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Assessment of Molecular Diversity and Population Structure of Pakistani Mulberry Accessions Using Retrotransposon-Based DNA Markers

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Abstract: Both morphological and molecular markers have been extensively used to evaluate genetic diversity; however, molecular markers are considered more reliable and can lead to improved reproductive efficiency. This study utilized inter-primer binding site (iPBS) markers to examine the genetic diversity and population structure of thirty mulberry accessions from the districts of Sahiwal and Faisalabad, Pakistan. These mulberry accessions belonged to three species: *Morus nigra* (n = 13), *Morus alba* (n = 12), and *Morus rubra* (n = 5). The use of nine iPBS primers in this study provided a comprehensive understanding of genetic diversity among the selected mulberry accessions. Nine iPBS primers were used in the study and generated 431 bands with allelic frequencies ranging from 21 to 75 and band sizes from 200 to 1500 base pairs. The primer 2230 showed the highest polymorphic information content (PIC) value of 0.47 and the highest Shannon's information index (I = 0.53). The *Morus nigra* accessions had the highest levels of expected heterozygosity (He = 0.30), unbiased expected heterozygosity (μHe = 0.33), and Shannon's information index (I = 0.45). The molecular variance analysis (AMOVA) revealed a high degree of genetic variation, as estimated by the pairwise PhiPT value of 0.21, which was significant at the $p < 0.001$ *** level. The neighbor joining tree, principal coordinate analysis, and structure analysis grouped the 30 mulberry accessions into four main clusters. The distinct grouping of accessions SWLS14, SWLS6, FSDS30, and SWLS7 validated their notable genetic distinctiveness. Overall, these findings contribute valuable insights into the genetic landscape of mulberry accessions, which are essential for conservation and breeding strategies.

Keywords: inter-primer binding site; genetic diversity; breeding and allelic frequency; conservation

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1. Introduction

Mulberry is a member of the *Morus* genus, which belongs to the Moraceae family. It is a tree that serves multiple purposes and possesses significant ecological, nutritional, and economic value [1,2]. Mulberries exhibit high adaptability to various soil and climatic conditions. They generally display a high tolerance towards drought, pollution, and poor soil quality. As a result, they are distributed across a wide range of tropical, sub-tropical, and temperate zones in Asia, Europe, North America, South America, and Africa [3,4]. The *Morus* genus encompasses a total of 24 species within areas with a Mediterranean climate [5]. The predominant species in regions with a Mediterranean climate include *Morus alba*, which exhibits fruit colors spanning from white to dark red; *Morus rubra*, characterized by mainly red/purple fruits; and *Morus nigra*, known for its dark purple to black fruits [6]. Due to changing climatic conditions in the arctic regions of Pakistan, such

as Azad Jammu and Kashmir, Chitral, and Quetta, growing mulberry tree species such as *Morus alba* L., *Morus nigra* L., *Morus laevigata* W., and *Morus rubra* L. is now possible [7].

The practice of cultivating white mulberry for the sustenance of silkworms originated over 4700 years ago in China and later spread to various other nations [8]. Evidence suggests that as early as 220 AD, Emperor Elagabalus adorned himself with a silk robe, highlighting the longstanding historical association between mulberry cultivation and silk production [9]. While efforts have been made to conserve the genomic background of mulberry for sericulture, it is crucial to note that mulberry plants are primarily consumed for their leaves [10]. While mulberry predominantly comprises diploid cultivars with 28 (2n = 28) chromosomes, natural polyploids are also cultivated [9]. Moreover, artificial polyploids, demonstrating enhanced vigor and adaptability, have been induced through mulberry breeding programs [8,11]. Extensive selection processes for mulberry species have been initiated from openly pollinated populations or single individuals generated through controlled hybridization and mutation. This has resulted in the development of over a thousand varieties, encompassing triploid, tetraploid, and hexaploid accessions [12]. Mulberry morphological characterization has been previously utilized to investigate possible genetic linkages, and this information has been used to enhance breeding traits [13].

