



Decoding genetic diversity and population structure of *Brassica* species by inter primer binding site (iPBS) retrotransposon markers

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Abstract Marker-assisted breeding serves as a potent tool for screening target germplasm, assessing genetic diversity, and determining breeding potential of a crop. Therefore, inter primer binding site (iPBS)-retrotransposons marker system was employed to evaluate a collection of 33 *Brassica* genotypes, including 10 *Brassica juncea*, 5 *B. oleracea*, 7 *Sinapis alba*, 5 *B. nigra*, and 6 *B. rapa*, were utilized to evaluate their genetic diversity and variations 10 polymorphic primers that generated a total of 144 bands. Various diversity indices were calculated in the studied germplasm, including polymorphism information content (0.13–0.30), effective number of alleles (1.217–1.689), Shannon's information index (0.244–0.531), and gene diversity (0.148–0.370).

These indices collectively affirmed substantial genetic variations within the germplasm. Molecular variance analysis revealed that the majority (62%) of genetic variations were present within populations. The *Brassica* accessions were categorized into three populations utilizing a model-based structure algorithm. Evaluation of diversity indices based on the structure indicated that populations III and II exhibited higher diversity. Principal coordinate analysis and neighbor-joining analysis further corroborated the three distinct populations, confirming the reliability of the STRUCTURE analysis. Notably, the genetic distance assessment identified BN1 and BN3 from *B. nigra* species and the genotypes BO1 and BO3 from *B. oleracea* as genetically diverse mustard accessions.

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The extensive genetic diversity observed within the *Brassica* germplasm underscores its significance as a valuable genetic resource for comprehensive *Brassica* breeding programs. Moreover, these accessions hold promise as suitable candidates for heterosis breeding initiatives aimed at improving mustard production.

Keywords *Brassica* · iPBS retrotransposon · Population structure · Genetic diversity

Introduction

The genus *Brassica* is characterized by a diverse array of plant species, and are extensively used for over 5000 years BCE, (Campbell et al. 2016; Watson and Preedy 2010). This family comprises 338 genera and 3709 species, displaying significant morphological diversity encompassing various crop species with commercial importance, including industrial oilseeds, spices, vegetables, and fodder crops (Li, et al. 2017), which offer a diverse array of end products due to their varied range of use.

Brassica rapa L. is a source of vegetable oil for cooking, food processing, and industrial applications (Chew 2020). The seeds of *Brassica arvensis* L. could be used for biofuel production (Gidik, et al. 2023). *Brassica nigra* L. is a popular spice used in many cuisines as well as its paste is used as a condiment (Kayacetin 2020). *Brassica juncea* L. is another popular spice used in South Asian cuisines (Kumar, et al. 2011). *Brassica napus* L. is a hybrid of *B. rapa* L. and *B. oleracea* L. and is a source of vegetable oil, which is used for animal feed and industrial purposes (Das, et al. 2022). Following U's triangle model, the interspecific hybridization of *B. rapa* × *B. nigra* produces the amphidiploid species *B. juncea* [AABB (n: 18)], derived from two fundamental diploid species, *B. rapa* [AA (n: 10)] and *B. nigra* [BB (n: 8)].

Mustard species, including *Sinapis alba* (white mustard or yellow mustard), *B. nigra* (black mustard), *B. juncea* (brown mustard), *B. rapa* (also known as *B. campestris*, field mustard, or turnip), represent a distinguished germplasm, which serve as primary raw materials for numerous agriculture-based industries globally including Turkey (Kayacetin 2020). *S. alba* is commonly utilized as a seasoning agent in its dry form, while *B. nigra* holds significance not only as a condiment but also for its medicinal purposes (Aslan

and Eryilmaz 2020; Kayacetin 2023a, b; Tomar and Shrivastava 2014). *B. juncea* is employed as a bio-fuel feedstock and spice, whereas *B. rapa* finds widespread use as a biofuel feedstock (Kayacetin 2023a, b; Kwon, et al. 2020).

