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# DEVELOPMENT AND APPLICATION OF SSR MARKERS FOR GENETIC DIVERSITY ASSESSMENT IN GARLIC (ALLIUM SATIVUM) GERMPLASM

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## ABSTRACT

Garlic (Allium sativum L.) is a globally significant but genetically complex species, propagated exclusively through vegetative means. This clonal propagation limits genetic improvement but has not prevented the accumulation of considerable intraspecific diversity. To facilitate molecular studies, this study aimed to develop a novel set of Expressed Sequence Tag-derived Simple Sequence Repeat (EST-SSR) markers and apply them to assess genetic diversity. A comprehensive in silico analysis of 289,142 unigenes identified 4,372 potential SSR loci. From these, a subset of 25 polymorphic SSR markers was used to genotype 30 diverse garlic accessions. These markers revealed 80 alleles, with an average of 3.2 alleles per locus. The newly developed EST-SSR markers proved to be highly effective for revealing genetic

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relationships and population structure, providing a valuable resource for future genetic conservation, characterization, and breeding programs in garlic.

**Keywords:** Garlic (Allium Sativum L.), EST-SSR Markers, Genetic Diversity, Population Structure, Molecular Markers

## Introduction

Agriculture forms the foundation of human civilization, providing food security and sustaining livelihoods across the globe (Afzal et al., 2025; Bangash et al., 2013). The cultivation of diverse staple crops supports billions, while specialty horticultural species contribute unique flavors and health benefits (Alam et al., 2025 & 2023; Aslam et al., 2021). Among these diverse species, garlic (Allium sativum L.) is a horticultural crop of immense global importance, valued for both its culinary and medicinal properties (Papaioannou et al., 2023; Begum et al., 2022; Farrukh et al., 2022). As an obligate apomictic species, it is propagated exclusively through vegetative means, which presents significant challenges for traditional breeding (Arif et al., 2024; Gul et al., 2011). Despite this clonal reproduction, garlic exhibits substantial phenotypic and genetic variability, leading to the development of numerous cultivars worldwide (Glala et al., 2025; Begum[a] et al., 2025 & 2019; Ahmed et al., 2021). This rich germplasm represents a critical resource for genetic improvement (Ren et al., 2025; Khan et al., 2025; Jamil et al., 2022; Mehmood[a] et al., 2021; Ahmed[a] et al., 2019).

Accurate characterization of this germplasm is essential for its effective management and utilization (Begum[b] et al., 2025; Raza et al., 2025; Khattak et al., 2022[a], Mehmood[b] et al., 2021). Traditional classification based on morphology can be unreliable due to phenotypic plasticity and the prevalence of synonyms and homonyms (Begum[c] et al., 2025; Qaiser et al., 2022; Mehmood[c] et al., 2021; Rehman et al., 2020). Molecular markers offer a robust solution to these challenges. Among them, Simple Sequence Repeats (SSRs) are particularly advantageous due to their co-dominant inheritance, multi-allelic nature, reproducibility, and high polymorphism.

However, the application of SSR markers in garlic has been constrained by their limited availability. Therefore, the objectives of this study were: (i) to develop a large set of novel EST-SSR markers through in silico mining of garlic transcriptome data, and (ii) to employ a selection of these markers to evaluate the genetic diversity and population structure within a collection of 30 garlic accessions.

## Materials and Methods

### Plant Materials and DNA Extraction

A diverse panel of 30 garlic accessions of global origin was used in this study. Total genomic DNA was extracted from approximately 0.5 g of freeze-dried young leaf tissue for each accession using the standard CTAB (cetyltrimethylammonium bromide) method. DNA quality and quantity were assessed via 1% agarose gel electrophoresis and a NanoDrop 2000 spectrophotometer. Final DNA concentrations were normalized to 50 ng/µL for subsequent analyses

### Identification of SSR Loci and Marker Development

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A previously assembled transcriptome dataset comprising 289,142 unigenes was utilized for SSR discovery. The Microsatellite Identification Tool (MISA) software was employed to identify SSR loci, with search parameters set to a minimum of 6 repeats for dinucleotide motifs and 5 repeats for tri-, tetra-, penta-, and hexanucleotide motifs. Primer pairs flanking the identified SSR regions were designed using Primer 3.0 software.

