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Assessing Intraspecific Genetic Variability in Cannabis sativa Populations of Punjab Pakistan Using RAPD and DNA Barcode Markers

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Cannabis sativa possess substantial amount of omega-3 fatty acids and several medicinal benefits with potential secondary metabolites. This study employed rbcL, matK, and trnH-psbA barcode markers and RAPD marker to explore genetic diversity in *C. sativa* populations in Punjab, Pakistan. In population genetic study using DNA barcode markers, cladogram showed investigated samples were genetically similar; however, demonstrated significant genetic diversity with the available DNA sequences of this plant from other regions and countries. The nucleotide sequence variation (% divergence) indicated high intraspecific variation in plants of different geographical zones. However, the RAPD marker analysis displayed genetic diversity among collected *Cannabis sativa* samples. The principal component analysis reinforced these findings by providing a spatial representation of genetic distances among the samples that revealed a considerable genetic diversity among the collected material and significant intraspecific variation was observed, indicating diverse genetic profiles across geographical regions. This research highlights the potential of molecular markers in germplasm selection for breeding programs.
Keywords: DNA barcoding, barcode *rbcL*, *matK*, *trnH-psbA*, RAPD markers, intraspecific variation, genetic diversity.

INTRODUCTION

Cannabis sativa L., a member of the *Cannabinaceae* family, is a monocotyledon diploid plant with 2n = 20 chromosomes. With a history of global cultivation and extensive growth in the wild, it ranks as one of the earliest plants to be domesticated. This adaptable plant is used for a variety of purposes (Malabadi *et al.*, 2023). There has been a rise in interest in cannabis for its agronomic potential, despite the fact that its growing is prohibited in several countries due to its potential application as a recreational narcotic (Schwabe and McGlaughlin, 2019; Siracusa *et al.*, 2023). *C. sativa* is a versatile plant, it shows a wide range of phenotypic variation. However, genetic profiling *indica*tes that *C. sativa* is an extremely structurally diverse plant that displays high levels of genetic polymorphism. Therefore, the phenotypic variations of these plants are not considered to define them as separate species or subspecies by traditional classification (McPartland, 2018).

C. sativa is growing majorly as a source of fiber, drugs, and seed oil. Mostly it has been cultivated in all temperate and tropical areas of the world. Historically, C. sativa has been utilized for producing rope, fibers, paper, and raw materials for various industries (food, drugs, and cosmetics). Moreover, its seeds are used for the production of oil that is used in soaps, paints, and cosmetics products. The seeds of *C. sativa* contain a high amount of omega-3 fatty acids (Pattnaik *et al.*, 2022). *C. sativa* has also medicinal benefits (Horne *et al.*, 2020) having potential secondary metabolites including delta-9-tetrahydrocannabinol (THC), cannabidiol (CBD), cannabinol (CBN) and, terpene compounds (Roman and Houston, 2020; Carvalho *et al.*, 2022). *C. sativa* is a native plant of Central and South Asian countries evident for the last 5000 years like Pakistan, India, Russia, China, and Iran. It has been originated from Central Asia (Pakistan Afghanistan and Turkmenistan) and spread throughout the world (Hurgobin *et al.*, 2021).

The key to every crop's improvement is the identification of diversity among its genotypes. The selection of these various genotypes involves the use of molecular markers. Currently, molecular tools and modern botanical techniques are employed to study the diversity of species in eukaryotes. Some molecular markers were utilized in the early phases of their development to study the genetic diversity in cannabis. These markers included inter simple sequence repeat amplification (ISSR), amplified fragment length polymorphisms (AFLP), and random amplified polymorphic

DNA (RAPD) (Kayis *et al.*, 2010). Although, these markers all have the flaws of weak reproducibility and dominance. DNA barcoding is one of the most modern molecular genetics methods which will identify the accurate species in eukaryotes and allow to determine the organism in doubtful position to a certain taxon. Moreover, it is fast, effective, and most accurate method for assessing the species diversity (Nikitina *et al.*, 2022). Further, intraspecific and interspecific variation in organisms were explained by the life history theory as both variables have an impact on an organism's population dynamics and individual fitness. Intraspecific variation comprised of genotypic and phenotypic variation of an organism within and among population (Des Roches *et al.*, 2021).

