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MICROSATELLITE-SIMPLE SEQUENCE REPEAT MARKERS BASED GENETIC DIVERSITY ANALYSIS OF CHICKPEA GENOTYPES (CICER ARIETINUM L.)

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ABSTRACT
The aim of this study was to identification of genetic diversity among germplasm accessions and breeding lines is essential for proper conservation and breeding for selected eight chickpea genotypes. Five published chickpea STMS marker sets in this study, using five genomic microsatellite (SSR) primers (TA2, TA59, TA71, TA96 and TA194). All five loci were found to have a different number of alleles, ranging from 2 for the locus TA96 to 5 for the locus TA71, with an average of 3.6 alleles per locus. PIC ranged from 0.359 in TA96 to 0.667 in TA2 with a mean PIC of 0.543 and a mean expected heterozygosity (gene diversity) of 0.648. The markers with the highest heterozygosity (0.875) and most discriminatory markers were primers TA71 and TA194. The eight genotypes were divided into three major clusters by Jaccard similarity coefficients and UPGMA cluster analysis with genotypes S4 and S8 being the most similar (Jaccard distance = 0.300) and S1–S7 being another close cluster (Jaccard distance = 0.111). The selected SSR markers, especially TA71 and TA194 were found to be informative and can be used for fingerprinting and diversity assessment of chickpea and can be used as reference for introgression of parents in future breeding programs.

KEYWORDS: Chickpea; SSR markers; polymorphism information content (PIC); genetic diversity

1. INTRODUCTION

Chickpea (Cicer arietinum L.) is the third most important pulse crop in the world, and an important source of dietary protein for resource-poor populations in the semi-arid tropics of South Asia, sub-Saharan Africa and Mediterranean basin. Chickpea is not only nutritious, but also has an important agronomic function in cereal cropping systems, as it enriches the soil with nitrogen and eliminates the need for chemical fertilizers. Despite this importance, productivity of chickpea is affected by a narrow genetic base due to domestication bottlenecks, self-pollination and limited number of elite parental lines used in breeding programs. This restricted diversity restricts the option to create cultivars with enhanced resistance to biotic and abiotic stresses, with higher yield potential and with better adaptability to changing climates (Arriagada et al. 2022; Singh et al. 2022).

Promises of sustainable improvement for chickpea rely on two important factors which are deeply intertwined: the availability of genetically diverse parental material and access to molecular tools that can accurately quantify and map the level and distribution of genetic variation within breeding lines and germplasm accessions. The assessment of diversity based on morphological and agronomic features is often hampered by environmental effect, low level of polymorphism and delayed expression of characters, and is therefore not sufficient for precise characterization. Molecular markers, which identify DNA level variation, have many advantages over these limitations because they are neither influenced by the environment, developmental stage, or tissue type (Zhang et al. 2025).

Simple sequence repeats (SSRs) or microsatellites are some of the most popular types of molecular markers that are used for genetic diversity analysis. They are popular because they possess a number of desirable properties: they are co-dominant, can be distinguished from homozygotes; they are highly polymorphic because there is variation in the number of repeat units; they are locus-specific and are easily mapped to defined genomic positions; and they can be easily and reproducibly amplified in PCR using simple, inexpensive protocols. The features of SSRs make them especially attractive for use in cultivar identification and genetic fingerprinting, linkage mapping, QTL (quantitative trait loci) mapping and marker-assisted selection (Muneer 2014; Park et al. 2009).

Chickpea-specific sequence-tagged microsatellite site (STMS)/SSR markers were developed from genomic libraries enriched with (TAA)n, (GA)n and (GT)n repeat motifs of which the TA series of primers were used in the present study. These markers have been extensively validated and used in various germplasm diversity studies, linkage map construction, identification of markers linked to genes for disease resistance and drought tolerance, and marker-assisted backcrossing programs on chickpea since their development. The repeatability of their behavior when analyzed across various genetic backgrounds and their public availability have made the TA-series markers a regular tool in chickpea molecular research. Even though these markers have been applied to a large number of genotypes, continued application to new sets of germplasm is worthwhile because it can confirm the performance of the markers in a particular germplasm set, and provide diversity information directly relevant to current breeding projects. The standard diversity parameters are used to estimate

the number of alleles per locus, observed and expected heterozygosity and polymorphism information content (PIC) which gives a quantitative measure of the informativeness of each locus and of the overall genetic relatedness of the genotypes under investigation. PIC values are especially useful for ranking markers for discriminatory power and values exceeding 0.5 are known to be very informative in diversity studies (Collard and Mackill 2008; Su et al. 2025).