Mustard and rape breeding programs require information on the diversity and relationships within and among landraces to facilitate germplasm recognition, preservation, and application (Rabbani et al. 1998; Ilyas et al. 2018). Previous research (Ali, et al. 2003; Azam, et al. 2013; Jan, et al. 2017; Naheed, et al. 2016; Neeru, et al. 2016) indicated that breeders extensively assess this germplasm exploring both morphological and genetic diversity before incorporating it into breeding programs. Nevertheless, molecular markers offer a more robust approach, representing a highly efficient, reproducible, and resource-saving strategy for selecting diverse genotypes.

Among the various molecular markers, retrotransposons stand out as versatile, reproductive, and efficient tools for determining genetic diversity in plant species. Retrotransposons, also known as jumping elements, constitute between 50 and 90% of plant genomes (SanMiguel, et al. 1996). There are two main types of retrotransposons: long terminal repeat (LTR) and non-LTR, serving as predominant type markers in plant genomes. Paradoxically Kalendar, et al. (2010) has proposed a universal DNA fingerprinting technique applicable to both plants and animals, termed as the "inter primer binding site (iPBS)," introducing a new marker system. This technique allows for the analysis of both LTR and non-LTR retrotransposons. Inter primer binding site primers are designed for reverse transcription during the retrotransposon replication cycle, based on primer binding site (PBS) sequences with conserved tRNA segments (Guo, et al. 2014; Kalendar, et al. 2010).

The efficiency of the iPBS-retrotransposon marker system has been demonstrated in a wide range of crops including laurel (Karik, et al. 2019), chickpea (Andeden, et al. 2013), pea (Baloch, et al. 2015b, a), lens (Baloch, et al. 2015b, a), quinoa (Barut, et al. 2020), Turkish okra (Yıldız, et al. 2015), safflower (Ali, et al. 2019), pepper (Yildiz and Arbizu 2022), Turkish wild and cultivated Emmer (Arystanbekkyzy, et al. 2019), Turkish bread wheat (Nadeem 2021), common bean (Aydın and Baloch 2019) and grapevine (Güler, et al. 2024). Despite the extensive use

of SSR markers to differentiate for species (Thakur, et al. 2017), genetic diversity (Hobson and Rahman 2016; Raza, et al. 2019; Yin, et al. 2023), yield potential (Wolko, et al. 2022), and breeding (Singh, et al. 2022); the iPBS marker system have not yet been employed for genetic diversity assessment.

As far as we know, this study constitutes the initial efforts to utilize iPBS markers for evaluating genetic diversity within Brassica species.

Materials and methods

Plant growth conditions

The Brassica accessions (shown in Table 1) were surface sterilized following the procedure as described previously (Aslam, et al. 2022). The seeds were washed in running tap water followed by surface sterilization using 20% (v/v) commercial bleach (Dometos) for 15 min, followed by 5×3 rinses with sterile distilled water. Subsequently, the seeds were placed in Petri plates containing sterile filter paper moistened with sterile water for germination of the seedlings. The growth chamber conditions were 16 h light/8 h dark, 25 °C temperature, and 65% humidity.

Plant genomic DNA extraction from Brassica plant

Brassica seedlings were crushed into a fine powder using a mortar and pestle that had been pre-chilled with liquid nitrogen. The genomic DNA was isolated following the plant genomic DNA kit protocol (Exgene™ Plant SV, GeneAll co. LTD, Seoul, South Korea). The DNA was stored at -80 °C for future use.

Genomic DNA gel electrophoresis

The gel electrophoresis was performed using TAE buffer at 100 V for 60 min in an agarose gel (1%) to determine the purity and quantity of the isolated genomic DNA. The gel was stained with ethidium bromide solution for 5 min and rinsed with distilled water to remove excess of the dye. The gel was visualized under the gel documentation system (Bio-Rad) and photographe.