Two region of plastid DNA (*rbcL* and *matK*) are utilized for DNA barcoding in plant and fungi. The genes of *rbcL* (ribulose 1, 5-bisphosphate carboxylase Oxygenases) located inside chloroplast (cpDNA) and often utilized by scientist for DNA barcoding in plants because these genes are proficient and facilitate amplifying and analyzing cpDNA region. These sequences of gene are used to study of intraspecific and phylogenetic variations in plants because these genes contain 1400bp, low level of mutation, and a high level of similarity between species and highly conserved regions as compared to DNA within the nucleus (Mello *et al.*, 2016). These two regions demonstrated high effectiveness in distinguishing and identifying plant species accurately.

Therefore, the main objective of this study was to assess the intraspecific diversity in *C. sativa* population using *rbcL*, *matK*, *trnH-psbA* barcode primers and RAPD markers.

MATERIALS AND METHODS

***Sample collection*:** The research was conducted at Center Agricultural Biochemistry and Biotechnology, University of Agricultural (CABB), Faisalabad, Punjab Pakistan. The leave samples of five *C. sativa* plants (Cs-Chi, Cs-CH-UAF, Cs-PH-UAF, Cs-PRIF, and Cs-HB_ck) were collected from various locations of Faisalabad.

***DNA Isolation*:** The genomic DNA was extracted from leaves by employing cetyl-trimethyl ammonium bromide (CTAB) method. The extracted DNA was run on 0.8 % agarose gel electrophoresis to evaluate the integrity and purity of DNA. DNA quantification was done using NanoDrop (8000 Spectrophotometer Thermo Scientific).

***PCR analysis and Sequencing*:** PCR analysis was performed using three barcode primers (*rbcL*, *matK*, and *trnH-psbA*) and ten RAPD primer in 96-well Thermo cycler (peqSTAR). The PCR product was run on 1% agarose gel electrophoresis and visualized on gel documentation system (BioRad, USA). For elution, PCR products were resolved on 1% agarose gel and stained with ethidium bromide for 20 minutes. Amplicon sizes were confirmed by comparing them with 1 Kb DNA ladder. The PCR products was purified and eluted using

FavorGen Gel Extraction Kit. The eluted purified PCR products were sequenced through Eurofins DNA sequencing services. The produced sequences were excised in BioEdit Software Program to get high-quality sequences. The resultant sequences were searched for homology using Basic Local Alignment Search Tool (BLASTn).

***Data analysis*:** For data analysis, sequences were analyzed using DARwin (Version 6.0.21) and STRUCTURE software (Version 2.3.4) for population genetic studies. .For RAPD marker, DNA bands were counted and scored in the form of binary matrix on excel (MS-toolkit), as "1" (presence) and "0" (absence). The collected data was aligned and analyzed using PAST software (Version 3.16), DARwin (Version 6.0.21), and POWERMARKER (Version 3.25).

RESULTS

***Assessment of intraspecific diversity in C. sativa population using rbcL, matK, trnH-psbA*:** DNA barcoding employing *rbcL*, *matK* and *trnH-psbA* region is molecular technique for species identification and discrimination within *C. sativa*. These regions refer to specific chloroplast DNA sequences used for this purpose. The primary goal of DNA barcoding using the *rbcL*, *matK* and *trnH-psbA* regions in *C. sativa* is to assess the intraspecific variations identifying and distinguishing different *C. sativa* samples based on their genetic diversity.

***DNA Isolation*:** The genomic DNA was extracted from leaves by employing CTAB method. The extracted DNA was run on 0.8 % agarose gel electrophoresis to evaluate the integrity and purity of DNA. DNA quantification was done using NanoDrop (8000 Spectrophotometer Thermo Scientific).

