina Faso, Togo, Ghana, and Benin. These lines exhibit early flowering and relatively lower photoperiod sensitivity. On the other hand, R-lines were developed by combining germplasm known for local adaptability, high productivity, high tillering, taller height, and smaller seed sizes (Rai et al., 2006; Singh & Gupta, 2019).

The development of hybrid-oriented heterotic populations and the application of schemes to improve the combining ability of parental lines should be an integral part of the hybrid breeding program in pearl millet. In a recent study involving 320 R-lines and 260 B-lines derived from six main pearl millet breeding programs in India including that of ICRISAT and public and private sector, two B-line heterotic groups/clusters (HGB-1 and HGB-2, where HGB is heterotic group B [seed parental groups]) and two R-line heterotic groups (HGR-1 and HGR-2, where HGR is heterotic group R [pollinator parental groups]) were identified based on heterotic performance and combining ability (Figure 1; Gupta et al., 2020). Grain yield heterosis of more than 10% was observed in hybrids from

Core Ideas

- There is significant variation observed in pearl millet germplasm owing to its combining ability patterns.
- The specific combining ability effect had shown to be useful in determining the heterotic grouping of the pearl millet populations.
- Heterotic group composite testers exhibited higher breeding efficiency in classifying new populations into heterotic groups.
- Testcross performance, general combining ability effect, and single nucleotide polymorphism based genetic similarity were helpful in assigning inbred lines to heterotic group.
- The introduction of new germplasm into heterotic group will broaden the genetic base and enhance genetic gain in hybrid breeding.

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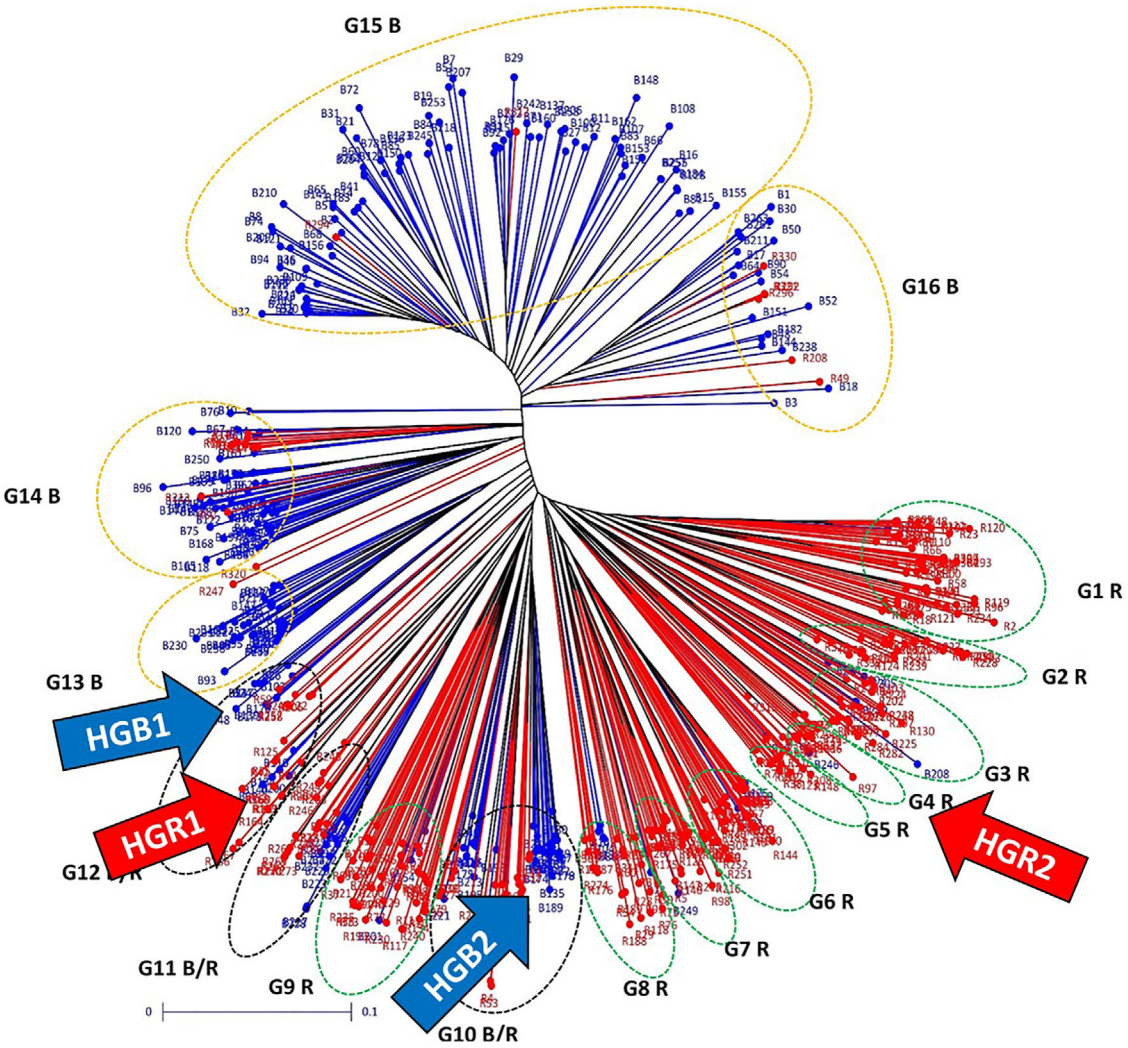

FIGURE 1 Marker-based groups in the clustering pattern of pearl millet hybrid parental lines (existing heterotic groups; figure adapted from Gupta et al. [2020]). Red and blue colors indicate restorer and seed parents, respectively. HGB, heterotic Group B (seed parental groups); HGR, heterotic Group R (pollinator parental groups).

these identified B × R heterotic groups compared to the best prevailing commercial hybrid checks. These pearl millet heterotic groups/clusters of hybrid parental lines were considered as the base material to initiate hybrid breeding in pearl millet via a heterotic grouping strategy. Since these founder pools are cluster based, they represent a set of closely related lines with limited diversity. To maintain the heterotic group further for an optimum and long-term sustainable hybrid breeding program, it is necessary to broaden the genetic base of founder heterotic pools.

Assigning a new inbred line into a heterotic group is a crucial step in hybrid crop breeding, and several approaches can be followed to introduce new germplasm into existing heterotic groups, either individually or in combinations. When the established heterotic patterns are available, selected elite genotypes from them can be used as testers for the classification of new germplasm into heterotic groups (Melchinger & Gumber, 1998). The "line × tester" analysis can be a valuable approach in this context of pearl millet heterotic grouping, just as it is in other crop species. Based on the testcross performance and combining ability analysis, populations or lines could be assigned to heterotic groups to enlarge their genetic base. The combining ability approach has been shown to be useful in assigning the population and inbred lines to a heterotic group to improve the efficiency of hybrid breeding in maize (Abera et al., 2018; Akinwale et al., 2014; Fan et al., 2009, 2010; Fato et al., 2012; George & Delacruz, 2009; Librando & Magulama, 2008; Melani & Carena, 2005; Menkir et al., 2004; Oyetunde et al., 2020; Singode et al., 2017; Vasal, et al., 1992, 1999). Also, molecular markers, like simple sequence repeats, single nucleotide polymorphisms (SNPs), or DNA sequence data, play a key role in assessing genetic relatedness between new inbred lines and existing ones within specific heterotic groups. They aid in

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quantifying genetic similarity through genetic distance calculations. If the markers of the new inbred line demonstrate higher genetic similarity to those of known parents within a specific heterotic group, it suggests compatibility due to shared genetic backgrounds. However, research in this area of classification of new germplasm into heterotic groups in pearl millet is lacking. Efforts should be made to identify new gene pools/populations, which are unexploited genetic diversity for classifying them into existing heterotic pools to enhance the frequency of heterotic loci in hybrid breeding programs. Hence, the current study investigates/explores the possibility of assigning different kinds of new germplasm (open pollinated varieties [OPVs] and inbreds) to established heterotic groups using various approaches to broaden the genetic base while maintaining the established heterotic pattern in pearl millet.