Table 1 *Brassica* genotypes for molecular characterization with inter primer binding site (iPBS) markers

Species	Serial No	Genotypes	Seed color
<i>Brassica juncea</i>	1	BJ1	Coffee color
	2	BJ2	Coffee color
	3	BJ3	Coffee color
	4	BJ4	Coffee color
	5	BJ5	Coffee color
	6	BJ6	Coffee color
	7	BJ7	Coffee color
	8	BJ8	Coffee color
	9	BJ9	Coffee color
	10	BJ10	Coffee color
<i>Brassica oleracea</i>	11	BO1	Coffee color
	12	BO2	Coffee color
	13	BO3	Coffee color
	14	BO4	Coffee color
	15	BO5	Coffee color
<i>Sinapis alba</i>	16	SA1	Yellow
	17	SA1 Fakülte	Yellow
	18	SA2 Fakülte	Yellow
	19	SA3 Fakülte	Yellow
	20	SA Fakülte	Yellow
	21	SA2	Yellow
	22	SA5	Yellow
<i>Brassica nigra</i>	23	BN1	Coffee color
	24	BN2	Coffee color
	25	BN3	Coffee color
	26	BN5	Coffee color
	27	BN6	Coffee color
<i>Brassica rapa</i>	28	BR1 Fakülte	Coffee color
	29	BR2 Fakülte	Coffee color
	30	BR3 Fakülte	Coffee color
	31	BR4 Fakülte	Coffee color
	32	BR5 Fakülte	Coffee color
	33	BR6 Fakülte	Coffee color

iPBS-retrotransposon analysis and agarose gel electrophoresis

The primers utilized were derived from the work of Kalendar, et al. (2010) in this study. Initially, 80 iPBS-retrotransposon primers were tested on a randomly selected group of 10 Brassica accessions. Thereafter, 10 most polymorphic primers were identified and subsequently employed for PCR amplification across all 33 Brassica accessions (refer to

Table 2 for details). The amplification conditions of iPBS-PCR were adapted based on the protocol outlined by Kalendar et al. (2010), with minor modifications (Saba, et al. 2020). The PCR reaction mixture included 30 ng of template DNA, 1×Taq reaction buffer, 200 µM of dNTPs, 0.2 µM primer for 11–15 length oligonucleotides or 18-bases long oligonucleotides, and 1.25 U Taq polymerase (Promega, Life Technologies, NEB) in a 25 µL reaction volume.

The PCR reaction procedure included an initial denaturation phase at 94 °C for 3 min, succeeded by 40 denaturation cycles at 94 °C for 15 s, an annealing step at a temperature ranging from 49 °C to 65 °C, based on the oligonucleotides used, for 1 min, and a final extension at 72 °C for 10 min (Kalendar et al. 2010). The amplified retrotransposon bands were subjected to analysis using 3% (w/v) agarose gel electrophoresis at 120 V. The resulting amplified bands were observed and documented using a gel documentation system (Bio-Rad GDS, California, USA).

Genetic diversity analysis

Retrotransposons were employed as markers to assess the relationships among individual plant genotypes within the collection. An iPBS marker-based neighbor-joining tree was constructed. The dissimilarity matrix was calculated using the Manhattan index in TASSEL software (Bradbury et al. 2007) to construct both the tree and principal coordinate analyses (PCoA). Different diversity parameters were also computed using GenAlEx V6.5 (Peakall and Smouse 2006) to investigate genetic differences between landraces and cultivars.

Results

Following PCR amplification of retrotransposon markers, the resulting products were separated on agarose gels and visualized using a gel documentation system. Thereafter the bands were scored, and the genetic diversity analysis was performed on the Brassica genotypes listed in Table 1. On average, each primer produced 14 bands, and the mean count of polymorphic bands was 13.5. Notably, iPBS 2376 exhibited the maximum rate of polymorphism (94.12%), followed by 88.24% bands by iPBS 2376 and minimum percentage of 64.17 was noted for iPBS 2074 primers. The values for Polymorphic Information Content (PIC) varied from 0.13 for iPBS 2074 to 0.30 for iPBS 2081. Additional details regarding the primer performance metrics are provided in Table 2. Across all primers, recessive alleles (q) were more frequent. The primers iPBS2376, and iPBS2081 exhibited the greatest allele count (Na), had values in close proximity to 1.882 and 1.824 correspondingly. Although iPBS 2074 exhibited the lowest values of Na at 1.294, the Shannon's information index (I) was the highest for iPBS 2081 and the lowest for iPBS 2074. The effective alleles (Ne) were the highest for iPBS 2081 and the lowest for iPBS 2074. The values of He (expected heterozygosity) in this study ranged 0.148 (iPBS 2074) to 0.370 (iPBS 2081).

