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MOLECULAR CHARACTERIZATION OF SIX GARLIC CULTIVARS FROM PAKISTAN BY USING ISSR METHOD

SABAHAT ANWAR[1*], HUMERA AFRASIAB[1], ZAKIA LATIF[2]

[1]*Institute of Botany, University of the Punjab, Lahore 54590, Pakistan*
[2] *Department of MicroBiology & Molecular Genetics, University of the Punjab, Lahore 54590*

Corresponding Author's Email: sabahatanwar41@gmail.com

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Abstract

In this current investigation, a DNA fingerprinting analysis was conducted to explore the genetic variation among six distinct garlic cultivars originating from Pakistan. This was achieved using six ISSR markers. The genetic material was extracted from the leaves of all cultivars and subsequently subjected to individual amplification with each ISSR primer in a PCR thermocycler. The resulting amplification products ranged in size from 340 to 860 base pairs. A total of 97 bands were generated using five primers, with each primer producing between 16 to 20 bands. On average, there were three bands per cultivar. All cultivars exhibited results with five out of the six primers. However, the (CA)8 primer did not yield any discernible banding pattern. Notably, the "White" cultivar displayed no bands with the ISSR primer (GACA)4, while the "Silver Skin" cultivar exhibited no bands with the ISSR primer (GTG)5. This study employed di-nucleotide (CA)8, tri-nucleotide [(GTG)5, (CAA)4], and tetra-nucleotide [(GACA)4, (GATA)4, (GGAT)4] repeats to achieve these findings. n this investigation, a total of 38 loci were identified, with 33 of them displaying polymorphism. A notable 89% polymorphism rate was observed among the six garlic cultivars that were subjected to amplification using five different primers. The Shannon diversity indices calculated for these primers revealed the highest value of 2.173 in the (GGAT)4 primer, signifying complete polymorphism among the garlic cultivars. Conversely, the (GTG)5 primer showed the lowest index of 1.598, attributable to the absence of bands in one garlic cultivar, specifically the "Silver Skin" variant. The ISSR markers proved to be highly effective in discerning genetic distinctions among the garlic cultivars, demonstrating the technology's significance in cultivar identification and the study of their genetic diversity.

Keywords: DNA fingerprinting, Garlic, ISSR, Molecular characterization, Polymorphism, Pakistan.

Introduction

Allium sativum L., commonly known as garlic, is a member of the Alliaceae family. This plant species is indigenous to Central Asia and has been integrated into the staple diet of the Mediterranean region (Ensminger, 1994). Throughout history, garlic has been cherished for its culinary and flavor-enhancing properties, finding its way into a wide array of dishes including soups, meat preparations, salads, pulses, garlic bread, and pickles. (Khoshtinat, 2017). Garlic is primarily cultivated for its consumable bulbs, which can be utilized in various forms such as fresh, cooked, processed, and can also be stored for future propagation. Additionally, garlic holds a place in ethnomedicine, with documented benefits in treating a range of health conditions. Research has highlighted its effectiveness in addressing hypertension and cardiovascular diseases (Ou *et al*., 2003), as well as providing relief for chest ailments, diabetes, rheumatism, and digestive disorders, among others.

Garlic is propagated asexually and exhibits significant diversity in various morphological aspects, including the arrangement, shape, color,

and size of cloves, as well as leaf size, presence of scapes, scape height, bulbil placement, and fertility (Pooler and Simon, 1993). Traditionally, garlic characterization has relied on these morphological traits, though it's worth noting that different varieties may also respond differently to environmental conditions.

Garlic is inherently sexually sterile, which necessitates its vegetative cultivation (Etoh, 1985). The propagation of garlic is slow because it primarily occurs through the division of bulbs, whether they are in the ground or aerial. Due to the challenges associated with flower induction, sexual reproduction and subsequent breeding programs have shown limited success in improving garlic varieties (Barandiaran *et al*., 1999).

The predominant threats to garlic cultivars are viruses, fungi, nematodes, and insect pests (Davies, 1994; Verbeek *et al*., 1995). Garlic varieties are categorized as either bolting or non-bolting types, referred to as hardneck and softneck garlic, respectively. However, some varieties may produce weak flower stalks, which can make classification less straightforward (Volk *et al*., 2004).