2 | MATERIALS AND METHODS

2.1 | Genetic material

The study employed two types of new breeding materials, as outlined in Table S1, which includes pedigree and other relevant information. These materials comprised 13 improved pearl millet populations, denoted as P-1 (AIMP 92901), P-2 (EC C6), P-3 (Sudan II), P-4 (SOSAT C88), P-5 (GB 8735), P-6 (Raj 171), P-7 (ICMV 221), P-8 (ICMS 7704), P-9 (ICMP 87307), P-10 (CZIC 618), P-11 (ICMP 87237), P-12 (ICTP 8203), and P-13 (GICKV 98771). These populations encompass genetic resources originating from both African and Asian sources, developed either at ICRISAT or through collaborative efforts with National Agricultural Research System partners.

Additionally, a set of 24 new hybrid parental lines with diverse pedigrees were selected from the pool of newly developed inbred lines from the pearl millet breeding program at ICRISAT, Patancheru. These new inbred germplasm, being assigned to heterotic groups, displayed unique diversity, as depicted in Figure S1, indicating the potential to enrich the genetic base and overall diversity within the existing heterotic groups. The selected lines comprises 12 seed parents identified as B-L1 (ICMB 100693), B-L2 (ICMB 100694), B-L3 (ICMB 101925), B-L4 (ICMB 100128), B-L5 (ICMB 100713), B-L6 (ICMB 101926), B-L7 (ICMB 100551), B-L8 (ICMB 100524), B-L9 (ICMB 100741), B-L10 (ICMB 100743), B-L11 (ICMB 12444), B-L12 (ICMB 14111), and 12 restorer parents: R-L1 (ICMR 14222), R-L2 (ICMR 15999), R-L3 (ICMR 101096), R-L4 (ICMR 101083), R-L5 (ICMR 100294), R-L6 (ICMR 101087), R-L7 (ICMR 101089), R-L8 (ICMR 101093), R-L9 (ICMR 13777), R-L10 (ICMR 100390), R-L11 (ICMR 101094), and R-L12 (ICMR 101129).

2.2 | Heterotic group testers

To assign the selected new germplasm to existing heterotic groups through test-cross evaluation, we used two types of testers. First, we utilized inbred testers (ITs), which included three testers from each of the established pearl millet heterotic groups (HGB-1, HGB-2, HGR-1, and HGR-2). These ITs were selected based on their combining ability, diverse pedigrees, and representation of existing heterotic group clusters. In addition to ITs, we employed composite testers (CTs) from each of the four established heterotic groups of pearl millet hybrid parents to classify populations. These CTs were developed by random mating inbred lines within each heterotic group cluster. For example, the heterotic group restorer-1 composite tester (HGR1-CT) was created at ICRISAT, Patancheru through three rounds of random mating involving 10 male parental lines (R-lines) from cluster G12R. (Figure 1; Gupta et al., 2020; Table S1). The schematic overview of genetic materials and crosses used in the classification of new germplasm into established heterotic groups in pearl millet are presented in the Figure 2a,b.

2.3 | Development of different sets of test crosses and their evaluation

2.3.1 | Pearl millet populations

Following a line × tester mating design, we created a total of 52 population hybrids. This was achieved by pollinating at least 15–20 panicles of a composite population tester with bulk pollen collected from 20 to 25 plants from each of the 13 populations. This approach was adopted to avoid the genotypic sampling effects within the parental populations and to ensure the production of sufficient seed for each population hybrid. These population hybrids make up the experimental materials for set 1, which involves four heterotic group-composite testers (HGB1-CT, HGB2-CT, HGR1-CT, and HGR2-CT) and 13 populations (P-1 to P-13). Similarly, a set of 52 top crosses were developed by crossing the four heterotic group ITs (HGB1-IT, HGB2-IT, HGR1-IT, and HGR2-IT) with bulked pollen from 20 to 25 random plants of each pearl millet population (P-1 to P-13) at ICRISAT, Patancheru, during summer (March–June) season of 2020, which forms the experimental material set 2. The two sets of population hybrids (set 1 and set 2), their parental populations, and checks were evaluated in the rainy season of 2020 at four different locations in India, namely Jamnagar (ENV-1, where ENV is environment), Dehgam (ENV-2), Gurugram (ENV-3), and Palem (ENV-4). The details of the experimental layout of hybrids (52), their parents (21), and check entries of set 1 and of set 2 evaluated in the trials are provided in Table 1.

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TABLE 1 Evaluation of different sets of pearl millet test crosses and their parents at different locations in India.

	Experimental material	Experimental design
1. Pearl millet populations as new germplasm		
Population parental trial	13 Populations (P-1 to P-13) and 4 HG composite testers (HGB1-CT, HGB2-CT, HGR1-CT & HGR2-CT) and checks (Dhanashakti, ICMB 04999, ICMR 14888) 4 HG inbred testers (HGB1-IT, HGB2-IT, HGR1-IT, and HGR2-IT); 4 HG composite testers (HGB1-CT, HGB2-CT, HGR1-CT, and HGR2-CT) Checks (Dhanashakti, ICMB 04999, ICMR 14888)	Randomized block design: It includes 24 entries in two replications (13 OPVs + three checks + four HG composite tester + four HG inbred tester)
Population hybrid trial (set 1)	13 Populations (P-1 to P-13) × 4 HG composite testers (HGB1-CT, HGB2-CT, HGR1-CT, and HGR2-CT)	Set 1: Alpha lattice: It consists of 54 entries (52 population hybrids + two checks) in two replications with each replication having nine blocks and six entries in each block.
	Checks (ProAgro 9444, Dhanashakti)	
Top cross hybrid (TCH) trial (set 2)	13 Populations (P-1 to P-13) × 4 HG inbred testers (HGB1-IT, HGB2-IT, HGR1-IT, and HGR2-IT)	Set 2: Alpha lattice: It consists of 54 entries (52 Top cross hybrids + two checks) in two replications with each replication having nine blocks and six entries in each block.
	Checks (ProAgro 9444, Dhanashakti)	
2. Inbred as new germplasm		
R-line parental trial	12 R-lines (R-L1 to R-L12) and 6 HGB inbred testers (HGB1-T1 to HGB2-T3)	Randomized block design: It includes 18 entries (12 R lines + six HGB testers) in two replications
Single cross hybrid (SCH) trial (set 3)	6 HGB testers (HGB1-T1 to HGB2-T3) × 12 R-lines (R-L1 to R-L12) Checks (ProAgro 9444, Kaveri Super Boss, 86M84)	Set 3: Alpha lattice: It consists of 75 entries (72 SCH + three checks) in two replications, each replication having 15 blocks and five entries in each block
B-line parental trial	12 B-lines (B L1 to B L12) and 6 HGR testers (HGR1 T1 to HGR2 T3)	Randomized block design: It includes 18 entries (12 B lines + six HGR testers) in two replications
Single cross hybrid (SCH) trial (set 4)	12 B-lines (B L1 to B L12) × 6 HGR-line testers (HGR1-T1 to HGR2-T3) Checks (ProAgro 9444, Kaveri Super Boss, 86M84)	Set 4: Alpha lattice: It consists of 75 entries (72 SCH + three checks) in two replications, each replication having 15 blocks and five entries in each block

Abbreviations: HG, heterotic group; HGB, heterotic Group B (seed parental groups); HGR, heterotic Group R (pollinator parental groups); OPVs, open pollinated varieties.