Hence, the present study was carried out to characterize a set of eight chickpea genotypes with the help of five TA-series SSR primers with three complementary aims. The selected primers were used to amplify the genotypes to produce consistent allelic profiles for each locus. Secondly, the resulting banding patterns were scored to determine allelic variation, and standard diversity parameters (number of alleles per locus, observed and expected heterozygosity and polymorphism information content (PIC)) were calculated to assess the discriminatory power and informativeness of each marker. Third, the combined SSR profile of the genotypes was clustered using cluster analysis to visualize and interpret the genetic relationships among the different genotypes. Overall, these three objectives were aimed to produce a combined assessment of marker informativeness and genotype relatedness, and thus to assess the usefulness of the TA-series SSR markers for the characterization of chickpea germplasm.

2. MATERIALS AND METHODS

2.1 Plant material

For the present study, eight genotypes of chickpea (Cicer arietinum L.), 15 chickpea varieties were planted at the agro-experimental farm, Rama University, Kanpur (India) and eight varieties were selected for detailed evaluation. Four standard centers namely IIPR Kanpur, IARI New Delhi, Durgapur (Rajasthan) and Madhya Pradesh were the points from where the cultivars were procured in 2021 cropping season were utilized. Young, healthy trifoliate leaves from 2-3 weeks old seedlings were harvested and genomic DNA was extracted either fresh or stored at –80 °C.

2.2 Genomic DNA extraction

The total genomic DNA was extracted from young leaf tissue according to the standard CTAB method, (Agbagwa et al. 2012) with some modifications for chickpea. The quality and quantity of DNA was determined on 0.8% agarose gel and spectrophotometry (A260/A280) and working stocks were diluted to ~25-50 ng/µL for PCR.

2.3 SSR primers and PCR amplification

Five genomic SSR primer pairs (TA2, TA59, TA71, TA96 and TA194), originally developed from chickpea genomic microsatellite libraries were used for amplification (Table-1).

The PCR reactions consisted of about 25 to 50 ng of genomic DNA, 1× PCR buffer, 2.0-2.5 mM MgCl2, 0.2 mM dNTPs, 0.3 µM of each forward and reverse primer, and 0.5-1.0 U Taq DNA polymerase. Amplification was carried out in a thermocycler with a denaturation step at 94 °C for 4–5 min, followed by 30–35 cycles of denaturation at 94 °C for 30–45 s, annealing at 50–55 °C (depending on the primer) for 30–45 s and extension at 72 °C for 45–60 s, with a final extension at 72 °C for 7–10 min. Amplified products were separated on 2–3% agarose gels with 1× TAE/TBE buffer, 100 bp DNA ladder, stained with a nucleic-acid fluorescent stain, separated with constant voltage and documented under UV/blue light gel imaging.

2.4 Allele scoring and Statistics analysis

Band sizes were determined according to the size range of the primer (Table 1) and bands that were clearly resolved were scored as present (1) or absent (0) in each of the eight genotypes giving a locus-wise genotype/allele pair for each sample. Allele frequency values were used to compute the number of alleles (Na), observed heterozygosity (Ho, the percentage of individuals with two different sized alleles) and Nei's (1973) unbiased gene diversity / expected heterozygosity (He) for each locus. The polymorphism information content (PIC) of each locus was calculated following Botstein et al.

$$PIC = 1 - \Sigma pi^2 - \Sigma_{i<j} 2pi^2pj^2$$

where pi and pj are the frequency of the i-th and j-th alleles at a locus, respectively. Other summary measures for marker efficiency included the marker index (MI = PIC × number of polymorphic alleles; Powell et al., 1996) and total resolving power (Rp = ΣIb, band informativeness; Prevost and Wilkinson, 1999). A matrix of pairwise genetic (dis)similarity between the eight genotypes was developed based on the Jaccard coefficient computed from the combined binary allele matrix of the five loci, and the genotypes were grouped using the unweighted pair-group method with arithmetic mean (UPGMA; Sneath and Sokal, 1973). IBM SPSS Statistics, version 24.0 (IBM Corp., Armonk, NY, USA) was used for statistical computation and cluster analyses. The binary (1/0) allele matrix was analysed in SPSS using Analyze → Classify → Hierarchical Cluster; the matrix was clustered by Jaccard's Coefficient (Similarity) using the Between-groups Linkage (UPGMA) method. The agglomeration schedule and dendrogram were exported from SPSS output (Figure 7). The frequencies and means were calculated for the allele scores and cross checked in SPSS Descriptives/Frequencies.