***PCR analysis and Sequencing*:** PCR analysis was performed using three barcode primers (*rbcL*, *matK*, and *trnH-psbA*) and one RAPD marker in 96-well Thermo cycler (peqSTAR). The PCR product was run on 1% agarose gel electrophoresis and visualized on gel documentation system (BioRad, USA). The distinct genomic DNA fragments of all selected *C. sativa* were successfully amplified through employing *rbcL*, *matK*, and *trnH-psbA* primers with an average length 750bp, 950, and 250bp, respectively (Figure 1A, B, and C). The PCR products were eluted employing FavorPrep Gel Purification Mini Kit (FAVORGEN, BIOTECH CORP., Taiwan), and these eluted purified PCR products were sequenced through Eurofins DNA sequencing services. The produced sequences were excised in BioEdit Software Program to get high-quality sequences. The resultant sequences were searched for homology using Basic Local Alignment Search Tool (BLASTn). The BLASTn showed 100% similarity with sequence of *rbcL*, *matK*, and *trnH-psbA* of Cannabis sativa. These sequences were then submitted to NCBI (National Centre for Biotechnology Information) for the acquisition of GenBank accessions.
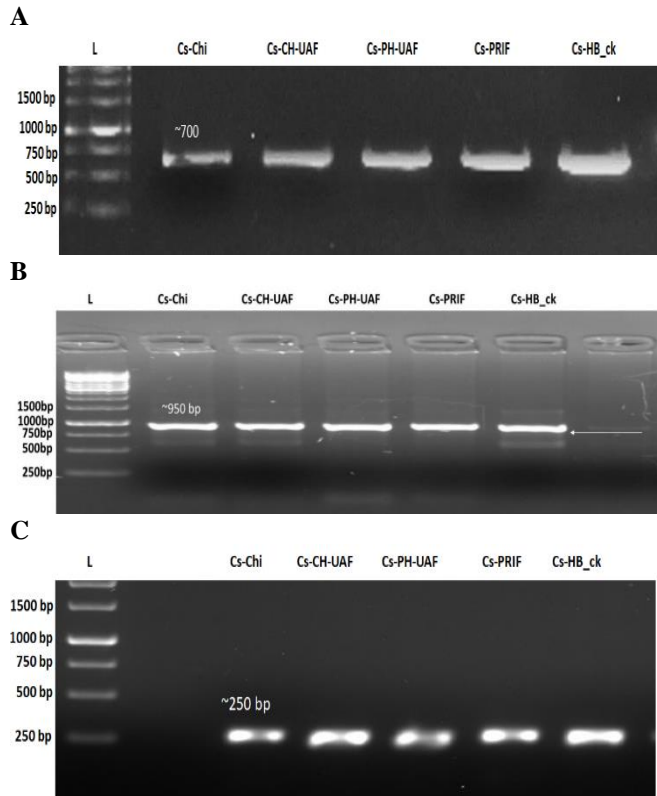
A

B

C

Figure 1. PCR amplifications with *rbcL* (A), *matK* (B), and *trnH-psbA* (C) primer resolved on 0.8 % agarose gel electrophoresis. L is DNA ladder while Cs-Chi, Cs-CH-UF, Cs-PH-UAF, Cs-PRIF, and Cs-HB_ck are representing collected sample of different location of Faisalabad, Pakistan.