Studying the genetic diversity of garlic cultivars is crucial due to the challenges posed by their vegetative propagation, hindering the development of improved traits. Screening germplasm is essential for identifying diverse cultivars, enabling the selection of potential donors for breeding programs (Sharma *et al*., 2016).

Somatic mutations can lead to variations in appearance and names of cultivars in different regions. Assessing genetic diversity helps resolve this ambiguity, ensuring accurate identification and classification of cultivars (Morales *et al*., 2013).

Molecular techniques like RAPD, AFLP, SSR, and ISSR markers are employed to detect differences at the molecular level. These markers do not require prior knowledge of gene sequences. Random primers, being cost-effective and efficient, yield prompt results (Al-Otayk *et al*., 2008).

In comparing closely related clones or cultivars, molecular methods surpass morphological data in reliability and efficiency. This aids breeders in making informed decisions regarding cross-breeding for superior traits (Al-Otayk *et al*., 2008).

ISSR stands out as a reliable method in genetic analysis. It can discern even closely related varieties or cultivars that may be indistinguishable by other markers. Additionally, ISSR proves valuable in genetic mapping, as it does not necessitate prior knowledge of DNA sequences for amplifying simple sequence repeats (Dagani *et al*., 2003; Salhi-Hannachi *et al*., 2005; Son *et al*., 2012).

Pakistan, there has been limited effort in the identification, classification, and characterization of various garlic cultivars. While morphological traits can be influenced by environmental factors, potentially leading to uncertain outcomes, both morphological and molecular (genetic) approaches are viable for characterization and identification. Given this context, this study places particular emphasis on the molecular analysis of six native garlic cvs. The ISSR method is employed to assess their resemblance or diversity.

Materials and Method

Source Material Acquisition

Six distinct indigenous varieties of garlic (*Allium sativum* L.) were gathered from various regions across Pakistan. These include Hazro from Attock, Chinese Cultivar from Lahore, Desi from Kasur, Pink local from KPK, Silver skin from Sindh, and White from Balochistan (see Figure 1).

Fig. 1: Six garlic cvs collected from different provinces of Pakistan.
A: Hazro; B: Chinese; C: Desi; D: Pink Local; E: Silver Skin; F: White

Genetic Diversity Analysis using ISSR Markers

Primer Selection: Six synthetic oligonucleotides (primers) were chosen for the microsatellite DNA fingerprinting. These primers were designed to be complementary to repetitive sequences found within the garlic genome. The selected primers were as follows:

1. $(GACA)_4$
2. $(GTG)_5$
3. $GATA)_4$
4. $(CA)_8$
5. $(GGAT)_4$
6. $(CAA)_5$

DNA Extraction: DNA was extracted from the leaves of all garlic cultivars using the Favorprep plant genomic DNA Extraction Kit provided by Favorgen Biotech Corp. USA.

Gel Electrophoresis: The isolated DNA from each variety was subjected to gel electrophoresis using 1% agarose gel and TBE buffer. This process was employed to verify the presence of DNA in each sample.

ISSR Procedure: The selected primers were used to amplify the DNA samples of garlic cultivars, aiming to estimate the level of polymorphism among the different varieties. These primers were obtained from the Microbiology & Molecular Genetics Department at the University of the Punjab, Lahore.

This comprehensive procedure was employed to assess the genetic diversity among the six studied garlic cultivars.

Table 1: Name and sequence of the primers used in ISSR analysis

Sr. No.	Sequence
1.	(GACA) 4: GACA GACAGACAGACA
2.	(GTG) 5: GTG GTGGTGGTGGTG
3.	(GATA) 4: GATA GATAGATAGATA
4.	(CA) 8: CA CACACACACACACA
5.	(GGAT) 4: GGAT GGATGGATGGAT
6.	(CAA) 5: CAACAACAACAACAA

Molecular Fingerprinting using ISSR method:

The ISSR procedure was carried out following the protocol outlined by Sharma *et al.*, (1995). This particular analysis relies on PCR techniques, and for each reaction, a total of 25 µl of mixture was prepared, comprising of:

1. **PCR Amplification**: DNA samples from different cultivars were underwent to polymerase chain reaction (PCR) for amplification of specific regions of interest.
2. **Agarose Gel Preparation.** A 1% agarose gel was created by blending agarose powder with a buffer solution and heating it until complete dissolution. The resulting molten agarose solution was subsequently poured into a gel mold, and a comb was introduced to form wells for loading the DNA samples.
3. **DNA Loading**: The PCR products, along with a DNA marker (1 kb ladder), were loaded into the wells created in the agarose gel. The DNA marker contains known DNA fragments of different

sizes, which serve as a reference for estimating the size of the amplified products.