3. RESULTS AND DISCUSSION

3.1 Amplification and polymorphism at individual SSR loci

In the eight chickpea genotypes (Table 1) all five SSR primers amplified clear scorable fragments in expected size ranges in all the samples (Figures 1–5). There were 18 alleles at five loci, ranging from 2 alleles at TA96 to 5 alleles at TA71 with the average of 3.6 alleles per locus (Table 2). Observed heterozygosity (Ho) ranged from 0.250 (TA2, TA59) to 0.875 (TA71, TA194), and Nei's gene diversity (He) ranged from 0.500 (TA96) to 0.767 (TA2), with locus means of Ho = 0.600 and He = 0.648.

PIC values ranged from 0.359 (TA96) to 0.667 (TA2) with the mean value of 0.543 among the five loci (Table 2). The markers TA2, TA59, TA71 and TA194 (PIC > 0.5) were very informative, the marker TA96 (PIC = 0.359) was moderately informative. The index values of the markers showed that the most effective ones were TA71 (2.930) and TA2 (2.670), and the total resolving power (Rp) of the whole panel of five primer was 8.00, which is good. The present study found a

PIC value of average 0. 543 which is moderate to high. Previous studies have reported that (TAA)n-enriched genomic library-derived SSR markers have high PIC values in chickpea. This confirms the well-known fact that TA-series markers are particularly suitable for resolving intraspecific diversity in this crop due to their creation from AT-rich microsatellite-enriched libraries, a very important trait as the crop already has a narrow genetic base due to evolution and domestication bottleneck (Hajibarat et al. 2015; Cuc et al. 2008).

Four of the five markers assessed in this study (TA2, TA59, TA71 and TA194) had PIC values greater than 0.5, the value that is typically considered the lower limit for a marker to be "highly informative" for germplasm characterization and marker-assisted selection (MAS). The consistency of these markers in respect to this benchmark implies that they explain a significant amount of allelic variation segregating in the genotypes sampled and thus should continue to be employed in chickpea genotyping panels. It is also important to recognize, however, that the PIC values are not only a function of the extent of mutability at a specific locus, but also of the allele frequency distribution of the particular sample of chickpeas being analyzed; thus the PIC values are useful for describing the discriminatory power of a particular genotype set, but should be viewed as suggestive rather than definitive measures of the universal discriminatory power of a marker across the entire chickpea gene pool (Yadav et al. 2022; Das and Rao 2015).

3.2 Primer-wise banding profiles

TA2 primer profile: SSR-PCR amplification profile of chickpea genotypes S1–S8 using primer TA2. Lane M: 100 bp DNA ladder. Bands were resolved mainly within the 180–260 bp expected range figure-1.

Primer TA2 amplified in all eight genotypes; most lanes carried a single allele, while genotypes S3 and S4 carried an additional lower-molecular-weight allele, giving Ho = 0.250 and PIC = 0.667 — the highest PIC value recorded among the five loci.

TA59 primer profile: Figure-2 SSR-PCR amplification profile using primer TA59. Amplified products were observed around the expected 150–300 bp region.

Primer TA59 showed variable banding, with a common lower allele present in most genotypes and additional upper alleles in S3, S4, S5, S7 and S8, giving four alleles overall (PIC = 0.605).

TA71 primer profile: Figure-3 SSR-PCR amplification profile using primer TA71. Multiple bands were observed within the expected microsatellite size range.

TA71 was the most allele-rich locus (Na = 5), combining a common lower allele shared by all genotypes with a highly variable upper allele; this yielded the highest observed heterozygosity (Ho = 0.875) and the largest marker index (MI = 2.930) of the panel.

TA96 primer profile: Figure-4 SSR-PCR amplification profile using primer TA96. Bands were observed mainly near 200 bp and 400 bp. TA96 produced a conserved lower band in all genotypes and a second, upper band in six of the eight genotypes (absent in S2 and S6), making it the least polymorphic locus examined (PIC = 0.359).