Similarly, the values of uHe (unbiased expected heterozygosity) for the same set of primers varied between 0.150 to 0.376. The Brassica genotypes were classified into three clades according to the Neighbor-joining clustering analysis. The first clade consisted of 11 genotypes all of which were from *B. juncea* species except one BO1 from *B. oleracea* clustered with

Table 2 Certain characteristics of the inter primer binding site (iPBS) molecular markers employed in the investigation

GC Guanine/cytosine ratio, Ta Annealing temperature, TB Total bands, PB Polymorphic bands, PIC Polymorphism information content

Primers	Band Freq	P	q	Na	Ne	I	He	uHe
iPBS 2381	0.4082	0.273	0.727	1.706	1.454	0.408	0.270	0.274
iPBS 2379	0.497	0.365	0.635	1.706	1.446	0.398	0.264	0.268
iPBS 2380	0.282	0.186	0.814	1.471	1.297	0.302	0.191	0.194
iPBS 2083	0.285	0.168	0.832	1.647	1.369	0.368	0.235	0.239
iPBS 2376	0.346	0.213	0.787	1.882	1.422	0.413	0.266	0.270
iPBS 2079	0.488	0.304	0.696	1.765	1.677	0.528	0.368	0.374
iPBS 2074	0.207	0.133	0.867	1.294	1.217	0.244	0.148	0.150
iPBS 2080	0.471	0.317	0.683	1.765	1.524	0.458	0.307	0.312
iPBS 2081	0.608	0.423	0.577	1.824	1.689	0.531	0.370	0.376
iPBS 2386	0.383	0.251	0.749	1.647	1.462	0.395	0.265	0.269

BJ10. The second group consisted of 10 genotypes both from *B. napus* and *rapa*. There were 6 *B. rapa* and 4 *B. napus* accessions in this clade. Out of total 12 members in the third clade, 7 genotypes belonged to *S. alba* and one to *B. nigra*. Rest of four from *B. oleracea* showed diverse nature of genotypes that were grouped under the same clade (Fig. 1) (Table 3).

STRUCTURE analysis using the Evanno method (Fig. 2) identified that three populations ($K=3$) had the optimal number of genetic clusters. This

conclusion was supported by the maximum ΔK value of 302.71 observed at $K=3$. The F_{ST} values, indicative of genetic differences, were computed as 0.64, 0.65, and 0.40 for the Brassica populations q1 to q3, correspondingly. Furthermore, the structure analysis depicted the proportions of genetic diversity within these three populations (Fig. 3). Notably, clustering analysis provided similar results in identifying distinct populations. The structural analysis

Fig. 1 The genetic tree of *Brassica* genotypes, constructed using neighbor-joining algorithm