The most successful tool for estimating genetic variation within and among populations is DNA fingerprinting [14]. DNA-based markers have proven useful in several types of studies ranging from population structure analysis, GWAS, genetic mapping fine mapping/gene cloning of QTLs and mendelian traits, and genomic selection [15]. Fingerprinting accessions, assessing seed quality, systematic germplasm sampling, and phylogenetic analysis are all applications of DNA marker technology [15]. Inter-primer binding sites (iPBS) are retrotransposon-based markers that are characterized by their high rates of polymorphism [16]. Due to their deficiency of reliance on sequence information, excellent repeatability, and capacity to identify polymorphism, iPBS markers are better suited to comparative genomic research [17,18].

iPBS markers were developed as an alternative tool to explore genetic diversity and interactions within plant species [19]. The iPBS marker system possesses the capability to survey large segments of plant genomes, with primers adaptable for various plants and animals. Its user-friendly nature and cost-effectiveness stem from its minimal requirement of basic laboratory facilities [20]. This research focuses on the genetic variability within and among three distinct mulberry species, namely *Morus alba*, *Morus nigra*, and *Morus rubra*, utilizing retrotransposon-based fingerprinting. The findings of this study encompass the genetic diversity of 30 mulberry accessions based on the iPBS analysis, which is validated by using statistical analysis.

2. Materials and Methods

2.1. Leaf Samples and DNA Extraction

Fresh leaves were collected from 30 mulberry accessions aged between 5 and 13 years, comprising *Morus nigra* (n = 13), 12 accessions of *Morus alba* (n = 12), and 5 accessions of *Morus rubra* (n = 5) from district Sahiwal and Faisalabad within the Punjab state of Pakistan (Table 1). The leaves were used to extract genomic DNA using a modified CTAB (Cetyl-trimethyl ammonium bromide) protocol developed by Murray and Thompson [21]. The quality and quantity of the extracted DNA were evaluated through 2% agarose gel electrophoresis and comparison with a known 1 kb size ladder (Thermo Fisher, Waltham, MA, USA).

Table 1. Genetic diversity analysis utilized 30 mulberry accessions comprising varieties from *Morus nigra*, *Morus alba*, and *Morus rubra*, sourced from Sahiwal and Faisalabad.