TA194 primer profile: Figure 5. SSR-PCR amplification profile using primer TA194. Banding was observed within the expected 150–350 bp range. TA194 amplified a common lower allele in all genotypes together with two alternative upper alleles distributed differentially among genotypes (Na = 3, Ho = 0.875, PIC = 0.496), making it, with TA71, one of the two most heterozygosity-rich loci. The one exception (TA96) had relatively low polymorphism, which is in line with the fewest number of alleles detected. This is not surprising, as repeat length, motif composition and the number of repeat units are important factors that influence microsatellite mutability, with short and/or more interrupted repeat arrays leading to more stable microsatellites and less microsatellite polymorphisms figure-6. It is not a surprising marker, however, but a valuable one because of its high conservation and consistent amplification; it could be used as an anchor or quality control marker in multiplex panels where consistent and unambiguous amplification is the most important. It represents a general principle of SSR marker selection: that an effective genotyping panel will contain a combination of very polymorphic and very conserved markers, depending on the goals of the project (whether to resolve fine scale diversity, or to provide strong, reproducible fingerprinting).(Cosson et al. 2014).

The markers that were the most informative overall were TA71 and TA194 that had the highest number of alleles and the highest observed heterozygosity. These features make them very useful in large-scale fingerprinting and diversity panel applications for the maximum chance of distinguishing closely related genotypes and detecting rare or even unique alleles which could have breeding value. Their high level of heterozygosity also suggests that they may be in regions of the genome which are not as strongly constrained as other regions and that they may be longer (and therefore more mutable) repeat tracts, both of which would be consistent with their superior discriminatory performance (Merritt et al. 2015; Muneer 2014). Summary of genetic diversity statistics in table-2 and Marker index (MI) of the five SSR loci, calculated as PIC × number of polymorphic alleles represented in table-3.

3.4 Genetic similarity and cluster analysis

Pairwise Jaccard similarity coefficients calculated from the combined five-locus allele matrix ranged from 0.25 (S2–S4) to 0.889 (S1–S7), indicating a moderate overall level of genetic similarity among the eight genotypes (Table 4). The one exception (TA96) had relatively low polymorphism, which is in line with the fewest number of alleles detected. This is not surprising, as repeat length, motif composition and the number of repeat units are important factors that influence microsatellite mutability, with short and/or more interrupted repeat arrays leading to more stable microsatellites and less microsatellite polymorphisms. It is not a surprising marker, however, but a valuable one because of its high conservation and consistent amplification; it could be used as an anchor or quality control marker in multiplex panels where consistent and unambiguous amplification is the most important. It represents a general principle of SSR marker selection: that an effective genotyping panel will contain a combination of very polymorphic and very conserved markers, depending on the goals of the project (whether to resolve fine scale diversity, or to provide strong, reproducible fingerprinting).

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A cluster analysis of the eight genotypes using UPGMA method and distance matrix based on Jaccard similarity coefficient (Figure 7) classified them into three main groups. The closest pair of genotypes was S4/S8 (Jaccard distance = 0.300); S1/S7 was a second tight group, while S6 and the more distant S2, S3 and S5 were a third group. This grouping broadly agrees with the average linkage cladogram derived directly from the combined banding profile (Figure 8, taken from the original band-scoring record) which also grouped S4 close to S8 and S3/S5 as a related pair, indicating the reliability of the clustering based on the SSRs.

In contrast, the relative lack of allelic richness of TA96 has not abated its usefulness, rather it has served as a reminder that marker usefulness depends on the application. TA71 and TA194 are recommended for studies that are primarily concerned with depth of resolution (parentage analysis, cultivar identification, detection of subtle population structure, etc.). A conserved, high-fidelity marker like TA96 continues to have great use for applications where amplification reliability is paramount in a wide range of potentially poor-quality DNA samples, e.g., large-scale seed-lot verification or quality control in the gene bank. This separation emphasizes an often-understated part of designing a marker panel: Informativeness and robustness do not always go hand in hand; a good marker panel should be designed to serve both purposes (Amiteye 2021; Rosenberg et al. 2000).

The results of cluster analysis indicated that the eight genotypes were divided into three major clusters, where the pair S4/S8 were closest. Applied breeding consequences of this kind of relatedness are obvious: crosses of genetically similar genotypes (e.g., S4 and S8) can be expected to produce progeny with a relatively restricted recombinant genetic basis and limited opportunities for transgressive segregation. Breeders who want to maximize heterosis and increase the genetic variability available for selection in segregating populations, on the other hand, would benefit from using genotypes from genetically distant clusters. Appropriate exploitation of this diversity information can thus be used to optimize crossing schemes, minimize redundancies in breeding programs and make the best use of available genetic variation (Mackay et al. 2021).