**PCR Amplification and Genotyping**

Polymerase chain reaction (PCR) was performed in 10 µL volumes containing 50 ng of template DNA, 5 µM of each primer, and 2× Taq PCR Master Mix. The touchdown PCR profile consisted of an initial denaturation at 95°C for 8 min; followed by 6 cycles of 94°C for 40 s, 68°C for 40 s (decreasing by 2°C per cycle), and 72°C for 40 s; 8 cycles of 94°C for 40 s, 58°C for 40 s (decreasing by 1°C per cycle), and 72°C for 40 s; 20 cycles of 94°C for 40 s, 50°C for 40 s, and 72°C for 40 s; with a final extension at 72°C for 5 min. Amplified PCR products were resolved on 3% agarose gels, visualized by silver staining, and documented. Clear and unambiguous bands were scored as alleles to create a co-dominant genotypic matrix.

**Results**

**Development and Characterization of EST-SSR Markers**

The in silico screening of 289,142 unigenes resulted in the identification of 4,372 SSR loci. Dinucleotide repeats were the most abundant (56.3%, 2,461 loci), followed by trinucleotide repeats (41.4%, 1,811 loci). The tetranucleotide, pentanucleotide, and hexanucleotide motifs were rare, collectively representing only 2.3% of the total. The most frequent dinucleotide motifs were TA/TA (20.4%), TG/CA (12.6%), and AC/GT (11.8%). Primer pairs were successfully designed for these loci, with amplicons ranging from 100 to 280 bp.

**SSR Marker Polymorphism and Genetic Diversity**

From the newly developed markers, 25 polymorphic EST-SSRs were selected to genotype the 30 garlic accessions. These markers generated a total of 80 alleles, with the number of alleles per locus ranging from 2 to 6 (average of 3.2).

**Marker Asa04**

The STS marker As04 was used to detect the genetic diversity in 30 Pakistani garlic lines. A total of 27 garlic lines showed 264bp fragment. The remaining 3 lines did not amplify any fragment as shown in (Fig.1).

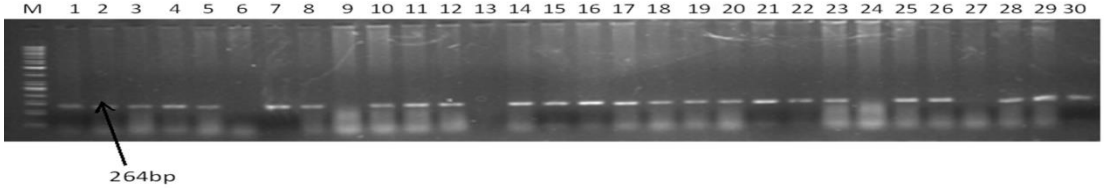



**Figure.1 PCR amplification of Asa04 locus in Pakistani garlic accessions with 100bp Ladder**

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**Marker Asa06**

The STS marker As06 was used to detect the genetic diversity in 30 Pakistani garlic lines. Overall 30 garlic lines showed 192bp fragment as shown in (Fig.2).