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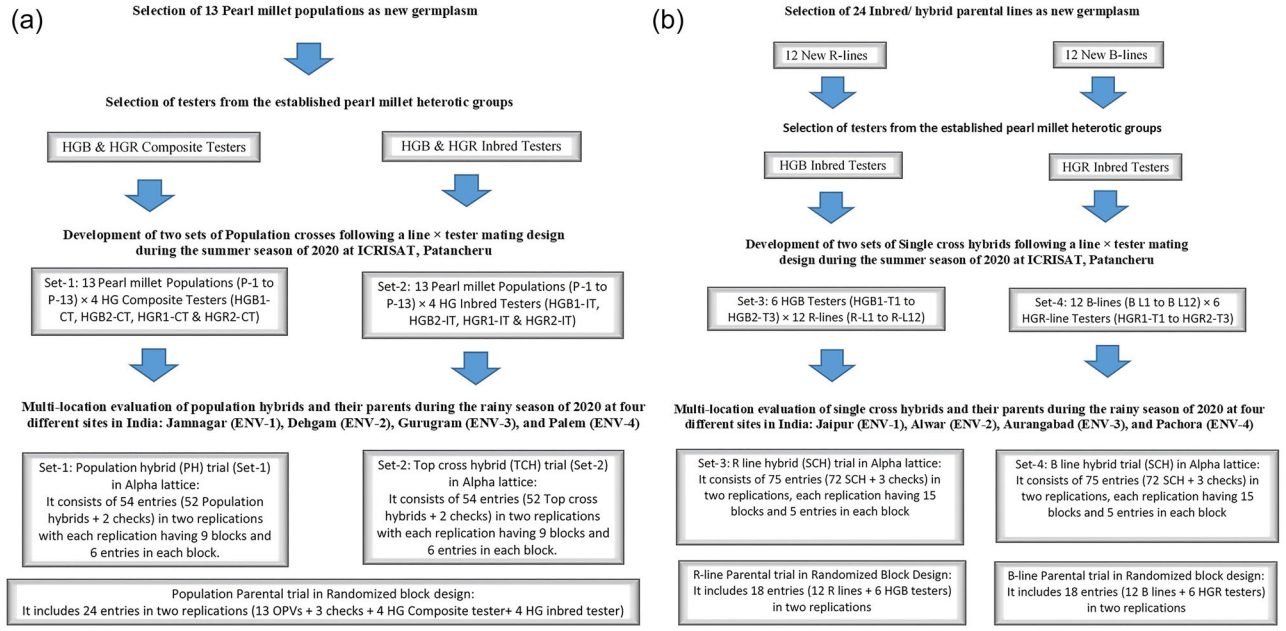

FIGURE 2 (a) Schematic overview of genetic materials and crosses used in the classification of pearl millet populations into established heterotic groups in pearl millet. (b) Schematic overview of genetic materials and crosses used in the classification of inbred/hybrid parental lines into established heterotic groups in pearl millet. CT, composite tester; HG, heterotic group; HGB, heterotic Group B (seed parental groups); HGR, heterotic Group R (pollinator parental groups); OPVs, open pollinated varieties.

2.3.2 | Inbred lines

The present study investigated B × R hybrids, although A × R hybrids must be developed for cultivation. Since A-lines of the CMS system are sterile but contain the exact same nuclear genotype as that of B-lines, we measured traits of B-line parents and genotyped them in place of A-lines to obtain consistent parent phenotypic values. The phenotype derived from hybrids of B-lines and R-lines can be used to represent the crosses between A- and R-lines. In pearl millet florets (during flowering), stigmas emerge first, and anthers appear 3–4 days later. This flowering behavior, known as protogyny, characterizes pearl millet as a highly cross-pollinated crop. The crop's protogynous nature facilitates both selfing and crossing; selfing is achieved by covering the spike with a paper bag as it emerges from the boot, while crossing does not require emasculation. Instead, it involves collecting pollen from the bagged panicles of one line and dusting it onto the panicle of the other line when stigmas have fully emerged. Hence, B × R hybrids were developed in this pearl millet study through crossing procedures.

During the rainy (June–September) season of 2019, a set of 72 single crosses was produced at ICRISAT, Patancheru, by crossing six testers from heterotic group B (three each from HGB-1 [HGB1-T1, T2, and T3] and HGB-2 [HGB2-T1, T2, and T3 as female parent]) with 12 R-lines (R L1–R L12 as male parent) in a line × tester fashion, forming the experimental material set 3. Similarly, the experimental material set 4 was created by crossing six testers from heterotic group R (three each from HGR-1 [HGR1-T1, T2, and T3] and HGR-2 [HGR2-T1, T2, and T3] as male parent) with 12 new B-lines (B L1–B L12 as female parent). These two sets of line × tester cross (set 3 and set 4), their parents, and commercial checks were evaluated in four different locations during the rainy season of 2020 in India, namely Jaipur (ENV-1), Alwar (ENV-2), Aurangabad (ENV-3), and Pachora (ENV-4). Table 1 shows the experimental layout of hybrids (72), their parents (18), and check entries (3) from set 3 and set 4 that were evaluated in the trials. In general, hybrids and parental genotypes were randomized separately and evaluated in adjacent blocks to avoid the suppressive effect of hybrids over parents. Hybrid entries were evaluated in Alpha lattice design with two replications, whereas parental genotypes were evaluated in randomized completed block design with two replications.

2.4 | Agronomic management practices

Standard agronomic management practices were followed at all the locations for good crop growth. A basal dose of 100 kg of DAP (Diammonium phosphate, containing 18% N and 46% P) was applied at the time of field preparation, and 100 kg of urea (46% N) was applied as a top dressing to meet the recommended dose of 64 kg of N ha^{-1} and 46 kg of P ha^{-1}; irrigations were given soon after sowing and then subsequently during the season as and when required. Seedlings

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were thinned 15 days after sowing to maintain seedlings at a uniform spacing of 15 cm. The other cultural practices like weeding, and protection against insects, pests, diseases, and birds were done throughout the growing period as and when required. All the panicles in a plot were harvested for each entry. The harvested material was sun-dried for 10–15 days, threshed and recorded for grain yield in kilogram, and converted to grain yield per hectare (kg ha^{-1}).

2.5 | Statistical analyses

The combined analysis of variance was carried out using PROC MIXED (SAS v9.4, SAS Institute Inc., 2018), considering location, genotypes, and replication as fixed and block as random. To pool the data across locations and to make the error variance homogeneous, individual location variances were estimated and modeled to error distribution using the residual maximum likelihood (REML) procedure. Testing of the homogeneity variances of environments has been done using advance mixed models of SAS PROC Mixed procedure with REML method and REPEATED statement. The REPEATED statement controls the covariance structure imposed upon the residuals or errors. PROC MIXED has a rich variety of structures to specify relationships among the errors. The GROUP = optional statement parameter permits different levels of the GROUP effect to have different structure parameters and defines an effect that specifies heterogeneity in the covariance structure of the "R" matrix. The main interaction effects of location and genotypes are fixed, and the block effect is random. *F*-statistic was estimated for the fixed effects (type-III) and variance components for the random effect (χ^2 statistic). The variance component for block effect (random) was tested using χ^2 statistic at 1 *df*.

The procedure of the line × tester analysis according to Kempthorne (1957) was used for estimating general and specific combining ability (SCA) effects. The variance due to general combining ability (σ^2GCA) and variance due to SCA (σ^2SCA) were estimated as described by R. K. Singh and Chaudhary (1977), and then the predictability ratio was computed following Baker (1978) to estimate the relative importance of GCA in explaining hybrid performance. Standard heterosis for grain yield was estimated as a hybrid yield advantage over the popular commercial check (ProAgro 9444) following Hallauer and Miranda (1988). Also, the correlation between combining ability effects and per se performance for different sets of testcross and its parents for grain yield (kg ha^{-1}) was conducted. This analysis was conducted to evaluate the predictive capacity of these parameters and to gain insights into selection strategies. We employed Pearson correlation coefficients to measure the strength and direction of the linear relationship between combining ability effects and per se performance in relation to grain yield.