***Population Genetic studies*:** For population studies, the high-quality sequences were analyzed for population genetic studies using DARwin (Version 6.0.21) and STRUCTURE software (Version 2.3.4). The Cluster analysis was performed with retrieved seqeunces from National Centre for Biotechnology Information (NCBI) employing UPGMA (unweighted pair group method with arithmetic mean) based on the Jaccard similarity coefficients procedure for assessing the intraspecific variations in *C. sativa* (Figure 2A, B, and C). Based on sequencing data from *rbcL* barcode primer, the UPGMA cluster analysis was performed that grouped all the accessions in one major cluster. UPGMA-based dendrogram showed that three accessions from Baluchistan and KPK (Kyber Pakhtunkhwa), Pakistan and Canada were genetically similar. Two accessions from Kenya and Italy were also genetically similar to each other but different from accession that is originated from China. However, five samples under study demonstrated significant genetic diversity with the retrieved sequences from NCBI (Figure 2A).
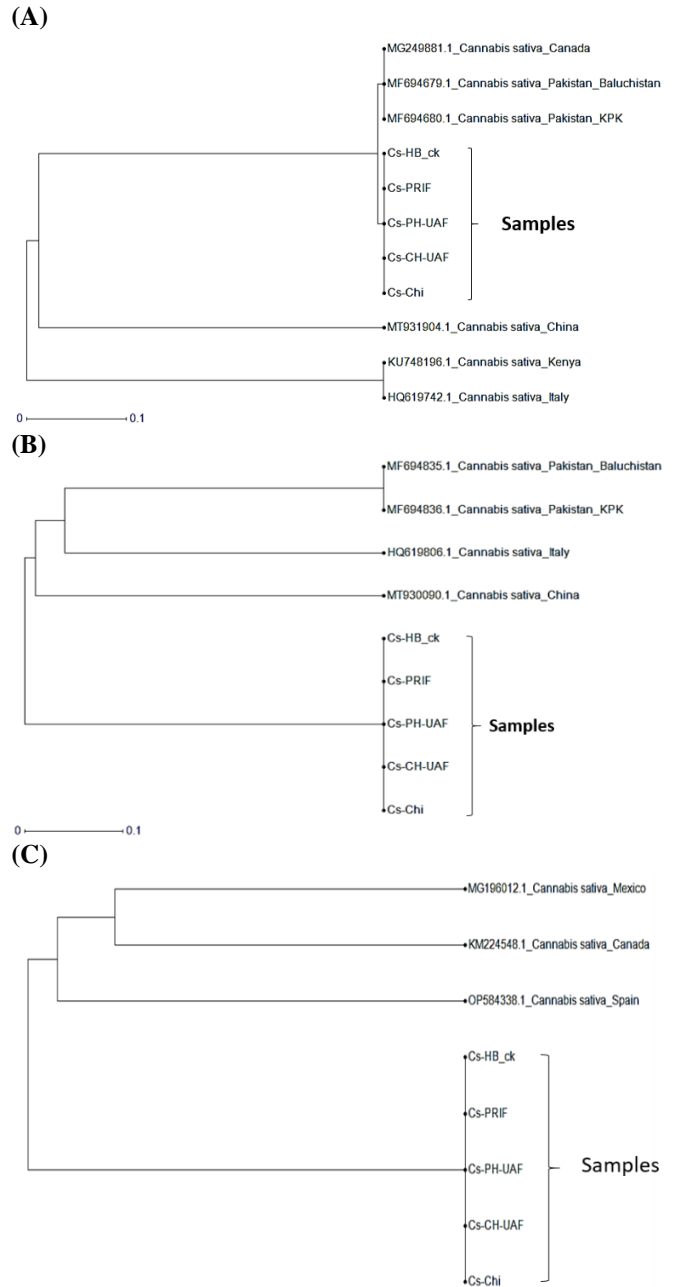
(A)

(B)

(C)

Figure 2. Unweight Pair Group Method with Arthimatic Mean (UPGMA) dendrogram of eleven accessions based on *rbcL* barcode (A), *matK* (B), *trnH-psbA* (C).

The cluster analysis was performed based on sequencing data from *matK* barcode primer that grouped all the accessions in one major cluster. The UPGMA-based dendrogram showed that two accessions from Baluchistan and KPK (Khyber Pakhtunkhwa) were genetically similar, while two accessions from Italy and China were also genetically distict. However,

five samples under study demonstrated significant genetic diversity with the retrieved sequences from NCBI (Figure 2B). The cluster analysis was performed based on sequencing data from *trnH-psbA* barcode primer that grouped all the accessions in one major cluster. The UPGMA-based dendrogram showed that two accessions from Mexico and Canada were genetically similar, while one accession from Spain was genetically distinct from these accessions. However, five samples under study demonstrated significant genetic diversity with the retrieved sequences from NCBI (Figure 2C).

Population Structure Analysis: STRUCTURE v.2.3.4, a computer program was employed for predicting population genetic structure (Figure 3A, B, and C). Results obtained from STRUCTURE software were subjected to CLUMPAK web server for the identification of optimal number of sub-populations that were identified as k=2.
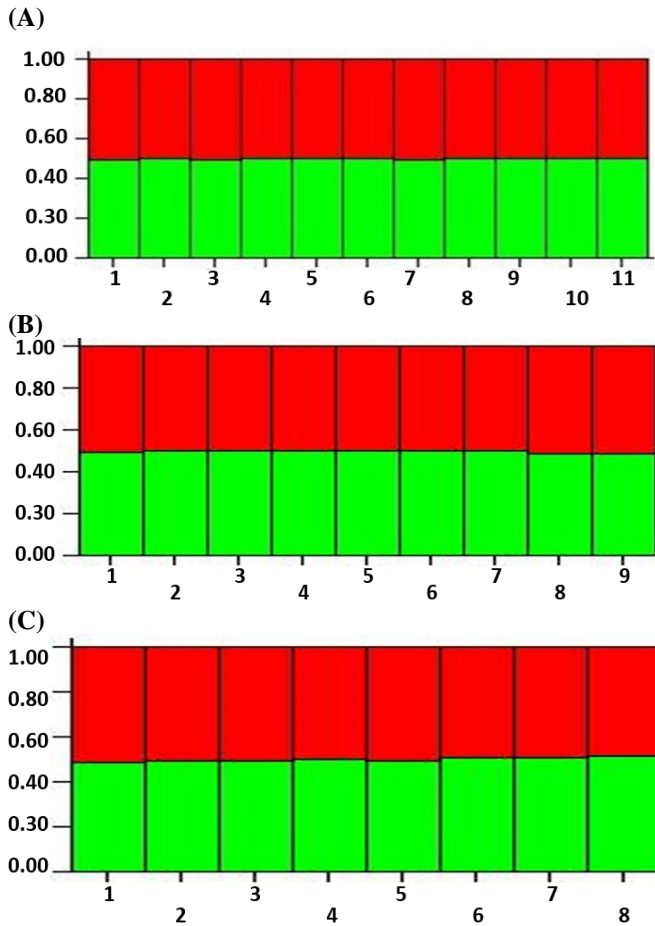
(A)