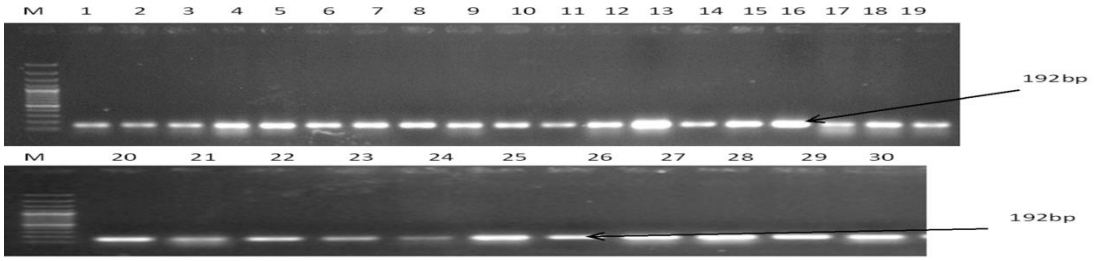



**Figure.2 PCR amplification of Asa06 locus in Pakistani garlic accessions with 100bp Ladder**

**Marker Asa23**

STS marker Asa23 amplifies 271bp fragment in 19 accessions out of 30 garlic lines and remaining 11 accessions did not amplified no amplification as shown in (Fig. 3)

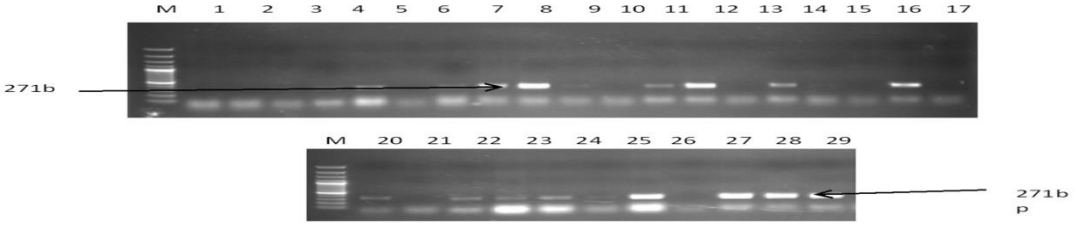



**Figure.3 PCR amplification of Asa23 locus in Pakistani garlic accessions with 100bp Ladder**

**Marker Asa08**

The STS marker Asa08 amplifies 209 bp fragments in 30 accessions out of 30 garlic lines as shown in (Fig. 4)

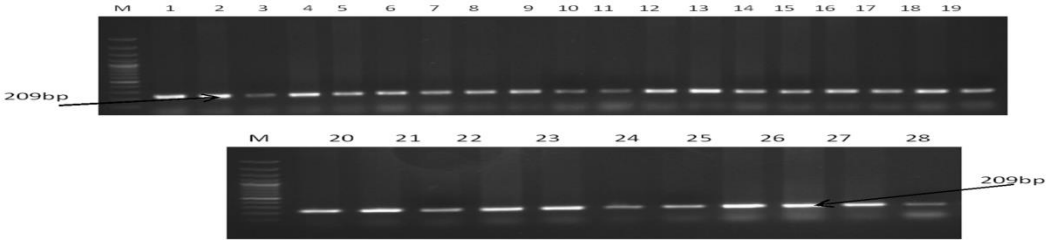



**Figure.4 PCR amplification of Asa08 locus in Pakistani garlic accessions with 100bp Ladder**

**Marker Asa16**

The STS marker Asa16 was used to detect the genetic diversity in 30 Pakistani garlic lines. A total of 28 garlic lines showed 154bp fragment. The remaining 2 lines did not amplify any fragment as shown in (Fig.5).