2.6 | The methodology followed for assigning pearl millet germplasm into heterotic groups

2.6.1 | Pearl millet populations

We followed Fan et al. (2009) and Oyetunde et al. (2020) criteria to assign populations (OPVs) into heterotic groups, with some modifications. The combining ability effects of the populations, when crossed to HGB and HGR testers, formed the basis for classifying them into heterotic groups. Populations with positive GCA effect and a high test cross mean were preferred, while those with a significant negative GCA were discarded. All populations that produced hybrids with negative SCA effects in crosses with a tester were allocated to that tester's heterotic group. However, when some populations were found to belong to two or more heterotic groups, the values of the SCA effects with the different testers were considered, and the populations were retained in the group with the lowest SCA effect. Finally, the classification based on the tester set, which exhibits higher breeding efficiency and proper differentiation of populations into a clear-cut heterotic group based on their SCA effect of grain yield, was considered for heterotic group classification. The concept of breeding efficiency (Fan et al., 2009) was applied in evaluating two kinds of tester sets in assigning the pearl millet populations into B and R heterotic pattern.

$$\text{Breeding efficiency } (\%) = \left[\text{number of high yielding intergroup crosses / total number of inter group crosses}\right] \times 100$$

Furthermore, the fertility restoration (%) of populations on three diverse, A1, A4, and A5, cytoplasmic male sterility systems were accessed for their utility in hybrid parental line development and to explore the opportunities for genetic diversification of CMS systems in pearl millet and improving the efficiency in seed parent breeding programs.

2.6.2 | Inbred lines

The inbred lines with significant negative GCA effects were discarded. New B lines showing positive GCA effect with HGR testers and those lines with average test cross mean yields equal to or greater than the mean yield of the check ProAgro 9444 were placed into the heterotic group HGB, the selected B-lines were further assigned to the individual group, that is, HGB-1 and HGB-2 based on genetic similarity (SNP-based) with the corresponding HGB testers. Similarly, new restorer (R) lines displaying a positive GCA effect with the HGB tester (negative nonsignificant to significant positive) were placed into the heterotic group HGR and were further

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assigned to HGR-1 and HGR-2 based on SNP-based genetic similarity with respective HGR testers.

Genotype-by-sequencing (GBS) data from hybrid parental lines were used to estimate their genetic similarity with corresponding heterotic group testers using Rogers's (1972) distance. The GBS dataset generated a total of 8017 markers, distributed evenly across all linkage groups (genomic regions), indicating robust genetic coverage to support the genetic similarity assays. The DNA extraction and genotyping of hybrid parental lines were carried out as described in Liang et al. (2018). A total of 30–35 seeds from each inbred line were sown in a 4-in. pot in a darkroom at ICRISAT Patancheru. The pots were maintained at a temperature between 18°C and 25°C. Etiolated leaf tissues were harvested 8 days after planting. Pooled leaf tissue from 20 to 25 seedlings per line was collected for DNA extraction. DNA was extracted using a modified DNA extraction method described by Mace et al. (2003). The DNA was stained by 5 ng μL^{-1} of ethidium bromide and checked using 0.8% (w/v) agarose gel electrophoresis in tris-acetate-EDTA buffer for 1 h at 90 V with visualization under ultraviolet light. GBS sequencing libraries for B-lines and R-lines were prepared following the protocol outlined in Ott et al. (2017) and GBS libraries were sequenced using an ion proton. Alignment of raw sequence data to the pearl millet reference genome version 1.1 was conducted using GSNAP (Wu & Nacu, 2010). SNP identification with 123SNP (Yu et al., 2012) involved strict criteria: five aligned reads, PHRED (Probability of a Base Call Error Given a Read) quality >20, and excluding the first/last 3 bp. Genotypes were assigned based on allele frequency criteria: homozygous if one allele had >0.9 frequency and heterozygous if five reads with >90% support for both alleles and >0.2 frequency were present; otherwise, data were marked as missing. Quality filters were applied to exclude SNPs with more than two alleles, single genotype calls, >10% heterozygous calls, and where the minor allele was not present in at least five samples or <20% had genotype calls. Beagle (Version: June 16, 2016) was used for SNP imputation to enhance data completeness. Subsequently, only SNPs with a minor allele frequency >0.05 were utilized, ensuring a focus on high-variation genetic markers for robust genetic similarity analysis.

3 | RESULTS AND DISCUSSION

3.1 | Analysis of variance

The combined analysis of the variance (ANOVA) of combining ability for grain yield productivity (kg ha^{-1}) is provided in Table 2 for different sets of line × tester crosses evaluated. ANOVA revealed significant variance ($p < 0.05$) due to environments (locations), indicating that the materials were evaluated under diverse environments. The genotypic variation due to hybrids was highly significant ($p < 0.05$) among all the sets, indicating the existence of significant genetic variation in the parents and thus in hybrids for grain yield productivity (kg ha^{-1}). The analysis reveals varying degrees of heritability across different sets and environments. While set 1 demonstrates consistent genetic influence, sets 2–4 highlight the interplay between genetic and environmental factors in shaping phenotypic variation. The low heritability observed in set 4 under ENV-2 is due to the genetic variation in the treatment being statistically nonsignificant, contributing only 9%, while the environmental variation is considerably high at 89%, as revealed by the mixed model analysis.

3.2 | Assessment of the hybrid breeding potential of African and Asian-origin pearl millet populations in relation to the heterotic pattern in seed (B) and restorer (R) groups of the hybrid parental lines

Based on the performance of their testcross with heterotic group testers, strategic classification of OPVs/populations was carried out to broaden the genetic base of seed (HGB) and restorer (HGR) hybrid parental pools of pearl millet. The SCA effect has been shown to be useful in determining the heterotic grouping of population and inbred lines to improve the efficiency of hybrid breeding in maize (Abera et al., 2018; Akinwale et al., 2014; Fan et al., 2009, 2010; Fato et al., 2012; George and Delacruz, 2009; Librando & Magulama, 2008; Melani & Carena, 2005; Menkir et al., 2004; Singode et al., 2017; Vasal, et al., 1992, 1999). In our present study, some populations belonged to two or more heterotic groups, and a few of the populations studied had shown both positive and negative SCA effects with the testers of the opposite heterotic groups. These deviations are apparent and common in any of the heterotic groups. Hence, for assigning populations into heterotic groups, the values of the SCA effects with the different testers were considered, and the populations with the highest positive SCA with one of the heterotic group testers were assigned to the heterotic group (primarily the opposite group) for which the SCA effect was least (Fan et al., 2009).

3.2.1 | Classification of populations (OPVs) into the heterotic group based on the SCA effect of grain yield with heterotic group B and R composite testers

Upon crossing a population with a tester from one of the heterotic groups, positive SCA effects indicate that the populations are in opposite heterotic groups. Negative SCA effects, on the other hand, indicate that the populations/lines belong to the same heterotic groups as the tester (Vasal et al., 1999). Thirteen populations were crossed with two types of testers from heterotic groups, namely inbred (four) and composite

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TABLE 2 Analysis of variance of combining ability for grain yield (kg ha^{-1}).