4. **Electrophoresis**: The gel was subjected to an electric current. Due to its negative charge, DNA migrated towards the positive electrode. Smaller DNA fragments traversed the gel matrix at a faster rate compared to larger ones, leading to their separation based on size. After electrophoresis, the gel was stained with a fluorescent dye like Ethidium Bromide. This dye intercalates with DNA, making the bands visible under UV light.
5. **Visualization**: The gel was placed under a UV trans-illuminator, which emits ultraviolet light. The Ethidium Bromide-stained DNA bands fluoresce under UV light, allowing them to be visualized.
6. **Data Recording**: The gel documentation system was used to capture an image of the gel with the UV-induced fluorescence. The presence or absence of bands for each cultivar was recorded. Typically, a band is recorded as "1" if it's present and "0" if it's absent.

Cluster Analysis:

This analysis was conducted to understand the genetic relationships and polymorphism among the tested garlic cultivars. **MINITAB 19 software** used to construct dendrograms. The dendrograms provided a visual representation of how genetically similar or dissimilar the garlic cultivars are.

Calculation of Shannon Indices:

Shannon indices provide a measure of genetic diversity within a population. They take into account both the number of different alleles and their frequencies**. POPGENE 32 software** was used to calculate the Shannon indices. The Shannon indices provided information about the genetic diversity within the tested garlic cultivars.

Statistical Analysis:

This analysis was performed to draw meaningful conclusions from the data and assess the significance of any observed differences or relationships. **SPSS release 16.0.0 software** was used to conduct the **Analysis of Variance (ANOVA**statistical analysis**.** The ANOVA results indicated if there were statistically significant differences between the groups.

Computation of Standard Errors:

Standard errors of mean values were calculated to provide an estimate of how much the sample mean vary from the true population mean. This was likely done using the statistical software used for the ANOVA, which in this case is SPSS release 16.0.0. Standard errors help in understanding the precision of the estimated means.

Results

The genetic assortment of six garlic cultivars was assessed using ISSR markers. Here are the findings for each primer used:

Primer (GATA)4:

PCR Results:

All cultivars displayed bands in the gel documentation system.

The amplified DNA motifs ranged from 400 to 700 base pairs (bps) in size.

Dendrogram Analysis:

Pink Local and White cultivars showed 0% variation, indicating that they are genetically identical or very closely related.

The Chinese cultivar exhibited 79.14% similarity with Pink Local and White. This suggests that the Chinese cultivar shares a significant portion of its

genetic makeup with Pink Local and White, but there are also differences.

Silver Skin showed 52.77% similarity with Chinese, Pink Local, and White cultivars. This indicates that Silver Skin shares genetic similarities with these cultivars, but there are also notable differences.

Hazro cultivar showed 26.39% similarity with Silver Skin, Chinese, Pink Local, and White cultivars. This suggests that Hazro has a lower genetic similarity with the other cultivars, indicating greater genetic divergence

Primer (CAA)5:

PCR Results:

The band sizes ranged from 340 base pairs (bp) to 820 bp, indicating a range of genetic diversity among the tested garlic cultivars.

All cultivars showed bands with the specific primer used, indicating successful amplification of the target DNA regions in all cultivars.

Cluster Analysis Results:

The cluster analysis revealed varying degrees of similarity among the different garlic cultivars.

For example, Silver Skin showed 82.74% similarity with the White cultivar. This suggests that Silver Skin shares a high proportion of its genetic makeup with the White cultivar.

Desi exhibited 64.49% similarity with Silver Skin and White. This indicates that Desi shares a significant portion of its genetic material with Silver Skin and White, but there are also genetic differences.

Hazro showed 43.67% similarity with Desi, Silver Skin, and White cultivars. This suggests that Hazro is genetically less similar to these cultivars compared to the similarity observed between Silver Skin, Desi, and White.

Primer (GTG)5:

PCR Results:

Banding patterns were observed in five cultivars, with the amplified DNA fragments ranging from 390 to 800 base pairs (bps). This indicates genetic diversity among the tested cultivars.

Notably, Silver Skin exhibited a complete absence of the observed motifs, suggesting that it may lack the specific DNA regions targeted by the primer used in this analysis.