The overall relation between the molecular clustering by Jaccard/UPGMA and the morphological banding-pattern cladogram (Figures 7 and 8, respectively) is promising, and suggests that the molecular markers used are reflecting genetic relationships more or less in agreement with the phenotypically observable variation. This agreement provides some confidence that the SSR based clustering reflects real underlying genetic structure and is not a sampling artifact or the result of marker choice. This concordance should therefore be read with due caution because of the small number of genetic markers and the small number of genetic genotypes used, statistical power to resolve fine-scale genetic relationships is inherently limited and the apparent agreement of the two clustering approaches is reassuring but cannot be used as definitive proof of the robustness or reproducibility of the genetic signal in a larger and more diverse germplasm collection. Bootstrap support values and other resampling based measures of stability of the observed groupings would also be calculated with a larger sample size, giving much greater confidence in the groupings (Elameen et al. 2011; Zhang et al. 2025).

This study presents some initial evidence for significant genetic variation within chickpea genotypes studied, but there are a number of limitations that should be recognized. First, the small number of genotypes in this study (n = 8) limits the generality of the clustering pattern and estimates of the allele frequencies to the entire chickpea gene pool that includes much higher geographic, agro-climatic and genetic diversity. Second, only five SSR markers were tested to demonstrate polymorphism at TA-series loci, which was adequate to prove the concept, but very few for a complete diversity assessment or for the development of a reliable matrix of genetic distance for cultivar protection and MAS. The estimated values of PIC, heterozygosity, and genetic distance based on such small number of markers are subject to greater sampling variance and may not be representative of the parameters of the entire population (Annicchiarico et al. 2018; Yadav et al. 2022).

Based on these constraints, the set of markers identified here is recommended for validation across a larger and more geographically diverse germplasm panel of chickpea, with a particular emphasis on landraces, wild relatives, elite germplasm breeding lines from various regions to reflect the entire genetic diversity of cultivated and wild chickpea. Adding other markers (both TA-series and non-TA-series) across the genome of chickpea, also known as gram, would provide better genome-wide coverage and minimize the possibility of clustering due to a few potentially non-representative markers. In addition to SSR-based analysis, higher density markers, such as SNP array or genotyping-by-sequencing, would provide further resolution and provide a means to validate the diversity patterns observed in this study, when possible (Dwivedi et al. 2017; Varshney et al. 2019).

4. CONCLUSION

A total of 18 alleles were amplified with a mean PIC of 0.543 using five SSR markers of chickpea (TA2, TA59, TA71, TA96 and TA194) across eight chickpea genotypes. TA71 and TA194 were the most informative and discriminatory markers and the cluster analysis resolved the genotypes into three genetically distinct groups, the most similar pairs being

S4/S8 and S1/S7. The results have shown that the TA-series SSR markers could be useful in the evaluation of genetic variations, germplasm fingerprinting and parental selection for chickpea improvement programs.

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Table 1. Microsatellite (SSR) primers used for PCR amplification in eight chickpea genotypes.

Primer	Forward primer (5'→3')	Reverse primer (5'→3')	Repeat motif	Expected size (bp)
TA2	AGAAAGGAGAGAGAGAGGGA	TGTGGTTTGATGTTGGTTGT	(TAA)n	180–260
TA59	GCTCTTCTTCTTCTTCTTGC	AGCAAGTGAAGGAGGAAGAG	(TAA)n	150–300
TA71	TCTTCTTCTTCTTCTTCCCA	GAGAGAGAGAGAGAGAGAGG	(ATT)n	180–350
TA96	CCTTCAACCAACCAACCAAC	TTGGAGAGAGAGAGAGAGGA	(TAA)n	200–400
TA194	AGTGGTGGTGGTGGTGTTGT	CCAACCAACCAACCAACTTC	(GA)n	150–350

Table-2 summary of genetic diversity statistics, Number of alleles (Na), observed heterozygosity (Ho), gene diversity (He) and polymorphism information content (PIC) at five SSR loci in eight chickpea genotypes.