Source of variation	*df*	Set 1	Set 2	*df*	Set 3	Set 4
	Pearl millet populations (OPVs)			Inbred as new germplasm		
Type-III fixed effects (*F*-statistic)						
Environment (ENV)	3	1480.69***	1290.12***	3	17.39***	9.29**
Replication (Loc.)	4	5.03**	1.4	4	10.00**	3.51*
Hybrids	51	4.53***	4.72***	71	2.46***	3.07***
Line	12	1.4	2.87**	11	2.45*	4.91***
Tester	3	2.29	1.18	5	2.98*	3.57**
Line × tester	36	4.03***	3.13***	55	1.76**	1.75**
ENV × hybrids	213	1.99***	1.59**	153	2.82**	2.56**
ENV × LINE (GCA)	33	2.22**	1.5	36	1.49	2.88***
ENV × TESTER (GCA)	15	2.87**	2.15**	9	2.29*	1.53
ENV × LINE × TESTER (SCA)	165	1.47*	1.33*	108	2.17**	1.55
Variance components						
Block(Rep × environment)		25,240	41,870		144.35	39,597
Predictability ratio		0.21	0.26		0.47	0.62755
Residuals of environments						
ENV-1		114,000	130,000		584,127	441,563
ENV-2		272,100	544,400		501,419	485,149
ENV-3		284,300	367,300		578,824	574,255
ENV-4		108,770	737,300		959,902	807,989
Broad-sense heritability (H^2)						
ENV-1		0.73	0.73		0.65	0.61
ENV-2		0.60	0.39		0.53	0.16
ENV-3		0.74	0.67		0.45	0.44
ENV-4		0.75	0.74		0.39	0.59

Abbreviation: OPVs, open pollinated varieties.

*, **, and ***denote significance at 0.05, 0.01, and < 0.001 levels of probability, respectively.

(four) testers. Two sets of test crosses, set 1 and set 2, were evaluated for combining ability analysis to estimate GCA and SCA effects for grain yield (kg ha^{-1}), and the results are shown in Tables 3 and 4, respectively. When the populations were assigned to specific heterotic groups (HGB-1, HGB-2, HGR-1, and HGR-2), two populations, P-12 and P-13, had negative significant GCA effects of −6.04** and −5.21**, respectively (Table 3). (* and ** denote significance at the 0.05 and 0.01 levels of probability, respectively.) The breeding value of a parent is directly related to GCA, and the negative significant GCA effect of these populations indicates poor average performance in hybrid combinations. As a result, they were excluded from heterotic pool classification. The population P-2 (6.94**), P-6 (7.47**), P-8 (4.67**), and P-9 (9.16**) had the highest positive SCA effect for grain yield in either one or both of the HGB CTs (Table 3), hence classifying them as opposite heterotic groups (HGR). Among these four populations, populations P-2 (−12.00**), P-6 (−5.57**), and P-8 (−3.22) showed higher negative SCA effects with the HGR-1 CT and were assigned to the heterotic group HGR-1, whereas population P-9 (−7.70**) showed higher negative SCA effects with the HGR2 CT and was assigned to the heterotic group HGR2. Seven populations, P-1 (8.06**), P-3 (5.06**), P-4 (2.49), P-5 (5.12**), P-7 (9.92**), P-10 (5.97**), and P-11 (3.69*), had the highest positive SCA effect for grain yield with the HGR CT (Table 3), indicating that these populations are in opposite heterotic groups (HGB). Among the seven populations, P-1 (−3.44*), P-3 (−3.65*), P-4 (−5.99**), P-5 (−4.86**), P-7 (−6.51**), and P-11 (−2.47) showed high negative SCA effects with the HGB2 CT, whereas P-10 (−3.05) showed high negative SCA effects with HGB1 CT and was classified into the respective heterotic group of the tester.

3.2.2 | Classification of populations (OPVs) into the heterotic group based on the SCA effect of grain yield with heterotic group B and R inbred testers

In the case of IT crosses, four populations with a negative significant GCA effect (Table 4) were excluded from

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TABLE 3 Classification of pearl millet populations into heterotic groups based on the specific combining ability (SCA) effect of grain yield with heterotic Group B (seed parental groups) (HGB) and heterotic Group R (pollinator parental groups) (HGR) composite testers.

Set 1: Population	GCA effect	SCA effect for grain yield (kg ha^{-1}) HGB1-CT	HGB2-CT	HGR1-CT	HGR2-CT	Heterotic pattern (B × R)	Heterotic group
P-1	−0.68	−1.67	−3.44*	8.06**	−2.96	B	HGB-2
P-2	0.59	6.94**	5.03**	−12.00**	0.02	R	HGR-1
P-3	−0.37	−1.99	−3.65*	5.06**	0.58	B	HGB-2
P-4	2.53**	1.19	−5.99**	2.49	2.31	B	HGB-2
P-5	0.06	−1.05	−4.86**	0.79	5.12**	B	HGB-2
P-6	3.85**	−1.45	7.47**	−5.57**	−0.44	R	HGR-1
P-7	−1.54	−1.17	−6.51**	9.92**	−2.23	B	HGB-2
P-8	1.28	1.57	4.67**	−3.22	−3.02	R	HGR-1
P-9	5.64**	5.64**	9.16**	−7.10**	−7.70**	R	HGR-2
P-10	−1.61	−3.05	0.19	5.97**	−3.11	B	HGB-1
P-11	1.5	−1.82	−2.47	0.59	3.69*	B	HGB-2
P-12	−6.04**	−2.26	−0.36	−1.65	4.27*	B	[a]
P-13	−5.21**	−0.88	0.76	−3.34	3.47*	R	[a]

Abbreviation: GCA, general combining ability.
[a]Unassigned to heterotic groups.
* and **denote significance at 0.05 and 0.01 levels of probability, respectively.

TABLE 4 Classification of populations into heterotic groups based on the specific combining ability (SCA) effect of grain yield with heterotic group B and R inbred testers.

Set 2: Population	GCA effect	SCA effect of grain yield (kg ha^{-1}) HGB1-IT	HGB2-IT	HGR1-IT	HGR2-IT	Heterotic pattern (B × R)	Heterotic group
P-1	−1.18	2.66	−0.76	−3.79	1.89	R	HGR-1
P-2	−4.86**	−0.52	2.5	−2.11	0.12	R	††
P-3	1.93	−2.55	−4.82*	−0.81	8.18**	B	HGB-2
P-4	1.42	−6.65**	−1.71	4.37*	3.98*	B	HGB-1
P-5	−2.08*	−3.6	4.67*	−2.17	1.1	B	[a]
P-6	6.07**	−5.17*	4.18*	1.85	−0.86	B	[a]
P-7	−0.36	3.25	−4.63*	−0.01	1.4	B	[a]
P-8	0.77	3.68	5.17*	0.82	−9.67**	R	HGR-2
P-9	3.21**	−1.28	−5.19*	2.81	3.66	B	HGB-2
P-10	5.75**	10.19**	−1.13	−3.41	−5.65**	R	HGR-2
P-11	−4.57**	1.7	3.62	0.97	−6.28**	R	[a]
P-12	−6.92**	−1.34	−3.31	6.38**	−1.72	B	[a]
P-13	0.83	−0.37	1.4	−4.88*	3.85	R	HGR-1

Abbreviations: GCA, general combining ability; HGB, heterotic Group B (seed parental groups); HGR, heterotic Group R (pollinator parental groups).
[a]Unassigned to heterotic groups.
* and **denote significance at 0.05 and 0.01 levels of probability, respectively.

heterotic pool classification: P-2 (−4.86**), P-5 (−2.08*), P-11 (−4.57**), and P-12 (−6.92**). Furthermore, P-6, P-7, and P-13 had the highest positive and negative SCA effects with the same heterotic group tester/s. P-6 had an SCA effect of +4.18* with HGB2-IT and −5.17* with HGB1-IT. Similarly, with HGB1-IT, population P-7 had an SCA effect of +3.24, and with HGB2-IT, it was −4.63*. Population P-13 had +3.85 with HGR2-IT and −4.88* with HGR1-IT. As a result, these three populations were not considered for heterotic group classification because they did not exhibit proper B and R heterotic patterns. Based on their SCA effects, six of the 13 populations were assigned to the respective heterotic

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groups of testers. The population P-1(2.66), P-8 (3.68), and P-10 (10.19**) had the highest positive SCA effect for grain yield with either one or both of the HGB ITs (Table 4), indicating that they represent opposite heterotic groups (HGR). Among the three populations, P-1 (−3.79) showed higher negative SCA effects with HGR1 ITs and was assigned to the heterotic group HGR-1, whereas P-8 (−9.67**) and P-10 (−5.65**) exhibited higher negative SCA effects with HGR2 ITs and were assigned to the tester's heterotic group. Three populations, P-3 (8.18**), P-4 (4.37*), and P-9 (3.66), had the highest positive SCA effect for grain yield with HGR IT/s, indicating that these populations represent opposite heterotic groups (HGB). Among the three populations, P-3 (−4.82**) and P-9 (−5.19**) were assigned to HGB-2 based on their highest negative SCA effect with the tester, whereas P-4 (−6.65**) showed high negative SCA effects with HGB1 IT and was assigned to group HGB-1. In line with these findings, Fato et al. (2012) also evaluated 36 top crosses produced by crossing with two ITs, ZM523 (Z) and Suwan-1 (S), and based on the SCA effect, 10 populations were assigned to Suwan-1 and eight to the ZM523 group.