Serial No.	Accessions	Species	Source	Morphological Characters	Ploidy Level
1	SWLS1	*Morus nigra*	Sahiwal	Black fruit	2n = 22x = 308 [22]
2	SWLS2	*Morus nigra*	Sahiwal	Small-sized, black fruit	2n = 22x = 308 [22]
3	SWLS3	*Morus nigra*	Sahiwal	Small-sized, black fruit	2n = 22x = 308 [22]
4	SWLS4	*Morus nigra*	Sahiwal	Large-sized, black fruit	2n = 22x = 308 [22]
5	SWLS5	*Morus nigra*	Sahiwal	Large-sized, black fruit	2n = 22x = 308 [22]
6	SWLS6	*Morus alba*	Sahiwal	White fruit	2n = 2x = 28 [23]
7	SWLS7	*Morus alba*	Sahiwal	White fruit	2n = 2x = 28 [23]
8	SWLS8	*Morus alba*	Sahiwal	White fruit	2n = 2x = 28 [23]
9	SWLS9	*Morus alba*	Sahiwal	Small-sized, white fruit	2n = 2x = 28 [23]
10	SWLS10	*Morus alba*	Sahiwal	Small-sized, white fruit	2n = 2x = 28 [23]
11	SWLS11	*Morus nigra*	Sahiwal	Large-sized, black fruit	2n = 22x = 308 [22]
12	SWLS12	*Morus nigra*	Sahiwal	Large-sized, black fruit	2n = 22x = 308 [22]
13	SWLS13	*Morus rubra*	Sahiwal	Small berry-sized reddish-black fruit	2n = 2x = 28 [24]
14	SWLS14	*Morus rubra*	Sahiwal	Small berry sized, reddish-black fruit	2n = 2x = 28 [24]
15	FSDS15	*Morus alba*	Faisalabad	Large-sized, white fruit	2n = 2x = 28 [23]
16	FSDS16	*Morus alba*	Faisalabad	Large-sized, white fruit	2n = 2x = 28 [23]
17	FSDS17	*Morus alba*	Faisalabad	Large-sized, white fruit	2n = 2x = 28 [23]
18	FSDS18	*Morus alba*	Faisalabad	Large-sized, white fruit	2n = 2x = 28 [23]
19	FSDS19	*Morus alba*	Faisalabad	Large-sized, white fruit	2n = 2x = 28 [23]
20	FSDS20	*Morus alba*	Faisalabad	Large-sized, white fruit	2n = 2x = 28 [23]
21	FSDS21	*Morus alba*	Faisalabad	Large-sized, white fruit	2n = 2x = 28 [23]
22	FSDS22	*Morus nigra*	Faisalabad	Large-sized, black fruit	2n = 22x = 308 [22]
23	FSDS23	*Morus nigra*	Faisalabad	Large-sized, black fruit	2n = 22x = 308 [22]
24	FSDS24	*Morus nigra*	Faisalabad	Large-sized, black fruit	2n = 22x = 308 [22]
25	FSDS25	*Morus nigra*	Faisalabad	Large-sized, black fruit	2n = 22x = 308 [22]
26	FSDS26	*Morus nigra*	Faisalabad	Large-sized, black fruit	2n = 22x = 308 [22]
27	FSDS27	*Morus nigra*	Faisalabad	Large-sized, black fruit	2n = 22x = 308 [22]
28	FSDS28	*Morus rubra*	Faisalabad	Small berry-sized, reddish-black fruit	2n = 2x = 28 [24]
29	FSDS29	*Morus rubra*	Faisalabad	Small berry-sized, reddish-black fruit	2n = 2x = 28 [24]
30	FSDS30	*Morus rubra*	Faisalabad	Small -sized, reddish-black fruit	2n = 2x = 28 [24]

2.2. PCR Amplification for iPBS Markers

The study used 15 iPBS primers, developed by Kalender et al. [25], to amplify genomic DNA from 30 mulberry accessions collected from Sahiwal and Faisalabad, Pakistan. In this setting, PCR reactions were conducted in 20 µL reactions, comprising 11.5 µL of double-distilled H_2O, 2 µL of 10× Taq buffer with $(NH_4)_2$ SO_4 (Thermo Scientific, Waltham, MA, USA), 2 µL of 20 mM $MgCl_2$ (Thermo Scientific), 1 µL of 2 mM dNTPs (Deoxyribonucleotide triphosphate), 1 µL (0.02 µM) of each iPBS primer (Macrogen, Seoul, Republic of Korea), 0.5 µL of Taq polymerase (Thermo Scientific), and 20 ng of template DNA. The PCR conditions were programmed with an initial denaturation step of 5 min at 94 °C followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at a temperature range of 30–50 °C (depending on the primer) for 1 min, and extension at 72 °C for 2 min. The reaction was finished with a final extension step of 10 min at 72 °C and stored for 1 h at 4 °C. The PCR products were then analyzed using 2% agarose gel electrophoresis, run for 60 min at 80 V, and visualized using a Gel Doc machine (Cleaver Scientific, Warwickshire, UK).

2.3. Statistical Analysis

To validate the consistency of the band patterns, three replicates of each primer pair were conducted in the PCR reaction. A binary matrix was generated based on the PCR results, where the presence of specific alleles was recorded as 1 and absence was recorded as 0. Further analyses, including polymorphic information content (PIC), Shannon's information index (I), heterozygosity (He), unbiased heterozygosity (µHe), number of distinct alleles (Na), number of effective alleles (Ne), principal coordinate analysis (PCoA), and