(B)

(C)

Figure 3. STRUCTURE analysis characterizes the *C. sativa* subpopulation into K2 based on *rbcL* barcode (A), *matK* (B), and *trnH-psbA* (C), represented in color codes in which each color illustrates the location of samples within its respective subgroup.

Genetic diversity analysis of Cannabis sativa using RPAD markers

PCR amplification and Data analysis: A total of ten RAPD marker from K series (Table 1) were used to amplify the DNA samples on 96-well thermal cycler. The PCR products were resolved on 1% agarose gel, and visualized on the Gel Documentation system. DNA bands were counted and scored in the form of binary matrix on excel (MS-toolkit), as "1" (presence) and "0" (absence). The collected data was aligned and analyzed using PAST software (Version 3.16), DARwin (Version 6.0.21), and POWERMARKER (Version 3.25).

Table 1. List of RAPD markers

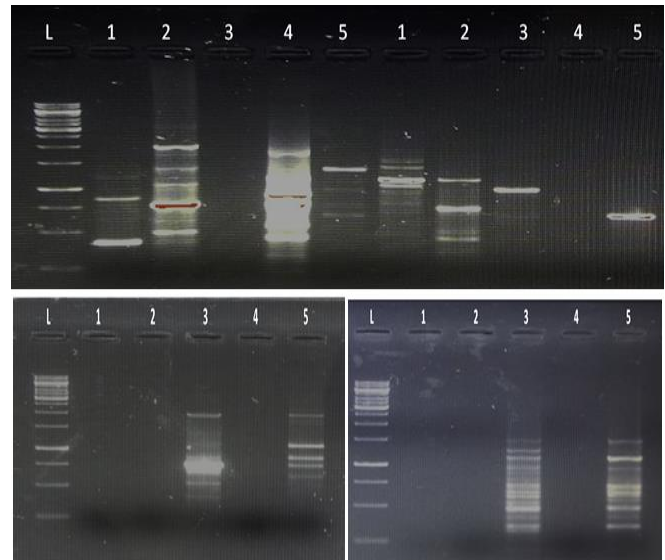| Sr.	RAPD primers	Sr.	RAPD primers
1	K1	6	K6
2	K2	7	K7
3	K3	8	K8
4	K4	9	K9
5	K5	10	K10

Figure 4. PCR amplification of Cannabis sativa with five RAPD primers of K series (K1-K10). L= Ladder, 1= Cs-PH-UAF), 2= Cs-HB_ck, 3= Cs-CH-UAF, 4= Cs-Chi, 5= Cs-PRIF.

Cluster Dendrogram: Cluster analysis was performed to generate UPGMA (unweighted pair group method with arithmetic mean) using PAST (Version 3.16). In this dendrogram, all samples were grouped into a major cluster (Figure 5). Two samples (Cs-PH-UAF and Cs-HB_ck) were grouped together into cluster 1 showing that they are genetically similar, while two samples (Cs-CH-UF and Cs-PRIF) were grouped in cluster 2, however, cluster 2 was rooted by Cs-Chi.
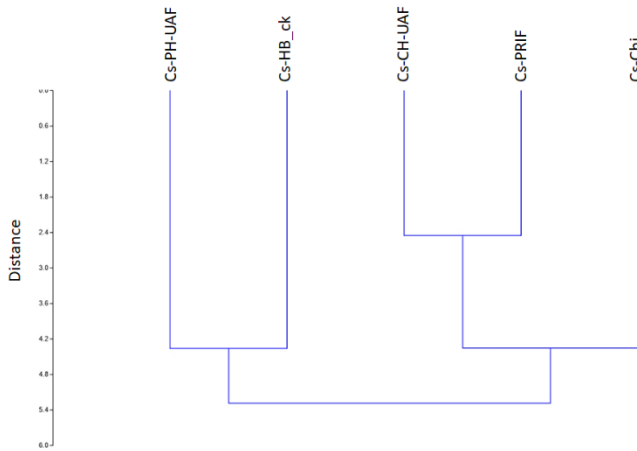
Figure 5. UPGMA (Unweighted pair group method with arithmetic mean) based Single linkage Euclidean Distance Cluster analysis of five *cannabis sativa* samples.