3.2.3 | Comparison of the efficiency of inbred and CTs in defining the heterotic pattern (B and R) of pearl millet populations (OPVs) based on the SCA effect of grain yield

The classification results from the two kinds of testers demonstrate certain similarities, but they also reveal evident differences. Both composite and ITs, for example, classified the populations P-3, P-4, P-7, and P-12 as B. Both testers assigned R to populations P-2, P-8, and P-13 in the same way. Some populations, such as P-1, P-5, P-10, P-11, and P-6 and P-9, were classified differently by composite and ITs. These differences emphasized the need of selecting the appropriate type of tester set for accessing the heterotic pattern of pearl millet populations (OPVs) used in the study. The heterotic group categorization criteria used by researchers have a substantial impact on how genetic material is assigned to a heterotic group, resulting in the development of superior hybrids from crossings between heterotic groups. Superior hybrids can, however, be obtained with a lower frequency from crossings done within heterotic groups (Akinwale et al., 2014). As a result, with a good heterotic group classification criterion, inter-heterotic group crosses create superior hybrids than within-group crosses (Fan et al., 2008). For the SCA effect of grain yield, breeding efficiency was estimated as the percentage of superior high-yielding hybrids obtained across the total number of inter-heterotic group crosses (Fan et al., 2009). The efficiencies of a heterotic grouping of pearl millet population were compared by arranging the yield performance of crosses of pearl millet populations with heterotic group composite (Table S2) and ITs (Table S3) in the decreasing order of their testcross mean grain yield (kg ha^{-1}). The total number of hybrids with each composite and ITs was divided into three yield groups, that is, high-yielding crosses, intermediate-yielding crosses, and low-yielding crosses.

TABLE 5 The heterotic pattern (B × R) of pearl millet populations defined by a set of heterotic Group B (seed parental groups) (HGB) and heterotic Group R (pollinator parental groups) (HGR) composite testers in set 1 and their breeding efficiency.

B (HGB1, HGB2-composite tester)	R (HGR1, HGR2-composite tester)
P-1	P-2
P-3	P-6
P-4	P-8
P-5	P-9
P-7	P-13
P-10	
P-11	
P-12	

Note: Breeding efficiency of the composite tester = 61.54%.

TABLE 6 The heterotic pattern (B × R) of pearl millet populations defined by heterotic Group B (seed parental groups) (HGB) and heterotic Group R (pollinator parental groups) (HGR) inbred testers in set 2 and their breeding efficiency.

B (HGB1, HGB2-inbred tester)	R (HGR1, HGR2-inbred tester)
P-3	P-1
P-4	P-2
P-6	P-5
P-7	P-8
P-9	P-10
P-12	P-11
	P-13

Note: Breeding efficiency of the inbred tester = 46.15%.

The results of the heterotic pattern (B and R) of the pearl millet population defined by the SCA effect of grain yield with heterotic group composite and ITs are given in Tables 5 and 6, respectively. The CT classified eight populations into B and five into R, whereas the IT classified six populations to B and seven to R. Crosses were later divided into intergroup and within-group crosses based on the heterotic pattern assigned to the population. Intergroup crosses are the crosses between populations with the testers of the opposite heterotic group, and within-group crosses are the crosses between populations with the testers of the same heterotic group. The results showed that CTs had higher breeding efficiency (61.54%) than ITs (46.15%). Populations showing negative SCA effects with HGB should show positive SCA effects when crossed with HGR and vice versa. Such populations can be classified into clear-cut heterotic groups. Most of

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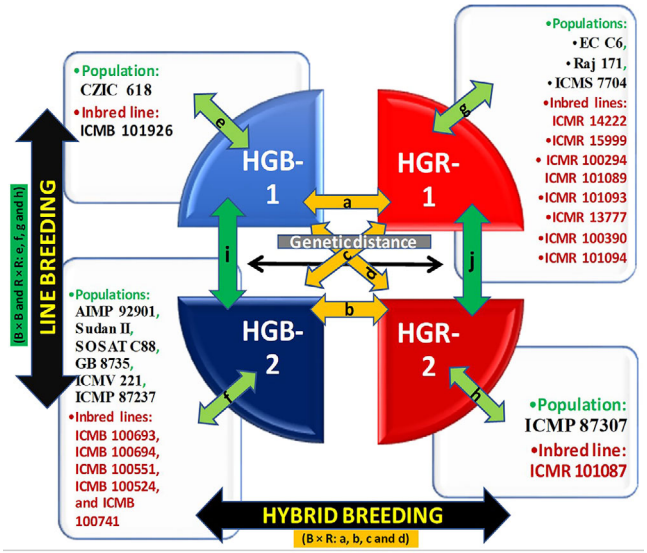

FIGURE 3 Heterotic group classification of new germplasm and its utilization in pearl millet hybrid breeding. HGB, heterotic Group B (seed parental groups); HGR, heterotic Group R (pollinator parental groups).

the populations exhibit clear-cut heterotic grouping with CTs compared to ITs, and also the breeding efficiency calculated indicated that the CTs were 15.39% more efficient than the ITs. Thus, in assigning pearl millet populations (OPVs), classification based on heterotic group CTs was more appropriate and considered for the final heterotic group classification (Figure 3).

3.2.4 | Utilization of new populations in hybrid breeding

Classifying these populations into heterotic groups will facilitate hybrid development. These populations could be utilized to broaden the genetic base of the hybrid parental lines within the established heterotic groups and to develop superior hybrid parents with higher productivity. In pearl millet breeding programs, seed and restorer parents were bred for a specific set of attributes separately in the development of B- and R-line hybrid parental lines. Maintainer or B-lines, for example, are commonly bred for shorter height (<100 cm) and larger seed size, compact panicles, lodging resistance, good seed set, and exertion. Whereas, different maturity types, plant height (grain vs. dual-purpose), tillering ability, seed color, and seed size are all regional preferences. The high grain yield potential of A-lines, both as lines per se as well as in hybrids (i.e., combining ability), is the most important consideration. Pollen parents must produce highly fertile hybrids, besides being able to produce high-yielding hybrids, the restorers should also be highly productive, which is important from the viewpoint of the seed production economy. It is desirable to breed pollinators of 150–180 cm in height, but no shorter than the A-line with built-in attributes of panicle, maturity, and tillering that will be preferred by farmers in the hybrids (Rai et al., 2006).

The genetic diversification of CMS systems in pearl millet, including the A1, A4, and A5 CMS systems, has been a significant focus of research (Athwal, 1961, 1966; Hanna, 1989; Marchais, 1985; Rai, 1995; Sujata et al., 1994). The A1 CMS system, widely used in the past, raised concerns about its stability and susceptibility to diseases and pests (Athwal, 1961, 1966), leading to the exploration of alternative sources. The A4 CMS system, identified from specific accessions, and the A5 CMS system from gene pools have been found to be more stable, with higher frequencies of maintainers and less association with negative agronomic traits (Rai et al., 2009). Additionally, their genetic backgrounds have a minimal impact on fertility restoration (Gupta et al., 2010), making them highly valuable for pearl millet hybrid breeding. As a result, there is a growing preference for prioritizing the A4 and A5 CMS systems, offering greater opportunities for genetic diversification and improved efficiency in seed parent breeding programs.