analysis of molecular variance (AMOVA), were conducted using the binary matrix data. The level of polymorphism in each dominant marker was calculated using the PIC formula: PIC = 1 − [f2 + (1 − f)2], where 'f' represents the frequency of the marker in the data set. Several statistical parameters, including I, He, µHe, Na, Ne and PCoA, were calculated using GeneAlex 6.5 software [26]. To analyze the genetic diversity and population structure of the 30 mulberry accessions, a binary matrix was utilized to construct a neighbor-joining (NJ) tree using MEGA 7.0.14 [27]. The population structure was determined using the software STRUCTURE v. 2.3.4, which applied a Bayesian approach [28]. Data from 11 iPBS markers were analyzed and the estimated number of clusters was determined by running 10 independent runs at each K value, with 10,000 steps followed by 50,000 simulations of a Monte Carlo Markov Chain (MCMC) [29]. The most probable K value was calculated by measuring the log probability of LnP(D). The Structure Harvester program was then used to perform computational analysis on the data and select the maximum number of Ln Pr (X | K) for bar plots among the 10 independent runs [30].

3. Results

3.1. Optimization and Reproducibility of iPBS for 30 Mulberry Accessions

To guarantee the optimal resolution and quality of genomic DNA, we conducted electrophoresis on a 2% w/v agarose gel at 80 V for one hour. Subsequently, UV transillumination was employed to capture images, revealing distinct and well-defined bands for all 30 samples. These bands serve as an indication of intact DNA without any signs of degradation. The banding pattern of the PCR products of 30 mulberry accessions using iPBS primer 2230 is shown in Figure S1. Out of a pool of 15 primers, distinct and discernible bands were obtained with nine specific iPBS primers, namely 2230, 2231, 2232, 2238, 2239, 2246, 2249, 2257, and 2277. In 30 mulberry accessions, a total of 431 bands were observed, with band sizes spanning from 200 to 1500 base pairs (Table 2). The allelic counts ranged from 21 to 75, with primer 2230 yielding the highest allelic number of 75 and primer 2238 showing the lowest at 21. Notably, there were no monomorphic bands among the 431 bands. Nine iPBS markers demonstrated polymorphic information content values ranging from 0.29 to 0.47. Primer 2257 exhibited the highest PIC value of 0.47, while primer 2230 displayed the lowest PIC value of 0.29.

Table 2. Details of 15 iPBS primers' amplification, used for the genetic diversity and population structure of 30 mulberry accessions.

Serial #	iPBS Primers	Primer Sequence (5′-3′)	Tm (°C)	Size Range (bp)	PM	PIC	I	He	µHe
1	2230	TCTAGGCGTCTGATACCA	46	200–800	75	0.29	0.34	0.21	0.22
2	2231	ACTTGGATGCTGATACCA	44	750–1400	56	0.40	0.46	0.31	0.32
3	2232	AGAGAGGCTCGGATACCA	48	400–1200	56	0.29	0.34	0.20	0.21
4	2238	ACCTAGCTCATGATGCCA	46	250–900	21	0.34	0.20	0.20	0.21
5	2239	ACCTAGGCTCGGATGCCA	50	250–1000	51	0.31	1.26	0.19	0.20
6	2246	ACTAGGCTCTGTATACCA	44	400–1000	35	0.33	0.34	0.20	0.21
7	2249	AACCGACCTCTGATACCA	46	600–1500	26	0.32	0.34	0.20	0.20
8	2257	CTCTCAATGAAAGCACCA	43	600–1000	41	0.47	0.53	0.35	0.36
9	2277	GGCGATGATACCA	46	400–1000	70	0.44	0.49	0.32	0.33
10	2251	GAACAGGCGATGATACCA	46		Did not amplify				
11	2398	GAACCCTTGCCGATACCA	48		Did not amplify				
12	2255	GCGTGTGCTCTCATACCA	48		Did not amplify				
13	2252	TCATGGCTCATGATACCA	43		Did not amplify				
14	2229	CGACCTGTTCTGATACCA	46		Did not amplify				
15	2377	ACGAAGGGACCA	46		Did not amplify				

Tm = melting temperature of primers; **PM** = number of polymorphic bands; **PIC** = polymorphic information content; **I** = Shannon's information index; **He** = heterozygosity; **uHe** = unbiased expected heterozygosity.