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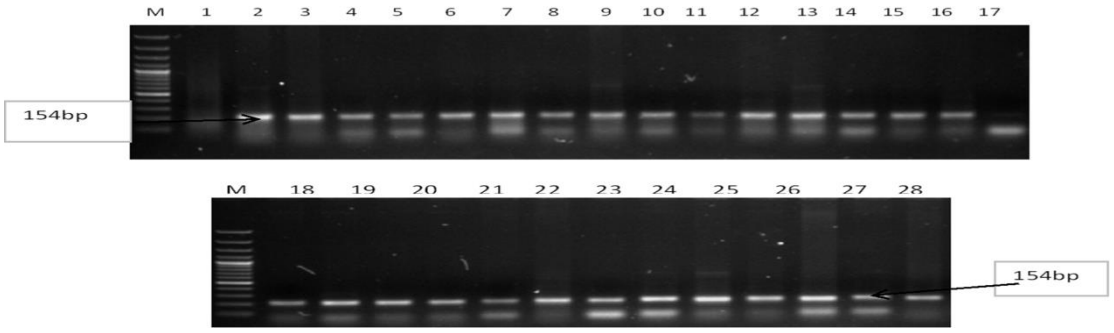



**Figure.5 PCR amplification of Asa16 locus in Pakistani garlic accessions with 100bp Ladder**

**Marker Asa27**

The STS marker Asa27 was used to detect the genetic diversity in 30 Pakistani garlic lines. A total of 28 garlic lines showed 127bp fragment. The remaining 2 lines did not amplify any fragment as shown in (Fig.6).

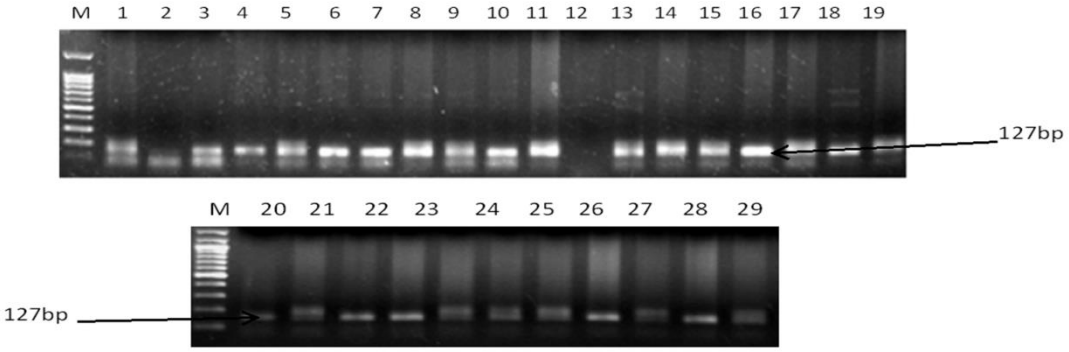



**Figure.6 PCR amplification of Asa27 locus in Pakistani garlic accessions with 100bp Ladder**

**Marker Asa25**

The STS marker Asa25 was used to detect the genetic diversity in 30 Pakistani garlic lines. Twenty eight garlic lines showed 117bp fragment. The remaining 2 lines did not amplify any fragment as shown in (Fig.7).

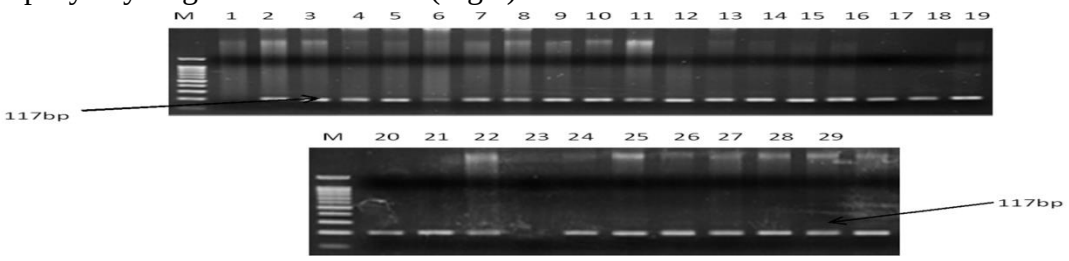



**Figure.7 PCR amplification of Asa25 locus in Pakistani garlic accessions with 100bp Ladder**

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**Marker Asa24**

The STS marker Asa24 was used to detect the genetic diversity in 30 Pakistani garlic lines. All garlic lines showed different bands range from 300-1300 bp fragment as shown in (Fig.8).