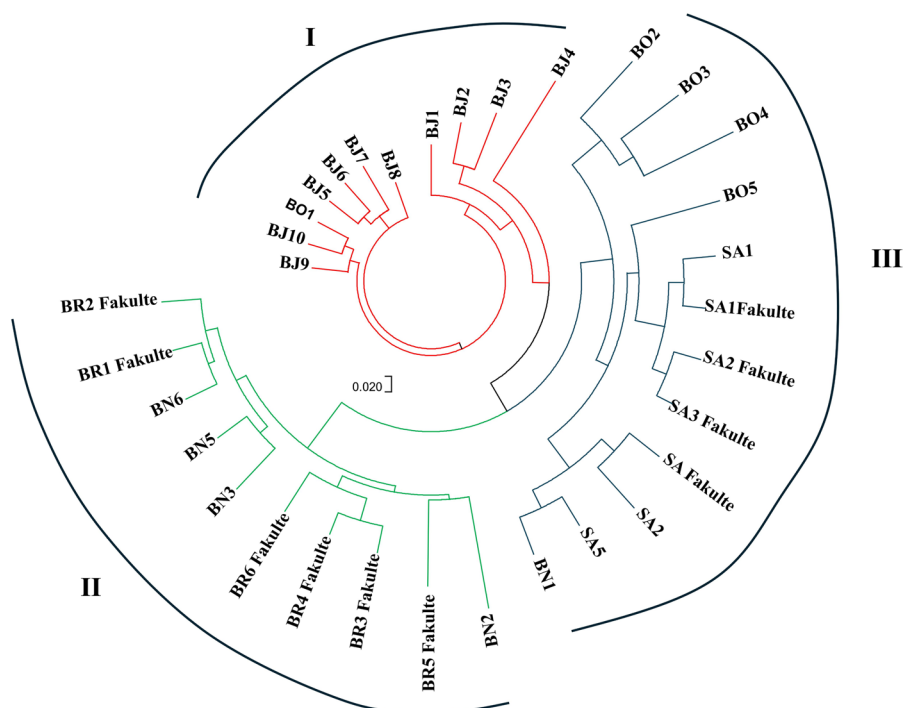


Table 3 Genetic diversity parameters for the inter primer binding site (iPBS) primers and populations used in this study

Primer IDs	Primer sequences	Ta(°C)	GC (%)	PIC	TB	PB	Ratio (%)
iPBS 2381	GTCCATCTTCCA	51	50.0	0.23	15	14	82.35
iPBS 2379	TCCAGAGATCCA	49.2	50.0	0.24	15	14	82.35
iPBS 2380	CAACCTGATCCA	51	50.0	0.17	13	13	70.59
iPBS 2083	CTTCTAGCGCCA	54.6	58.3	0.18	14	13	82.35
iPBS 2376	TAGATGGCACCA	52	50.0	0.21	16	14	94.12
iPBS 2079	AGGTGGGCGCCA	65.2	75.0	0.27	15	14	88.24
iPBS 2074	GCTCTGATACCA	51	50.0	0.13	11	11	64.71
iPBS 2080	CAGACGGCGCCA	63.3	75.0	0.25	15	14	88.24
iPBS 2081	GCAACGGCGCCA	64	75.0	0.30	16	16	88.24
iPBS 2386	CTGATCAACCCA	51	50.0	0.21	14	12	82.35
Total					144	135	
Average/primer				0.22	14	13.5	

P estimated dominant allele frequencies, q estimated recessive allele frequencies, Na Number of alleles, Ne number of effective alleles, I Shannon's information index, He Expected heterozygosity, uHe Unbiased expected heterozygosity

Fig. 2 The Delta K value indicates the existence of three populations in the *Brassica* species germplasm based on the inter primer binding site (iPBS)-retrotransposons marker system