Cluster Analysis Results:

Hazro and Chinese cultivars showed 61.72% similarity and were grouped together in cluster 1. This indicates a relatively high level of genetic similarity between Hazro and Chinese cultivars in the specific DNA regions analyzed.

Pink Local and White cultivars showed 33.99% similarity and were grouped together in cluster 2. This suggests that Pink Local and White cultivars have a less genetic similarity in the analyzed regions compared to Hazro and Chinese.

No similarity was recorded between clusters 1 and 2. This means that the genetic characteristics analyzed in cluster 1 are distinct from those in cluster 2, further highlighting the genetic diversity among the cultivars.

Primer (GGAT)4:

PCR Results:

The bands ranged in size from 380 to 750 base pairs (bps).

All six cultivars displayed banding patterns, indicating genetic diversity among the tested cultivars.

Cluster Analysis Results:

Chinese and Pink Local cultivars showed 100% similarity and were grouped together in cluster 1. This indicates that these two cultivars share

identical genetic characteristics in the specific DNA regions analyzed.

Hazro showed 76.56% similarity with Chinese and Pink Local in cluster 1. While Hazro shares a high proportion of its genetic material with Chinese and Pink Local, there are some genetic differences as well.

White cultivar exhibited 53.11% similarity with Hazro, Chinese, and Pink Local in cluster 3. This suggests that White shares genetic similarities with these cultivars, but there are also notable genetic differences.

Desi and Silver Skin showed 17.24% similarity and were placed in separate cluster 4. This indicates that Desi and Silver Skin have a relatively low level of genetic similarity in the analyzed regions.

Primer (GACA)4:

PCR Results:

The White cultivar did not show any amplified motifs with the specific primer used. This suggests that the White cultivar may not possess the specific DNA regions targeted by this primer.

The amplified bands ranged in size from 500 to 860 base pairs (bps) among the other cultivars.

Cluster Analysis Results:

In Cluster 1, Hazro and Silver Skin exhibited 100% similarity. This indicates that Hazro and Silver Skin share identical genetic characteristics in the specific DNA regions analyzed.

In Cluster 2, Chinese and Desi showed 80% similarity. This means that Chinese and Desi share a high proportion of their genetic material in the analyzed regions, but there are also some genetic differences.

Both Clusters 1 and 2 showed 70% similarity with each other in Cluster 3. This suggests that Clusters 1 and 2 have a significant portion of genetic material in common.

In turn, Cluster 3 showed 25% similarity with Pink Local. This indicates that Cluster 3 and Pink Local have a lower level of genetic similarity in the analyzed regions.

A total of 97 bands were amplified by five different primers. The bands number ranged from 16 to 20 per primer. On average, each cultivar showed 3 bands. The similarity indices among all cultivars ranged from 0% to 100%. This indicates an extensive range of genetic heterogeneity among the tested cultivars. This analysis helps to quantify the genetic relationships among the different cultivars based on the ISSR markers used. White cultivar did not show any banding pattern with the ISSR marker (GACA)4. This suggests that the White cultivar lacks the specific DNA regions targeted by this particular primer. Silver Skin exhibited no bands with the ISSR marker (GTG)5. This indicates that Silver Skin also lacks the specific DNA regions targeted by this primer.

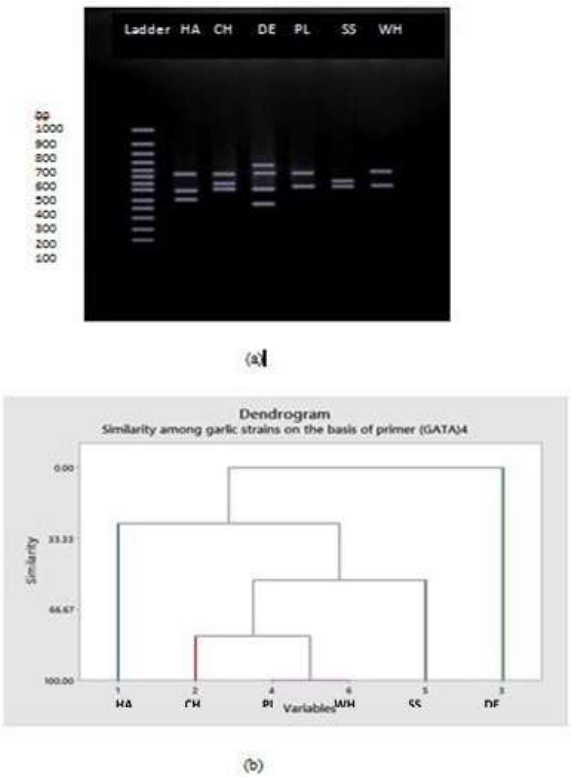

Figure1: a) gel electrophoresis of PCR products using (GATA) as primer b) Dendrogram constructed using binary system in minitab 19 software HA (Hazro), CH (Chinese), PL (Pink local), SS (Silver skin, WH (white)