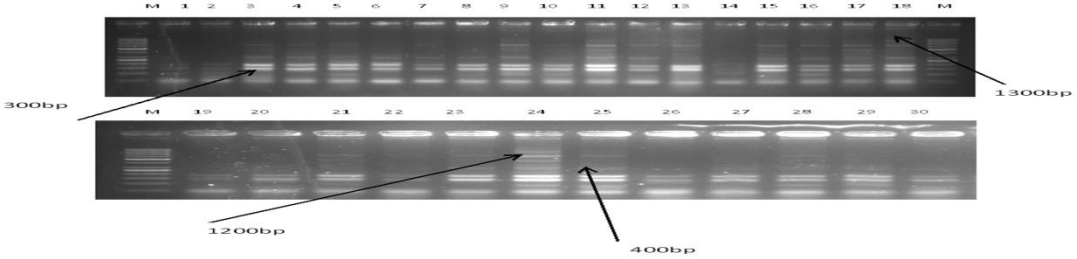



**Figure.8 PCR amplification of Asa24 locus in Pakistani garlic accessions with 100bp Ladder**

**Marker Asa03**

The STS marker Asa03 was used to detect the genetic diversity in 30 Pakistani garlic lines. A total of 19 garlic lines showed 300 bp fragments as shown in (Fig.9).

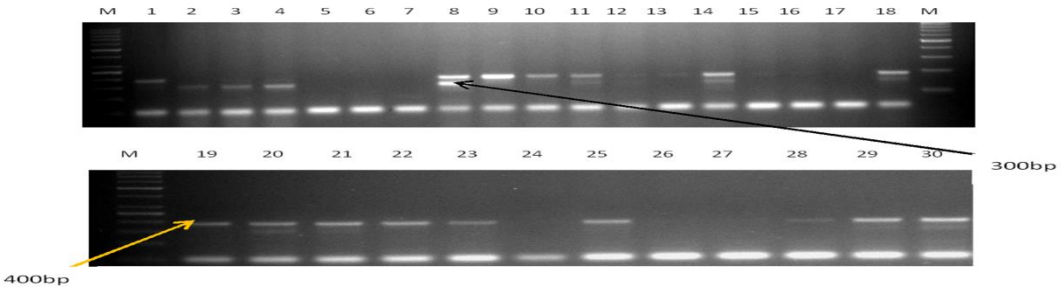



**Figure.9 PCR amplification of Asa03 locus in Pakistani garlic accessions with 100bp Ladder**

**Marker Asa02**

The STS marker Asa02 was used to detect the genetic diversity in 30 Pakistani garlic lines. Overall 19 garlic lines showed 300 bp fragments as shown in (Fig.10).