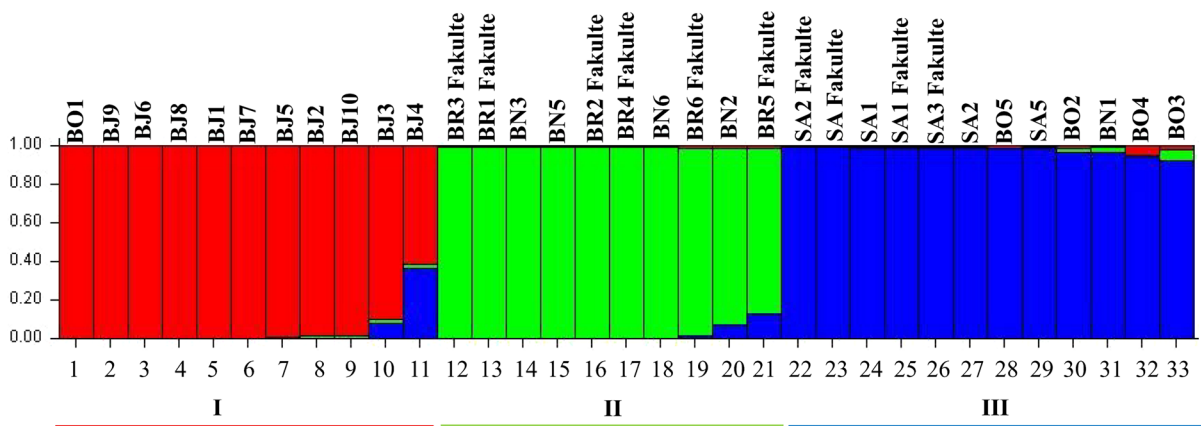
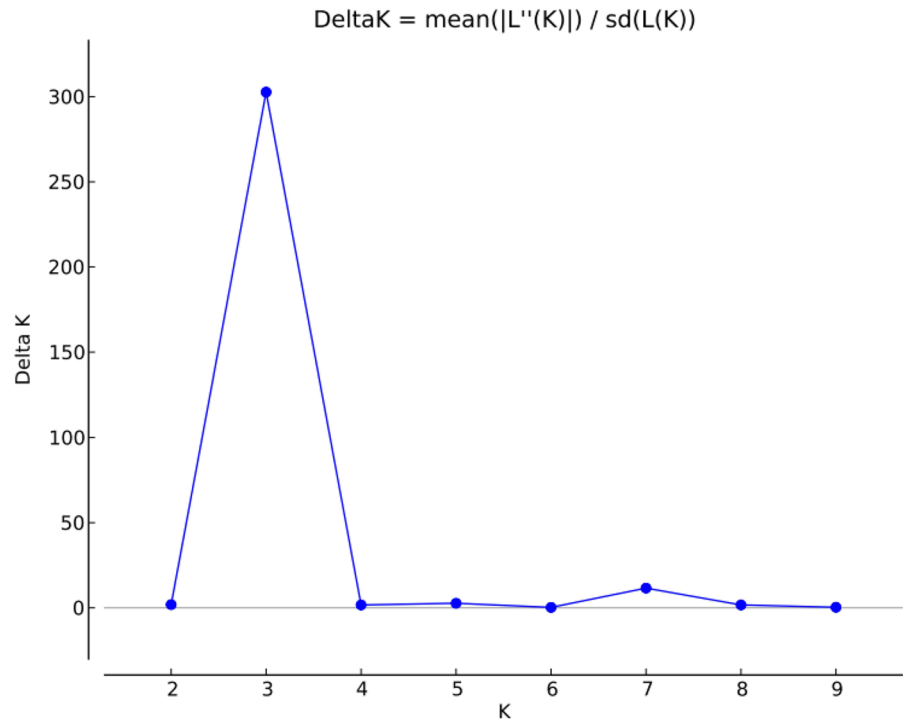


Fig. 3 The population structure analysis of *Brassica* germplasm, utilizing the inter primer binding site (iPBS)-retrotransposons marker system, reveals the presence of three distinct

groups: Group 1 (represented in red), Group 2 (represented in green), and Group 3 (represented in blue)

offered the added advantage of visualizing the proportional contributions of genetic diversity.

PCoA further confirmed the clustering observed with the model-based structure algorithm, as evidenced by the separation of the 33 *Brassica* accessions into three populations (Fig. 4).

Genetic variation among the genotypes was assessed through Analysis of Molecular Variance

(AMOVA). As presented in Table 4, approximately 38% of the total variation was attributed to disparities among populations accounted for, while the remaining 62% of the variation was observed within the populations.