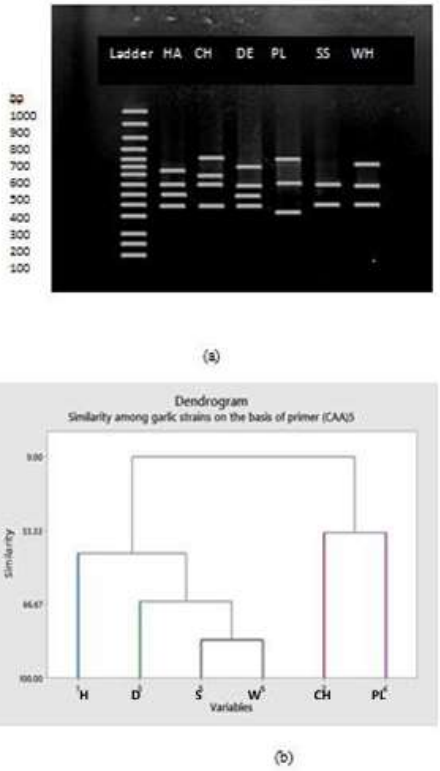

Figure 2: a) gel electrophoresis of PCR products using (CAA) as primer b) Dendrogram constructed using binary system in minitab 19 software HA (Hazro), CH (Chinese), PL (Pink local), SS (Silver skin, WH (white)

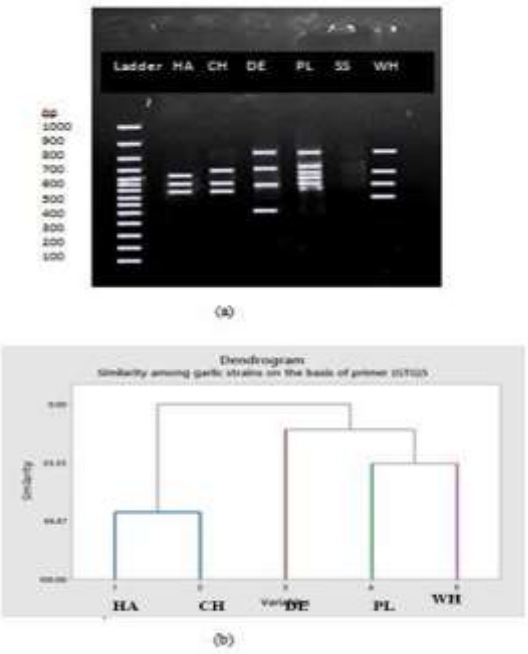

Figure3: a) gel electrophoresis of PCR products using (GTG) as primer b) Dendrogram constructed using binary system in minitab 19 software HA (Hazro), CH (Chinese), PL (Pink local), SS (Silver skin, WH (white)

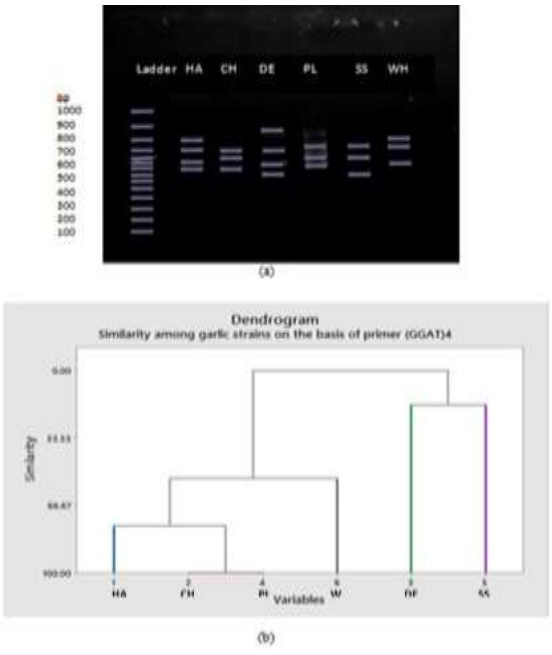

Figure4: a) gel electrophoresis of PCR products using (GGAT) as primer b) Dendrogram constructed using binary system in minitab 19 software HA (Hazro), CH (Chinese), PL (Pink local), SS (Silver skin, WH (white)