3.2. Genetic Diversity and Heterozygosity Interpretation

GenAlex was used to determine % polymorphism, I, He, μHe, Na, and Ne. Unbiased expected heterozygosity values ranged from 0.23 (FSD) to 0.26 (SWL), with an average value of 0.24. Expected heterozygosity (He) values ranged from 0.22 (FSD) to 0.25 (SWL), averaging at 0.23 (Table 3). AMOVA revealed significant variation both across and within populations (Table 4). The AMOVA, conducted using GeneAlex 6.503, demonstrated that the variance within populations is notably higher (96%) than the variation among populations (4%). A significant PhipT score of 0.04* further indicated minimal population variation (Table 4). The calculated genetic distances, derived from the dissimilarity index and nine iPBS marker data, were used to construct a NJ phylogram which distributed 30 mulberry accessions into four groups (Figure 1). Group one comprised seven *M. nigra* accessions, namely FSDS26, FSDS25, FSDS22, FSDS27, SWLS11, FSDS24, and FSDS23 and three *M. rubra* accessions included FSDS29, FSDS28, and SWLS14. Group two had two accessions of *M. rubra*, FSDS30 and SWLS13. Group three contained four *M. nigra* species, SWLS1, SWLS2, SWLS3, and SWLS5, and group four was characterized by high diversity and included twelve *M. alba* accessions: SWLS6, SWLS7, SWLS8, SWLS9, SWLS10, FSDS15, FSDS16, FSDS17, FSDS18, FSDS19, FSDS20, FSDS21 and two *M. nigra* accessions, which were SWLS4 and SWLS12. Within the *Morus rubra* population, the accessions FSDS14 (18.07) and SWLS30 (17.52) exhibited the highest genetic distance, whereas the accessions FSDS29 (8.13) and SWLS13 (9.35) demonstrated the lowest genetic distance. In the *Morus nigra* population, the accessions SWLS3 (12.18) and FSDS27 (11.04) displayed the highest genetic distance, while SWLS4 (6.15) and SWLS12 (7.34) exhibited the lowest genetic distance. Among the *Morus alba* population, the accessions SWLS6 (18.07) and SWLS7 (17.21) presented the highest genetic distance, whereas FSDS15 (7.86) and FSDS16 (7.07) showed the lowest genetic distance.

Table 3. Summarized results of 30 mulberry accessions, including *Morus nigra* (13 accessions), *Morus alba* (12 accessions), and *Morus rubra* (5 accessions) with nine iPBS primers.

Population	N	Na	Ne	I	He	uHe	%P
Morus nigra	13	1.65	1.53	0.45	0.31	0.33	82.4%
Morus alba	12	1.29	1.44	0.37	0.25	0.27	64.7%
Morus rubra	5	1.26	1.50	0.39	0.27	0.34	60.8%
Mean	30	1.88	1.36	0.38	0.24	0.25	69.3%

N = number of sample size; **Na** = number of different alleles; **Ne** = number of effective alleles; **I** = Shannon's information index; **He** = heterozygosity; **uHe** = unbiased expected heterozygosity; **%P** = percentage of polymorphic loci.

Table 4. Summary of AMOVA and pairwise comparisons using PhiPT values among and within subgroups.

Source	df	SS	MS	Est. Var.	%	PhiPT
Among subgroups	2	52.57	26.29	1.97	20	0.029 *
Within subgroups	27	211.78	7.84	7.84	80	
Total	29	264.37		9.81	100	

df = degrees of freedom; SS, sum of squares; **MS** = mean sum of squares; **Est. Var.** = estimate of variance; **%** = percentage of total variation; **PhiPT** = values below diagonal; **Probability** based on 999 permutations is shown above the diagonal, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