Locus	Product size range (bp)	No. of alleles (Na)	Ho	He	PIC	Marker type
TA2	180–260	4	0.250	0.767	0.667	Polymorphic
TA59	150–300	4	0.250	0.700	0.605	Polymorphic
TA71	180–350	5	0.875	0.667	0.586	Polymorphic
TA96	200–400	2	0.750	0.500	0.359	Polymorphic
TA194	150–350	3	0.875	0.608	0.496	Polymorphic
Mean	—	3.60	0.600	0.648	0.543	Total alleles = 18

Table-3 Marker index (MI) of the five SSR loci, calculated as PIC × number of polymorphic alleles

Locus	PIC	No. polymorphic alleles	Marker Index (MI)
TA2	0.667	4	2.670
TA59	0.605	4	2.420
TA71	0.586	5	2.930
TA96	0.359	2	0.718
TA194	0.496	3	1.487

Table 4. Pairwise Jaccard similarity coefficients among eight chickpea genotypes based on combined SSR allelic data from five loci.

Number	S1	S2	S3	S4	S5	S6	S7	S8
S1	1.00	0.40	0.55	0.31	0.45	0.50	0.89	0.45
S2	0.40	1.00	0.36	0.25	0.40	0.44	0.36	0.27
S3	0.55	0.36	1.00	0.50	0.42	0.60	0.64	0.31
S4	0.31	0.25	0.50	1.00	0.31	0.45	0.29	0.70
S5	0.45	0.40	0.42	0.31	1.00	0.36	0.42	0.33
S6	0.50	0.44	0.60	0.45	0.36	1.00	0.45	0.36
S7	0.89	0.36	0.64	0.29	0.42	0.45	1.00	0.42
S8	0.45	0.27	0.31	0.70	0.33	0.36	0.42	1.00

Figure-1 Figure-2 Figure-3 Figure-4 Figure-5

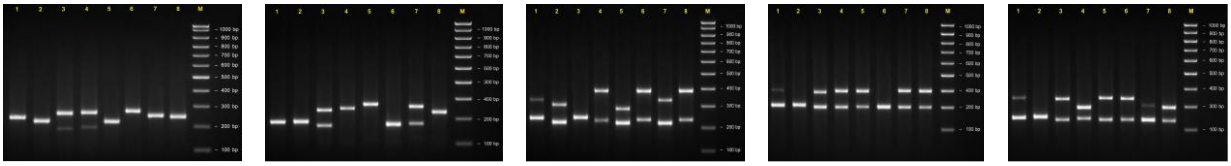

Figure 1. SSR-PCR amplification profile of chickpea genotypes S1–S8 using primer TA2. Lane M: 100 bp DNA ladder. Bands were resolved mainly within the 180–260 bp expected range. Figure 2. SSR-PCR amplification profile using primer TA59. Amplified products were observed around the expected 150–300 bp region. Figure 3. SSR-PCR amplification profile using primer TA71. Multiple bands were observed within the expected microsatellite size range Figure 4. SSR-PCR amplification profile using primer TA96. Bands were observed mainly near 200 bp and 400 bp.Figure 5. SSR-PCR amplification profile using primer TA194. Banding was observed within the expected 150–350 bp range.

Figure-6 Figure-7 Figure-8

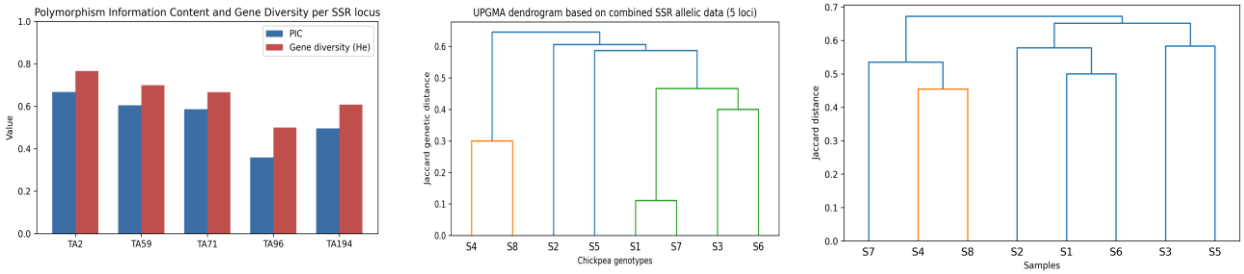

Figure 6. Polymorphism information content (PIC) and gene diversity (He) at each of the five SSR loci. Figure 7. UPGMA dendrogram of eight chickpea genotypes based on Jaccard genetic distances calculated from combined allelic data at five SSR loci (TA2, TA59, TA71, TA96, TA194). Figure 8. Average-linkage cladogram of eight genotypes based on the original combined SSR banding profile (binary band-scoring), shown for comparison with Figure 7.