***Principle component analysis*:** PCA was performed to assess the spatial distribution of genetic distance among selected *cannabis* samples. The 2-D (two dimensional) plot revealed first two components accounted for total of 77.73 % genetic variation. The comomponent 1 accounted 49.61 % while component 2 showed 28.12% genetic variation (Figure 6). PCA plot showed that two samples (Cs-PRIF and Cs-CH-UAF) were closely clustered hance genetically similar. While, three samples (Cs-Chi, Cs-PH-UAF, and Cs-HB_ck) were distantly placed in separate coordinates showing that they are genetically diverse depicting high genetic diversity
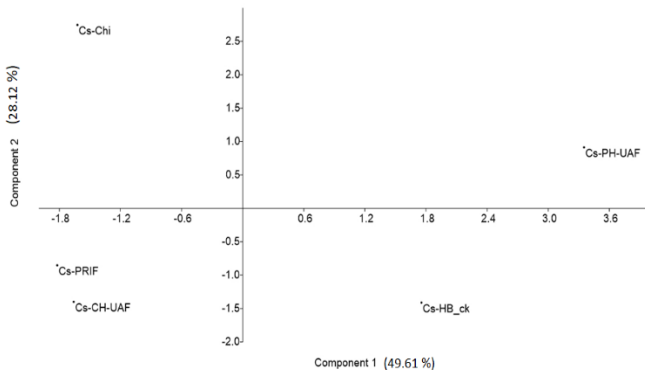

Figure 6. Spatial representation of selected 5 samples using PCA analysis in PAST (Version 3.16).

***Principle coordinate analysis*:** Principle Coordinate Analysis was performed to understand spatial representation of genetic distance among selected cannabis population from different regions of Pakistan. For this analysis, DARwin (Version 6.0.21) was used to evaluate the constancy of genetic differentiation among selected samples as well. However, the

PCoA gave a better resolution in terms of revealing the genetic relatedness and diversity. The 2-D (two dimensional) plot revealed first two coords accounted for 42.58% of genetic variation total. The Coord I accounted 23.38% while Coord II showed 19.2% genetic variation (Figure 7). These results of cannabis genotypes were distributed in plot, in accordance to the results of UPGMA cluster analysis. PCoA plot showed Cs-PRIF and Cs-CH-UAF were closely clustered hance genetically similar. While, Cs-PH-UAF and Cs-HB_ck were closely clustered hance genetically similar to each other and placed in separate coordinates showing that they are genetically diverse depicting high genetic diversity from samples (Cs-PRIF and Cs-CH-UAF). Further, Cs-Chi showed high genetic diversity among all samples.
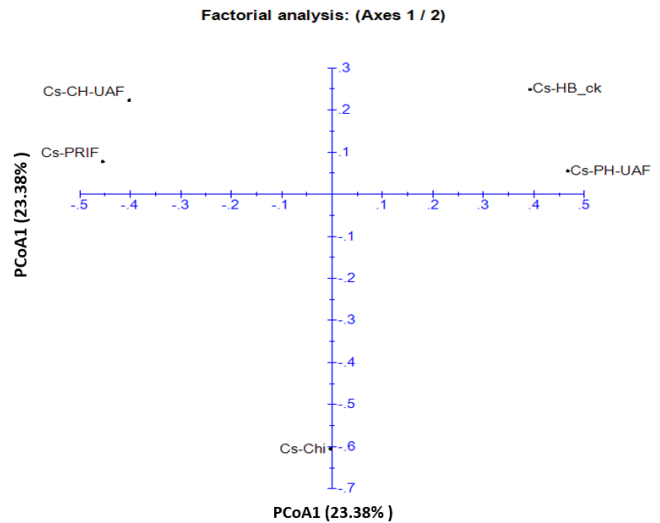

Figure 7. Spatial representation of selected 5 samples using PCoA analysis in Darwin6 (Version 6.0.21).