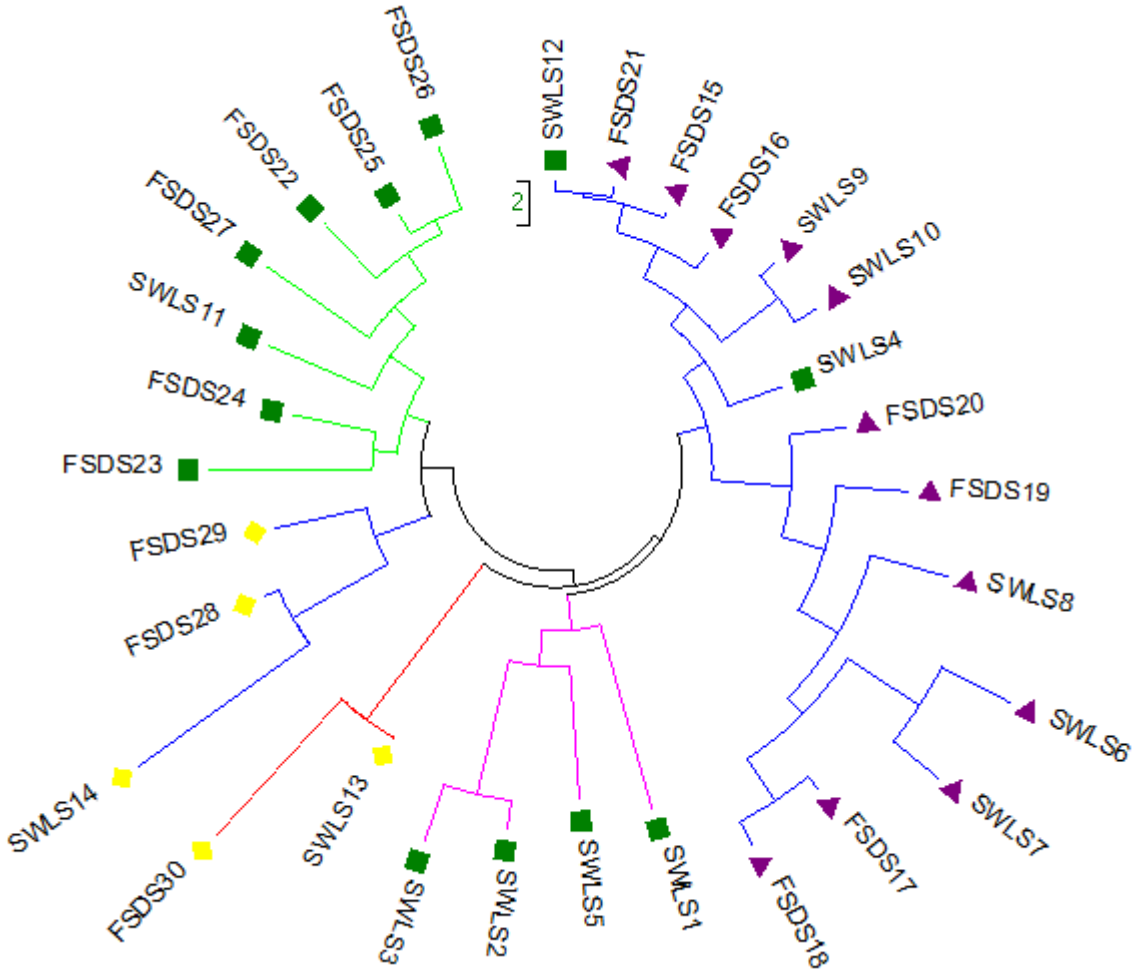

Figure 1. Neighbor-joining tree constructed using data from nine iPBS primers for 30 mulberry accessions, resulting in the formation of four clusters. These clusters represent three *Morus* species, namely *Morus alba* (purple triangles), *Morus nigra* (green squares), and *Morus rubra* (yellow diamonds).