In the present study, seven populations were assigned for HGB for seed parental line development and most of them can be deployed as sources for developing maintainers for A4 and A5 CMS systems (Patil & Gupta, 2022). Five populations P-1, P-3, P-4, P-5, and P-7 have low maintainer frequency for the A1 CMS system (13%–22%) (Table S4), and they can be subjected to recurrent selection for sterility/maintainer reaction to increase the frequency of maintenance to derive new maintainer lines. Rai et al. (2006) had shown an increase in maintainer frequency among Asian and African populations for multiple CMS systems employing recurrent selection. In the Asian population Raj 171, one cycle of selection in favor of sterility/maintainer reaction increased the frequency of maintainers from 18% (C0 bulk) to 98% (C1 bulk) for the A1 CMS system and 49% (C0 bulk) to 99% (C1 bulk) for the A4 CMS system. Given that all of these seven populations have greater plant heights of >160 cm, donor lines from the respective HGB must be used to develop diverse maintainer lines with shorter plant height (Bidinger & Raju, 1990; Kumar & Adrews, 1993).

The four populations P-2, P-6, P-8, and P-9 assigned to HGR had a high restoration frequency of 89%–94% for the A1 CMS system and three populations P-6, P-8, and P-9 had a restoration frequency of 32%–63% for A4 CMS, whereas one population P-9 had relatively higher restoration frequency of 16% for A5 CMS system. Excellent restorers of the A1 CMS system are available with India's public and private sector hybrid programs. However, there is a lack of A4 restorers everywhere and the restorers of A5 in elite agronomic backgrounds are rare and are yet to be developed (Rai et al., 2006). These populations could be subjected to recurrent selection to improve the frequency of restoration in order to generate restorer parents for the specific CMS system.

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Similarly, the populations categorized into their respective heterotic groups could be utilized in selection of individuals with superior performance for yield and its component traits, facilitating the isolating of inbred lines that exhibit heterotic performance from the opposite heterotic pools. These populations also displayed higher values of SCA compared to GCA values, indicating that hybrid performance was more influenced by SCA. This observation is consistent with previous findings reported by Sattler et al. (2019) and Patil et al. (2021) in pearl millet population hybrids. Recurrent selection processes have been effective in achieving gradual genetic improvements for complex traits in genetically diverse populations. Estimates of GCA and SCA have been extensively employed in maize population improvement programs, as outlined by Hallauer in 1999. The populations assigned to each heterotic group can undergo population improvement methods, such as reciprocal recurrent selection or modified reciprocal recurrent selection for one or two cycles, which can significantly assist in identifying superior inbred lines. Hallauer (1999) recommended the use of reciprocal recurrent selection strategies to enhance the heterotic pattern in breeding programs that aim to develop inbred lines and hybrids from populations originating from diverse heterotic groups.

3.3 | Classification of new hybrid parental lines (new B and R lines) into heterotic groups

To undertake classification into established heterotic groups, it is essential to identify genotypes with high combining ability with the opposite heterotic group of the established pattern. This study was designed to introduce new hybrid parental lines (new B and R inbred lines) into the four established heterotic groups—HGB-1, HGB-2, HGR-1, and HGR-2—based on combining ability analysis and hybrid performance with selected heterotic group testers. The selected inbred lines were advanced breeding lines resulting from diverse B × B and R × R parental crosses, with preliminary information suggesting the existence of B-lines and R-lines as two separate broad heterotic pools. Consequently, new inbred lines were directly crossed with their opposite heterotic group testers to assess their combining ability (hybrid performance), serving as eligibility criteria for heterotic group classification. The new R lines were crossed with heterotic group B testers, three from HGB-1 (HGB1-T1, HGB1-T2, and HGB1-T3) and three from HGB-2 (HGB2-T1, HGB2-T2, and HGB2-T3), forming set 3. Similarly, the new B lines were crossed with three HGR-1 testers (HGR1-T1, HGR1-T2, and HGR1-T3) and three HGR-2 testers (HGR2-T1, HGR2-T2, and HGR2-T3), constituting set 4. The results of heterotic group classification, with new R lines into HGR-1 and HGR-2, and new B lines into HGB-1 and HGB-2, are presented in Tables 7 and 8, respectively.

The R lines R-L3, R-L4, and R-L12, displaying significant negative GCA effects with HGB testers, were excluded from the heterotic group classification. Among the new R lines, namely R-L1, R-L5, R-L7, R-L10, and R-L2, which exhibited positive GCA effects with the opposite heterotic group (HGB) testers and an average standard heterosis of >10% over ProAgro-9444 (a widely used commercial hybrid in India), were considered the most promising candidates. These lines are potential sources of valuable alleles for enhancing yield productivity. Additionally, R-L6, R-L8, R-L9, and R-L11, with an average standard heterosis of >5% over ProAgro 9444, were also considered for heterotic group classification.

Our ultimate objective is to exploit heterosis (hybrid breeding) between HGB (B) and HGR (R) while line breeding within the HGB (B) and HGR (R). In this context, any new R line meeting the eligibility criteria and identified as heterotic and promising can be categorized into either of the heterotic subgroups (HGR1 and HGR2) based on any suitable criteria, such as marker-based genetic similarity. Melchinger et al. (1991) established that Roger's distance estimates between two homozygous inbreds are linearly related to the coefficient of coancestry, making Roger's distance suitable for studying relationships between inbreds based on allelic informative marker data. Genetic similarity of new R lines with HGR-1 and HGR-2 testers was assessed using SNP marker data. The selected new R lines—R-L1, R-L2, R-L5, R-L7, R-L8, R-L9, R-L10, and R-L11—demonstrated numerically higher genetic similarity values with HGR-1 testers compared to HGR-2, suggesting a closer genetic relationship and thus assignment to the same group. Similarly, R-L6 was assigned to HGR-2 based on relatively higher genetic similarity with HGR-2 testers. Assessing genetic distance or similarity through molecular markers proves beneficial for identifying related germplasm, offering a method to introduce new R lines into heterotic pools and enhance heterotic response in pearl millet.

B lines B-L3, B-L10, and B-L11, exhibiting significant negative GCA effects, were excluded from heterotic group classification. On the other hand, B lines B-L1, B-L2, B-L6, B-L7, B-L8, and B-L9, demonstrating positive GCA effects with HGR testers and achieving average test cross mean yields equal to or greater than the mean yield of the commercial check ProAgro 9444, were incorporated into the HGB. Further categorization within HGB was based on genetic similarity with the respective heterotic group B testers using SNP markers, assigning B-L6 to HGB-1 and B-L1, B-L2, B-L7, B-L8, and B-L9 to HGB-2 (Table 8). These newly introduced B and R lines hold potential for utilization in line development; these selected new B and R lines could provide a source of new desirable alleles to broaden the genetic base. With an appropriate strategy, it might increase the genetic divergence between heterotic pools, thereby improving hybrid performance and/or heterotic pool response.

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TABLE 7 Assigning new R lines into heterotic groups HGR-1 and HGR-2, where HGR is heterotic Group R (pollinator parental groups).