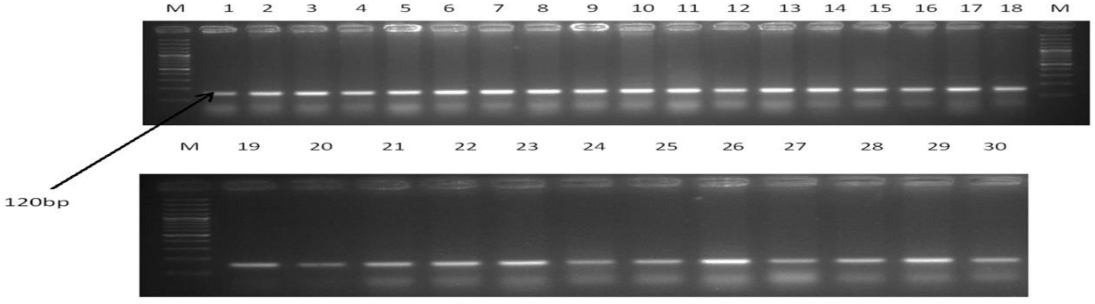



**Figure.10 PCR amplification of Asa02 locus in Pakistani garlic accessions with 100bp Ladder**

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## Discussion

Despite the predominantly asexual propagation of most garlic varieties, significant morphological diversity exists both within and between them (Kirac et al., 2022; Gomes et al., 2021; Khattak et al., 2020; Ahmed[b] et al., 2019). Assessing the genetic variation among cultivated garlic accessions is crucial for accurate phenotypic identification and the establishment of core germplasm collections (Glala et al., 2025; Shaheen et al., 2023; Aslam et al., 2018; Siddiqui et al., 2017). Furthermore, the analysis of genetic diversity and relatedness between accessions is essential for informed garlic selection and breeding strategies (Begum[d] et al., 2025; Yang et al., 2024; Tahir et al., 2023; Figliuolo et al., 2022). Early investigations into the variation of different garlic clones primarily focused on morphology and enzyme analysis (Ren et al., 2025; Saleem et al., 2024; ur Rehman et al., 2020; Afzal et al., 2017). High levels of morphological diversity have been reported in garlic based on several phenotypic traits, including bulb size, shape, rind color, clove rind color, bolting habit, and clove number and weight Kumari, S. (2021).In the present study, the 30 garlic accessions exhibited considerable diversity across the investigated morphological traits (Glala et al., 2025; So et al., 2021; Thangasamy et al., 2021;Waqar et al., 2018; Aslam[a] et al., 2017). However, reliance solely on morphological traits did not permit the accurate identification of potentially redundant accessions, highlighting the limitations of assessing genetic diversity in garlic germplasm from a purely morphological perspective. Previous studies reported that the garlic diversity assessed based on morphological traits is highly influenced by both the underlying genetic composition and environmental factors such as cultivation practices, soil properties, and fertilization regimes (Begum[d] et al., 2025; Polyzos et al., 2019). This context likely explains the inherent disadvantages of using morphological traits alone for crop analysis (Glala et al., 2025; Yang et al., 2024; Ahmed et al., 2014, 2012 & 2011).

For genetic diversity assessment, Simple Sequence Repeat (SSR) markers have been extensively utilized in numerous plant species for evaluating genetic diversity, constructing genetic maps, and determining species lineages (Hanci et al., 2025; Khattak et al., 2024 & 2022[b]; Zahid et al., 2022). However, a limited number of available SSR markers poses a significant constraint for related genetic studies in garlic (Liu et al., 2020; Ahmed[b] et al., 2017). Among these newly identified SSR markers, dinucleotide repeat motifs were the most abundant type, followed by trinucleotide and tetranucleotide repeat motifs. This observation contrasts with the previous findings, who reported trinucleotide repeat motifs as the most frequent, followed by dinucleotide repeats (Begum[e] et al., 2025; Liu et al., 2015). This discrepancy could be attributed to variations in SSR search criteria, dataset size, and the bioinformatic tools employed (Aggarwal et al., 2007). Furthermore, only 244 consistent SSR markers were shared between the study by Liu et al. (2015) and the markers reported herein, potentially reflecting differences in the assembled unigene sequences between the two studies. To evaluate the quality of these newly developed SSR markers, 50 randomly selected primer pairs were used to genotype 10 garlic accessions. Of these, 78% of the markers (39) successfully amplified the target DNA fragments, while the remaining 22% (11) failed to produce any amplification products. The unsuccessful amplification of these SSRs could potentially be due to

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primer design across splice sites or large intronic regions.