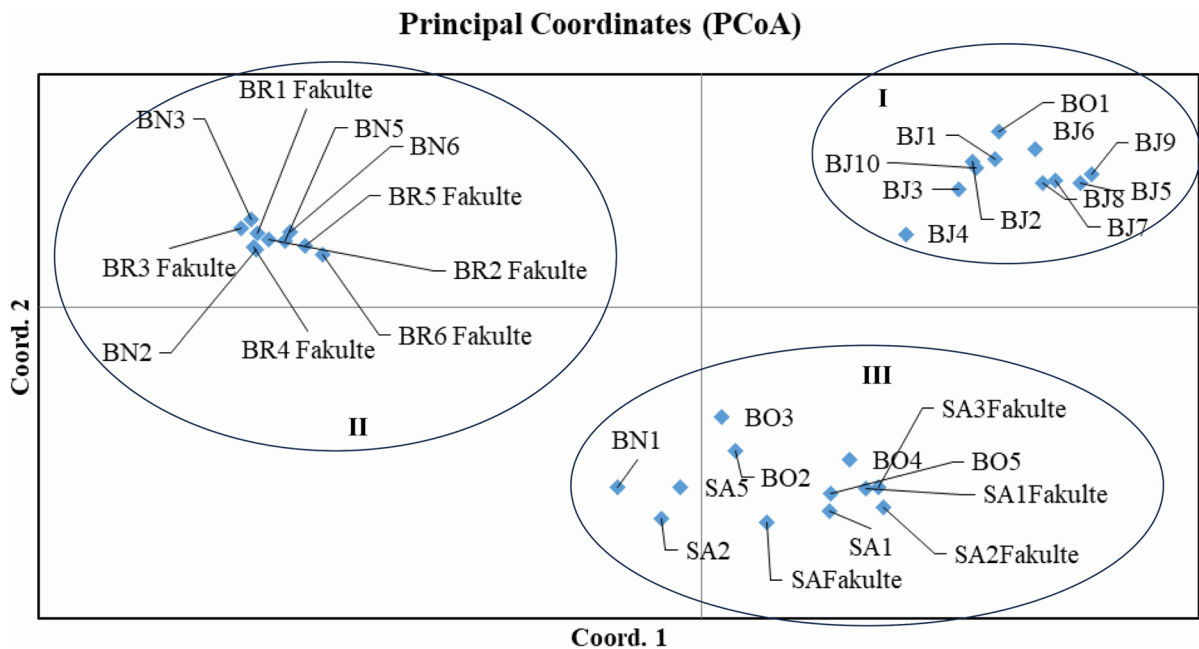


Fig. 4 Principal Coordinate Analysis (PCoA) of *Brassica* accessions was performed utilizing the inter primer binding site (iPBS) retrotransposons marker system

Table 4 Analysis of Molecular Variance (AMOVA) results of 10 inter primer binding site (iPBS) primers

Source	Df	SS	MS	Est. Var	Var. (%)
Among Pops	2	237.085	136.542	10.828	38
Within Pops	30	533.036	17.768	17.768	62
Total	32	806.121		28.595	100

Df Degree of freedom, *SS* Sum of squares, *MS* Mean squares, *Est. Var.* Estimated variance, *Var.* Variance

Discussion

Genetic variation within plant populations is a crucial resource for breeders. It allows them to develop improved cultivars with traits desired by farmers, industry, and consumers (Govindaraj, et al. 2015). Numerous research endeavors have explored relationships within Brassicaceae family, employing SSR markers (El-Esawi, et al. 2016; Singh, et al. 2018; Thakur, et al. 2017). Similarly, several investigations have utilized ISSR markers for assessing genetic associations within the Brassicaceae family (Khalil and El-Zayat 2019; Safari, et al. 2013; Shen, et al. 2016; Wang, et al. 2017). Nevertheless, to the best of our information, this study marks the first instance

where the iPBS-retrotransposon marker system has been employed to evaluate genetic diversity in 5 different *Brassica* species populations.

A set of 10 highly polymorphic iPBS-retrotransposon primers was employed to investigate the genetic variability and structure of populations of *Brassica* species. These iPBS primers generated a total of 144 bands, with 135 of them identified as polymorphic (refer to Table 2). The total and polymorphic bands identified in this study surpassed those reported by Raza, et al. (2019) using SSR markers. However, it is worth noting that the PIC value was lower in the case of iPBS.

In contrast to another study utilizing ISSR markers with a set of 7 primers, where the number of polymorphic bands was higher (20) compared to iPBS (13), this discrepancy could be attributed to the limited number of primers and the marker system employed. Similarly, in another study of ISSR, the average number of polymorphic bands in *B. rapa* var. *chinensis* was 6.3, which is lower than the findings in the present study (Shen, et al. 2016). Furthermore, a study applying ISSR markers to Amphidiploid lines of *B. juncea* ($2n=4x=36$), reported a lower PIC value (0.18) compared to our iPBS system, highlighting

the influence of species and marker choice on genetic diversity estimates.

This findings of current study on genetic diversity are comparable to those obtained by Wang et al. (2017) using ISSR markers. The average diversity index (I), effective allele number (N_e), and average genetic diversity (H_e) were 0.4045, 1.4557, and 0.2684, respectively. While a study by Thakur et al. (2018) reported a higher number of alleles (N_a) using SSR markers for *B. napus*, these values were close to those obtained with iPBS markers in this study. However, the lowest allele count observed in the SSR study exceeded compared to iPBS markers. These variations likely stem from differences in both plant species analyzed and the marker systems employed.