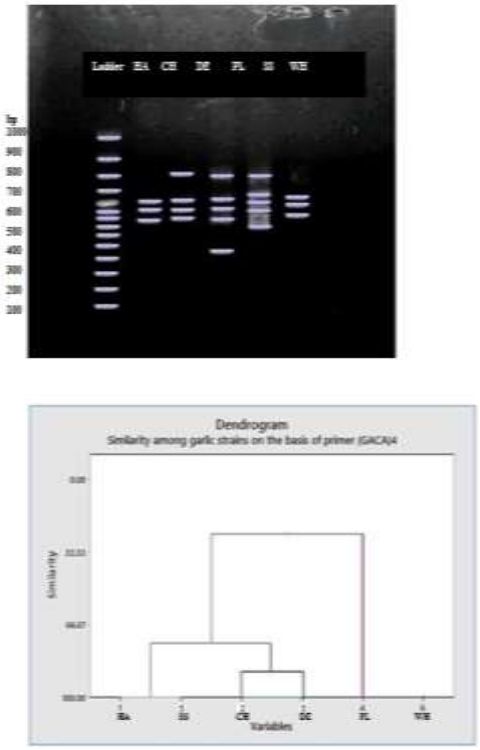

Figure5: a) gel electrophoresis of PCR products using (GACA) as primer b) Dendrogram constructed using binary system in minitab 19 software HA (Hazro), CH (Chinese), PL (Pink local), SS (Silver skin), WH (white)

Polymorphic content

The study evaluated the polymorphic content of six ISSR primers in six different garlic cultivars. The primer (GATA)4 produced PCR products ranging from 400 to 700 base pairs (bp), amplifying a total of six loci, of which five were found to be polymorphic, resulting in a polymorphism rate of 85.7%. The Shannon index for this primer was calculated at 1.908. Similarly, the primer $(CAA)_4$ exhibited a comparable polymorphism rate of 85.7%, amplifying six loci with five of them being polymorphic. The range of PCR product sizes for this primer was 340 to 820 bp, and its Shannon index was 2.041. Primer (GTG)5 showed a slightly higher polymorphism rate at 87.5%, with eight loci amplified, seven of which were found to be polymorphic. However, its Shannon index was relatively lower at 1.598, primarily due to the absence of bands in one garlic cultivar (Silver Skin). On the other hand, primer (GGAT)4 demonstrated the highest polymorphism rate at 100%, with all eight amplified loci being polymorphic. The length of PCR products ranged from 380 to 750 bp, and Shannon index was recorded at 2.173. Lastly, primer (GACA)4 exhibited a polymorphism rate of 88.8%, amplifying nine loci, eight of which were polymorphic. The size range of PCR products for this primer was from 500 to 860 bp, and its Shannon index was 1.744. It's worth noting that the garlic cultivar White did not exhibit amplification with this particular primer. These findings provide valuable insights into the genetic diversity and polymorphism within the six garlic cultivars under investigation, as assessed through the application of the six ISSR primers.

Table 2. Polymorphic products of six garlic cultivars amplified by using six ISSR primers

Primer	Sequence	Tm (°C) *	Size Range (bp)	TAL **	PL ***	Shannon Index	Polymorphism (%)
I.	$(GATA)_4$	35.0	400-700	6	5	1.908 ± 0.288	85.7
II.	$(CAA)_4$	34.0	340-820	6	5	2.041± 0.243	85.7
III.	$(GTG)_5$	42.0	390–800	8	7	1.598 ± 0.202	87.5
IV.	$(GGAT)_4$	48.0	380-750	8	8	2.173 ± 0.254	100
V.	$(GACA)_4$	48.0	500-860	9	8	1.744 ± 0.277	88.8
VI.	$(CA)_8$	42.4	-	-	-	-	-
6	–	–	340-860	38/7.6	33/6.8	1.892 ± 0.247	89.06

*Tm= temperature
*TAL= Number of Total amplified loci
**PL= Polymorphic loci

Discussion

In the realm of garlic classification, conventional methods rely on observable agricultural traits or phenotypes. However, this approach can lead to the occurrence of both homonyms (different cultivars sharing the same name) and synonyms (identical genetic material bearing different cultivar names). This issue is particularly pronounced in species like garlic, which exhibit similar outward appearances and considerable phenotypic adaptability. Consequently, it becomes crucial to accurately identify and distinguish between different cultivars to effectively manage and preserve these genetic resources (Govindaraj *et al*., 2015).