***Similarity Index*:** The similarity matrix was generated using Popgen32 v.1.32. Results showed similarity value of Cs-Chi (49%), Cs-CH-UAF (49%), Cs-PH-UAF (94%), Cs-PRIF (37%), and Cs-HB_ck (81%) (Table 2).

Table 2. Nei's genetic identity (above diagonal) and genetic distance (below diagonal) using Popgen 32 v.1.32.

PopID	Cs-Chi	Cs-CH-UAF	Cs-PH-UAF	Cs-PRIF	Cs-HB_ck
Cs-Chi	*****	49.247	94.446	37.806	81.093
Cs-CH-UAF	49.247	*****	89.794	11.778	49.247
Cs-PH-UAF	94.446	89.794	*****	89.794	43.363
Cs-PRIF	37.806	11.778	89.794	*****	49.247
Cs-HB_ck	81.093	49.247	43.363	49.247	*****

Genetic diversity: The provided RAPD data table contains information about different markers and their genetic diversity measures for a set of samples labeled K1 to K10. Each row in the table represents a specific marker, and the columns contain various genetic diversity metrics for that marker. For polymorphic information content (PIC), the values ranged from 0.8284 to 0.1806 with an average of 0.4896, while gene diversity ranged between 0.4998 and 0.1845 with an average of 0.3415. The values of major allele frequency ranged from 0.8868 to 0.2456 with an average of 0.6027 (Table 3).

Table 3. Major allele frequency, expected gene diversity, and PIC of K-series RAPD primers (K1-K10).

Marker	Major allele frequency	Gene diversity	PIC
K1	0.7170	0.4058	0.3235
K2	0.8491	0.2563	0.2235
K3	0.5094	0.4998	0.3749
K4	0.8868	0.2008	0.1806
K5	0.6792	0.4357	0.3408
K6	0.7181	0.4168	0.3347
K7	0.2456	0.1978	0.8284
K8	0.4598	0.3642	0.6356
K9	0.2648	0.1845	0.7489
K10	0.6972	0.4537	0.3048
Total	**6.0270**	**3.4154**	**4.8957**
Mean	**0.6027**	**0.3415**	**0.4896**

DISCUSSION

The current investigation discovered that *matK*, *rbcL*, and *trnH-psbA* are trustworthy identification markers and that the DNA identification technique successfully distinguished all samples up to the species level (Caetano Wyler and Naciri, 2016; Naim and Mahboob, 2020). DNA can be amplified and sequenced using this technique with great accuracy, and plastid DNA plays a key role in establishing evolutionary connections and defining plant species boundaries (Srivastava *et al.*, 2022). Plastid DNA has a compact size, a unique structure, and a gene arrangement since it is often inherited from a single parent and shows little to no recombination (Olmstead and Palmer, 1994). Understanding the processes through which genetic variances within species result in genetic imbalances across species and promote genetic diversity is crucial. By looking at nucleotide amounts and patterns of nucleotide variation both within and within species, this is accomplished. These differences have an effect on population structures, recombination, mutation, and selection in evolutionary processes (Kipkiror *et al.*, 2023).

The DNA barcoding research has yet not been well demonstrated in cannabis population. The efficacy of any DNA barcoding approach hinges on the degree of intraspecific and interspecific variation seen at a single genetic locus or a collection of genetic loci (Liu *et al.*, 2014).

Additionally, the *rbcL* and *matK* markers demonstrated species identification efficiency of 32.1% and 38.6%, respectively (Huang *et al.*, 2015). Based on previous research, it has been shown that because to their high success rates in amplification and sequencing, *rbcL* and *matK* are both acceptable for species-level plant identification (Kang *et al.*, 2017). The success rates for identifying plant genera and families using these two DNA fragments are exceptionally good (Makhadmeh *et al.*, 2022).