The observed variations in scores for different diversity indices in this investigation may have arisen from disparities in germplasm and the inherent characteristics of the utilized molecular markers. The iPBS-retrotransposons marker system, known for its high reproducibility and universal applicability, has been validated in several investigations (Shimira, et al. 2021; Yildiz and Arbizu 2022). Hence, the marker system could be the adopted choice for molecular profiling of Brassica genotypes over co-dominant and dominant marker systems.

Importantly, AMOVA findings emphasized that the highest genetic variances in *Brassica* germplasm exist within populations. These findings align with previous research that also reported higher genetic variations within populations (Jozová, et al. 2023; Tesfaye, et al. 2023).

Nei's genetic distance analysis suggests that *B. nigra* accessions BN1 and BN3 belong to a distant clade. This genetic distance makes them potential parents for cross-pollination breeding programs aimed at improving mustard quality. For the accessions of species *B. oleracea* such as BO1 and BO3 could be desired candidate for heterosis research. These combinations could enhance mustard pungency and glucosinolate contents and yield potential. The use of iPBS in marker-assisted breeding is particularly advantageous for gaining insights into genetic potential and diversity among species or cultivars, expediting molecular-based breeding.

The model-based structure algorithm categorized 33 Brassica genotypes into three distinct populations primarily based on their species level (refer to Fig. 3). Population III, comprising 36.36% (12) accessions,

is a mixed population of *S. alba* and *B. oleracea*. Population I, accounting for 33.33% (11) accessions, mainly consists of *B. juncea*, except for one that is from *B. oleracea*. Population II was the smallest and included 30.30% (10) genotypes, encompassing *B. rapa* and *B. nigra*. The presence of *B. nigra* and *B. rapa* in Population II, and *S. alba* and *B. oleracea* in Population III, contributed to the high diversity of these populations. Neighbor-joining analysis, as depicted in Fig. 1, aligns with the population structure (Fig. 3), showing congruence and support the diversity pattern with multiple lines of evidence. Furthermore, PCoA echoes the distribution pattern of genotypes seen in the neighbor-joining tree and structure analysis, underscoring the consistency and reproducibility of the analyses.

STRUCTURE analysis revealed higher genetic variations within populations III and II, which was further confirmed by AMOVA analysis. Importantly, AMOVA analysis confirms that most genetic variation resides within *Brassica* germplasm populations. This high within-population diversity holds promise for future breeding efforts aimed to improve mustard paste quality and yield. PCoA analysis further supports the clustering observed by the model-based structure algorithm, segregating Brassica accessions into three populations (refer to Fig. 4).

Conclusion

The current investigation offers a comprehensive understanding of genetic variations within a germplasm consisting of 5 *Brassica* species utilizing the iPBS-retrotransposons marker system. The study identified BN1, BN3, BO1, and BO3 as genetically diverse accessions within the germplasm, making them promising putative candidates for breeding programs. AMOVA findings emphasized increased genetic variations within populations compared to variations among populations. Notably, population II and III, as identified through structure clustering, exhibited higher diversity, suggesting that accessions within these populations should be prioritized for future Brassica breeding initiatives. The structure algorithm based on a model and PCoA successfully separated the examined germplasm into distinct populations, primarily attributed to their diverse genetic potential and increased heterozygosity. This study

further corroborated the practicality and widespread applicability of the retrotransposons markers, positioning them as valuable tools for exploring genetic diversity in Brassica crops.

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Author contributions MS: Investigation, Methodology, Laboratory research, Data curation, Visualization, Software, Writing—original draft. FK: Resources supply, KMK: Supervision, writing, review and editing, AYP: Laboratory research and experimentation, SM: Laboratory research and experimentation, MTW: review & editing, VÇ: Resources supply.

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Declarations

Conflict of interest The authors declare that they have no known conflict of interests for the work reported in this paper.

Ethical approval The research in this article does not involve any human or animal subjects therefore it does not need any ethical approval.

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