Set 3: R-line	GCA	Average grain yield (kg ha^{-1})[a]	Heterosis (%)[b]	Genetic similarity[c] HGR-1	HGR-2	Heterotic group classification
R-L1	410.87**	4749	20.1	0.7721	0.7638	HGR-1
R-L2	59.62	4398	11.22	0.7736	0.7662	HGR-1
R-L3	−268.15*	4070	2.93	0.7895	0.7839	[d]
R-L4	−355.19**	3983	0.73	0.7773	0.7676	[d]
R-L5	147.67	4486	13.44	0.7883	0.7880	HGR-1
R-L6	−151.67	4186	5.87	0.7814	0.7830	HGR-2
R-L7	132.77	4471	13.07	0.7654	0.7578	HGR-1
R-L8	−136.03	4202	6.27	0.7764	0.7678	HGR-1
R-L9	−35.97	4302	8.8	0.7776	0.7713	HGR-1
R-L10	525.72**	4864	23.01	0.7770	0.7681	HGR-1
R-L11	−103.46	4234	7.09	0.7750	0.7656	HGR-1
R-L12	−226.20*	4112	3.99	0.7872	0.7808	[d]

Abbreviations: GCA, general combining ability; HGR, heterotic Group R (Pollinator parental groups).
[a]Average grain yield (kg ha^{-1}) of a line across six HGR testers.
[b]Heterosis (%) over Check ProAgro 9444.
[c]SNP-based genetic similarity with HGR testers.
[d]Unassigned to heterotic group.
* and **denote significance at 0.05 and 0.01 levels of probability, respectively.

TABLE 8 Assigning new B lines into heterotic groups HGB-1 and HGB-2.

Set 4: B-line	GCA	Average grain yield (kg ha^{-1})[a]	Standard heterosis (%)[b]	Genetic similarity[c] HGB-1	HGB-2	Heterotic group classification
B-L1	642.46**	4695	16.76	0.7564	0.7795	HGB-2
B-L2	66.71	4120	2.44	0.7574	0.7783	HGB-2
B-L3	−287.39**	3765	−6.36	0.8046	0.7809	[d]
B-L4	−142.44	3910	−2.76	0.7629	0.7797	HGB-2
B-L5	−49.26	4004	−0.44	0.7964	0.8124	HGB-2
B-L6	139.54	4192	4.26	0.7801	0.7784	HGB-1
B-L7	246.00*	4299	6.9	0.7539	0.7730	HGB-2
B-L8	401.67**	4454	10.77	0.7605	0.7839	HGB-2
B-L9	63.53	4116	2.37	0.7574	0.7783	HGB-2
B-L10	−217.60*	3835	−4.63	0.7647	0.7822	[d]
B-L11	−771.85**	3281	−18.41	0.7654	0.7784	[d]
B-L12	−91.39	3961	−1.49	0.8111	0.7898	HGB-1

Abbreviations: GCA, general combining ability; HGB, heterotic Group B (seed parental groups).
[a]Mean grain yield (kg ha^{-1}) of a line across six HGR testers.
[b]Heterosis (%) over Check ProAgro 9444.
[c]SNP-based genetic similarity with HGB testers.
[d]Unassigned to heterotic group.
* and **denote significance at 0.05 and 0.01 levels of probability, respectively.

3.4 | Combining ability and selection strategies

The association between GCA and per se performance of parents for grain yield was positive but nonsignificant (Table 9), indicating that high general combiners are equally likely or even more likely to occur in lines with average to high grain yield per se. Selecting high GCA lines in high-yielding lines increases the profitability of seed production (Rai & Virk, 1999), and preliminary selection for line yield (per se) is easier and less expensive to evaluate than GCA evaluation (Rai et al., 2006). The selected lines can then be evaluated for GCA and SCA at the later stages. The high positive significant association between the sum of parental GCA effects and hybrid

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TABLE 9 Correlation between combining ability effects and per se performance of parents and hybrids for grain yield (kg ha^{-1}).

Parameter	Set 1	Set 2	Set 3	Set 4
	Pearl millet populations		Inbred/hybrid parental lines	
Per se performance of parents and their GCA effects	0.189	0.355	0.465	0.322
The sum of GCA effects of parents and their hybrid per se performance	0.635**	0.706**	0.658**	0.752**
SCA effect of hybrid and its per se performance	0.772**	0.708**	0.753**	0.659**

Abbreviations: GCA, general combining ability; SCA, specific combining ability.
* and **denote significance at 0.05 and 0.01 levels of probability, respectively.

per se performance for grain yield in this study indicates that hybrid per se performance can be predicted based on parental GCA, which is attributable to additive effect genes (Falconer & Mackay, 1996). As a result, the GCA of parents can be used as a predictive tool for developing hybrids with superior per se, reducing the use of input resources and increasing breeding efficiency. The four heterotic groupCTs used in the study are the broad-based testers, which showed a relatively better ability to classify the new germplasm into heterotic groups in the current study and would be the best candidates for early generation testing as stage-1, which enables the selection of good combiners and the maintenance of the heterotic pattern in the hybrid breeding programs.

According to Melchinger et al. (1987), heterotic groups can improve GCA/SCA variance, which could be one of the approaches to improve prediction accuracy by employing GCA in the long term. This also means that superior hybrid parents could be identified based on combining ability effects. Hybrid prediction accuracy is determined by the predictability ratio. In general, grain yield (kg ha^{-1}) has a lower predictability ratio in our study, indicating that prediction accuracy of hybrid performance based on GCA alone would be relatively less reliable to support early testing and indicated that hybrids performance increases with improvement in both general and SCA of the populations. Also, a positive correlation was observed between hybrid grain yield and the hybrid's SCA effect, indicating that both GCA and SCA effects are important for predicting hybrid performance. Thus, after the pre-selection of potential hybrid parents based on the top cross performance with heterotic group CT (stage-1), the evaluation of crosses of the selected lines with heterotic group ITs as advanced generation testing stage-2 can be adopted to identify the best-performing parents based on both GCA and SCA.

4 | CONCLUSIONS

The study aimed to classify different types of new germplasm, including 13 populations of African and Asian origins, along with 24 new inbred parents from ICRISAT's breeding program, into existing heterotic groups of pearl millet. Different approaches were employed for assigning new germplasm to broaden the existing genetic base while maintaining the established heterotic pattern. The new population of pearl millet (OPVs) was grouped based on GCA and SCA effects, and CTs were found to be more effective for assigning new pearl millet populations into existing heterotic groups. New inbred lines were assigned to HGB and HGR based on their GCA with opposite heterotic group testers and also using genetic similarity estimated from GBS data. These new germplasms have the potential to be utilized in the development of superior hybrid parents, contributing to the expansion of the genetic base of hybrid parental lines. The pearl millet populations, categorized into specific heterotic groups, exhibited varying frequencies of fertility maintenance and restoration across A1, A4, and A5 cytoplasmic male sterility systems. A recurrent selection for one or two cycles can increase the frequency of these traits, providing a pathway to explore genetic diversification possibilities in cytoplasmic male sterility systems in pearl millet and enhance seed production efficiency. Both GCA and SCA effects were deemed significant in predicting hybrid performance in different sets of testcrosses. A two-stage testing strategy was proposed, involving a preliminary screening with broad-based heterotic group testers for GCA, followed by testing with heterotic group ITs to enhance genetic gains in the pearl millet hybrid breeding program. The classification of suitable new germplasm into existing heterotic groups, guided by combining ability studies, was recognized as a method to enhance the genetic base and increase the frequency of heterotic loci in hybrid breeding programs. The emphasis on assigning appropriate new germplasm into heterotic groups and implementing strategies to enhance combining ability between heterotic groups remains crucial for the long-term success of pearl millet hybrid breeding programs.

AUTHOR CONTRIBUTIONS

Rakshith Papanna: Conceptualization; formal analysis; investigation; methodology; visualization; writing—original draft; writing—review and editing. **I. Shanker Goud**: Conceptualization; investigation; supervision; validation; writing—review and editing. **Anilkumar Vemula**: Data curation; formal analysis; investigation; methodology; software; validation; writing—review and editing. **B. V. Tembhurene**:

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Supervision; validation; writing—review and editing. **M. K. Meena**: Writing—review and editing. **Shashi Kumar Gupta**: Conceptualization; formal analysis; funding acquisition; investigation; methodology; resources; supervision; validation; writing—original draft; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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