In present study three chloroplast regions (*rbcL*, *matK*, and *trnH-psbA*) were targeted and after sequencing the selected cannabis genotypes from different locations were appraised using BLAST method. A phylogenetic tree based on maximum likelihood model was created for assessing the intraspecific variations in *C. sativa*. Based on sequencing data, all the accessions were grouped into one major cluster. Based on *rbcL* gene sequences, three accessions from Baluchistan and KPK (Kyber Pakhtunkhwa), Pakistan and Canada were genetically similar. Two accessions from Kenya and Italy were also genetically similar to each other but different from accession that is originated from China. Based on *matK* gene sequences, the cluster grouped all the accessions in one major cluster where two accessions from Baluchistan and KPK (Khyber Pakhtunkhwa) were genetically similar, while two accessions from Italy and China were also genetically distict. However, five samples under study demonstrated significant genetic diversity with the retrieved sequences from NCBI. The cluster analysis was performed based on sequencing data from *trnH-psbA* barcode primer that showed two accessions from Mexico and Canada were genetically similar, while one accession from Spain was genetically distinct from these accessions. These results inferred that five genotypes under study demonstrated significant genetic diversity with the retrieved sequences from different genographical areas.

In previous study DNA barcode markers including *rpl32-trnL* and *trnS*-trnG have been employed to investigate genetic diversity among cannabis population (Roman *et al.*, 2019). There are significant differences in dissimilarity values that may be related to genetic differences, according to previous study, and it is feasible to measure the genetic distances between various accessions of plant species using these barcode markers (Algarni, 2022). In another research study, intergenic spacer regions (*trnL* and *trnF*) have been employed in cannabis population to assess interaspecific variation (Kohjyouma *et al.*, 2000). However, no preceding study has been performed yet to assess the intraspecific variation in *Cannabis* species. Moreover, using both coding as well as noncoding genetic markers together is the most efficient method for barcoding plant DNA. Among these, the *matK* gene, which displays more diversity, and the *rbcL* gene, recognized for its excellent conservation, have both undergone considerable research and are frequently used in multiple studies (Yu *et al.*, 2021).

Further, the study employed Random Amplified Polymorphic DNA (RAPD) analysis to investigate the genetic variations within Cannabis sativa populations collected from different locations in Faisalabad, Pakistan. RAPD analysis, a molecular technique, involves the amplification of random DNA segments using short, single-stranded primers (Pendi *et al.*, 2022). RAPD analysis using PCR was extensively utilized to obtain informative genetic markers and assess genetic variation among the studied plant populations. This systematic approach ensured the reliability and traceability of the genetic data obtained (Yadav *et al.*, 2021). Cluster analysis, grouped the samples into two distinct clusters, Pakistani hemp and hemp from Baba Chak 55JB formed one cluster, suggesting genetic similarity, while hemp from China, Punjab Research Institute, and Cs-Chi from Jaminaabad Tehsil Bhawana District Chiniot constituted another cluster, indicating genetic divergence. This clustering pattern underscored the presence of significant genetic heterogeneity within the *Cannabis* population.

Principal Component Analysis (PCA) reinforced these findings by providing a spatial representation of genetic distances among the samples. It revealed that two samples, Cs-PRIF and Cs-CH-UAF, were closely clustered, indicating genetic similarity. In contrast, Cs-Chi, Cs-PH-UAF, and Cs-HB_ck were dispersed, signifying higher genetic diversity within this population. Finally, the study evaluated genetic diversity metrics of RAPD primers within the *Cannabis* population. The mean major allele frequency (0.7283), gene diversity (0.3597), and Polymorphic Information Content (PIC) (0.2887) collectively indicated a moderate level of genetic diversity within the population. In preceding study, the genetic characterization of cannabis accessions have been performed using RAPD markers (Kayis *et al.*, 2010).

***Conclusion*:** This research study effectively utilizes a suite of molecular techniques to unravel the genetic diversity and relationships within Cannabis sativa populations. It highlights the significance of genetic diversity for the species' adaptation and potential applications in breeding and conservation. The findings contribute valuable insights into the genetic structure of *Cannabis* populations, which could guide future research and practical applications in agriculture and medicine.

***Conflict of Interest*:** We clarify that the submitted manuscript is our original research work and has not been published previously. Additionally, there is no competing interest.

Ethical statement: This article does not contain any research with human participants or animal performed by any of the authors.

***Availability of data and material*:** We declare that the submitted research article is our own work which has not been published before and is not currently considered for elsewhere publication.

***Code availability*:** N/A

***Consent for publication*:** All authors have given the consents to publish this manuscript in Phytopathogenomics and Disease Control (PDC).

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