Twenty polymorphic markers, selected from reported and validated SSR markers were employed to assess the genetic diversity of 30 garlic accessions. The average Polymorphism Information Content (PIC) value for these 20 SSR markers was 0.36, ranging from 0.22 to 0.49. This mean PIC value is comparable to the average PIC value (0.38, range 0.30–0.54) obtained by Barboza et al. (2021) using 10 EST-SSRs to evaluate the genetic diversity of a collection of 73 garlic accessions. However, Ipek et al. (2015) reported a higher mean PIC value of 0.60 for 26 EST-SSRs used in 31 garlic accessions. The elevated mean PIC value in the study by Ipek et al. (2015) likely resulted from the intentional selection of the 31 garlic accessions based on prior Amplified Fragment Length Polymorphism (AFLP) analysis (Ipek et al., 2003) to maximize observed genetic variation. In contrast, the 30 accessions used in the present research were not subjected to pre-selection or classification. Furthermore, the number and type of markers used, as well as the population size and the inherent genetic variation within the evaluated germplasm collection, can all contribute to variations in PIC values (Papaioannou et al., 2023; Barboza et al., 2020; Ahmed et al., 2010).

When assessing the genetic diversity and population structure of a germplasm collection, the SSR or other DNA markers were able to overcome the problem in the nomenclature of garlic accessions and the redundancy. The guaranteed identification of potential duplicates is helpful for reducing costs in the maintenance of garlic germplasm. Based on the genetic distance and the SSR marker-based dendrogram, 10 potential duplicates were identified among the 30 garlic These redundancy garlic accessions may be due to the farmers in different areas tend to exchange their garlic varieties/produce from year to year, to protect yield against degeneration caused by continuous cropping using the same variety, and each farmer might name the same garlic variety with obviously different names; thus, some different garlic varieties were improperly given a same name (Aslam[b] et al., 2017; Wang et al., 2016; Ahmed[b] et al., 2010). In addition, both the cluster analysis of 30 accessions and the population structure analysis of 10 unique garlic accessions revealed that three major clusters existed for the garlic germplasm used in this study. However, the garlic accessions that grouped into the same cluster were not completely consistent with their geographical origins. Moreover, the Mantel test results also verified that there was no significant correlation between genetic distance and geographic distance, which was also found by Ipek et al. (2003), Pooler, M. R., & Simon, P. W. (1993)., Wang et al. (2014), and Morales et al. (2013). Furthermore, the frequent introduction of garlic cultivars has led to serious homonymy, making genetic analysis crucial to identify redundancy (Glala et al., 2025; Ullah et al., 2025; Benke et al., 2021; Ahmed[b] et al., 2011). This study successfully identified 10 duplicate accessions using SSR markers, confirming this issue. While studies report varying genetic variation levels within populations (Barboza et al., 2020; Aslam[b] et al., 2017; Zhao et al., 2011), a high Fst value (0.18) here suggests valuable diversity. Despite a large genome hindering research (~16 Gb); Meryem et al., 2015; Ahmed[b] et al., 2011), the new genome assembly and these SSR markers are vital tools for developing a core germplasm collection, reducing maintenance costs, and aiding future breeding.

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## Conclusion

In conclusion Reported EST-SSRs were developed from assembled unigene sequences for garlic, in which 12 polymorphic markers combined with other 8 SSR markers were successfully used for the genetic diversity and population structure analysis of a garlic germplasm collection. There were 10 duplications among the evaluated 30 garlic accessions, unique garlic accessions can be divided into three clusters. Among this garlic germplasm collection, some interdependent traits such as bulb weight clove number, bulb height, and width exhibited significant positive correlations with each other. Overall, SSR markers will be of great value for the related genetic studies in garlic, and the assessment of genetic diversity and population structure of the involved garlic accessions will also provide valuable information for further utilizing these garlic germplasm.

## Author contributions

Dr. Sania Begum conducted the majority of the reported research. Sahir hameed khattak helped with genotyping of SSR markers. Muhammad Aqeel Aslam helped with the morphological data collection. Dr Ghulam Muhammad Ali conceived and supervised the research. Dr. Sania Begum wrote the manuscript with the input of Dr. Ghulam Muhammad Ali and Saida Komal. All authors reviewed and approved the final submission.

## Declaration of competing interest

None

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