In this study, six primers were employed as markers, yielding bands ranging from 300 to 1000 base pairs in size. Each primer produced between 16 to 20 bands on average, with approximately 3 bands detected per cultivar. While all garlic cultivars exhibited results with five out of six primers, none displayed any banding pattern with the (CA)8 primer.

In total, 38 loci were identified in this study, of which 33 demonstrated polymorphism. This amounted to an 89% polymorphism rate among the six garlic cultivars examined, which were amplified by five of the primers. Notably, none of the garlic cultivars displayed amplified products with the (CA)8 primer. Among the six primers used, the (GGAT)4 primer produced the highest Shannon index reading.

The plant genome predominantly features dinucleotide repeats in microsatellites, with mono, tri, and tetra nucleotide repeats being less commonly found (Wang *et al*., 2016). Anwar *et al*. (2016) tested 21 garlic varieties for genetic diversity assessment by utilizing ISSR and SSR

techniques. Three ISSR primers displayed complete polymorphism, while 16 SSR primers exhibited polymorphism ranging from 33% to 100%. Bradley *et al*. (2001) investigated genetic resemblence among 20 Australian garlic varieties through DNA fingerprinting, revealing a range of similarity from 58% to 97%, indicating notable genetic variation.

In a study by Paredes *et al*. (2008), 60 garlic clones were analyzed using 40 RAPD markers, revealing 87% polymorphism and a 94% similarity index among the garlic clones. Jabbes *et al*. (2011) calculated Nei's coefficient of genetic diversity for thirty Tunisian and French garlic accessions, obtaining values of 0.45 and 0.34, respectively. They used seven ISSR primers, generating 73 bands and highlighting a high level of diversity, particularly among the Tunisian accessions, ranging from 38% to 78%.

These findings align with previous studies on garlic utilizing various molecular markers (Ipek *et al*., 2003, 2005, 2008; Lampasona *et al*., 2003; Volk *et al*., 2004), affirming the substantial diversity present among garlic accessions. While traditional morphological characteristics have been employed for diversity assessment, their reliability is constrained by environmental influences, mutations, and phenotypic adaptability. Thus, the use of DNA polymorphism markers through diverse techniques such as RAPD, AFLP, SSR, and ISSR proves to yield more dependable results (Bachmann *et al*., 2001).

The presence of a substantial number of polymorphic ISSR markers highlights the genetically diverse nature of the garlic genetic material and underscores the effectiveness of the ISSR technology in identifying variations in both sexually and vegetatively propagated varieties. The multiplicity observed within garlic populations may also be caused by the mutations with the duration of time, likely due to the selection pressure of grower. (Simon and Jenderek, 2003).

Upon conducting DNA fingerprinting of six garlic cultivars, it was evident that all six were genetically distinct from each other. While cultivars Desi, Hazro, and Pink Local may share similarities in morphology, such as color and clove size, this study conclusively demonstrates their genetic differentiation.

Conclusion

The traditional methods employed for characterizing garlic cultivars have proven to be unreliable. Molecular techniques, such as ISSR, offer a more accurate and dependable means of assessing diversity at the molecular level. In present study, different garlic cultivars were distinguishable based upon the banding pattern produced by standard amplification conditions which yielded numerous polymorphic bands. Specifically, the primer sequence (GGAT)4 proved to be the most effective for amplifying DNA samples across all cultivars, producing the highest number of bands and facilitating more robust comparisons.

The results clearly indicated that while certain cultivars exhibited similarities to one another to some extent, none of them shared 100% genetic similarity. This underscores the fact that they are indeed distinct cultivars, each possessing a unique genetic makeup.

DNA fingerprint databases serve as vital and indispensable tools in plant molecular research. They offer robust technical support and valuable information for endeavors such as crop breeding, quality control of varieties, protection of variety rights, and the application of molecular markers in breeding practices.

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