3.3. Principal Coordinate Analysis (PCoA) and Population Structure of 30 Mulberry Accessions

The 30 mulberry accessions were categorized into four distinct groups through the PCoA, as illustrated in Figure 2. The first and second coordinates contributed to 22% and 21%, respectively, collectively representing 42% of the total genetic variation. The population structure of mulberry accessions was assessed, revealing the identification of four clusters as the most probable K number. This implies the existence of four distinct populations, as determined by ΔK values calculated through the Structure Harvester program. The analysis identified four genetic clusters as appropriate for these mulberry accessions. Delta K, representing the maximum number of genetic clusters in a particular sample, is illustrated in Figure 3. Bayesian analysis was employed to generate a model-based cluster among 30 mulberry accessions, using a set of nine iPBS primers. The resulting clusters, denoted by colors (yellow, red, blue, and green), are depicted in Figure 4. If a genotype possesses a membership coefficient of 80% in K = 4, it is classified within that population. Each vertical line in a multi-locus genotype signifies the cluster's most likely ancestry, indicating the origin of the accessions or admixture accessions. Concerning the population structure of specific mulberry accessions (SWLS4, SWLS6 to SWLS10, SWLS12 to SWLS14, FSDS15 to FSDS18, FSDS21, FSDS22, FSDS25, FSDS26, and FSD30), it is suggested that they lack a common ancestor and represent pure genetic material. Concurrently, accessions displaying multiple colors are inferred to be a mixture of inherited material from other accessions within the same genus, including SWLS1, SWLS5, SWLS11, FSDS23, FSDS27, and FSDS30.

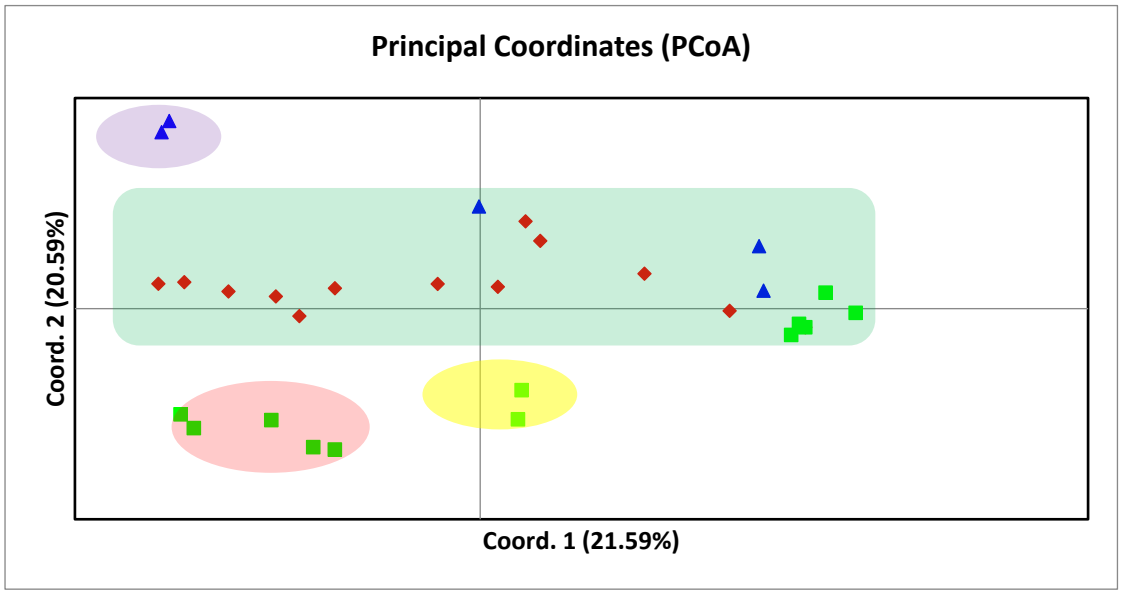

Figure 2. Principal coordinate analysis distributing 30 mulberry accessions into four groups using nine iPBS primers. Coordinate 1 showed 21.6% genetic diversity and coordinate 2 showed 20.6% genetic diversity, meaning a 42.2% total genetic diversity. Red diamonds: *Morus nigra* (thirteen accessions); green squares: *Morus alba* (twelve accessions); blue triangles: *Morus rubra* (five accessions).

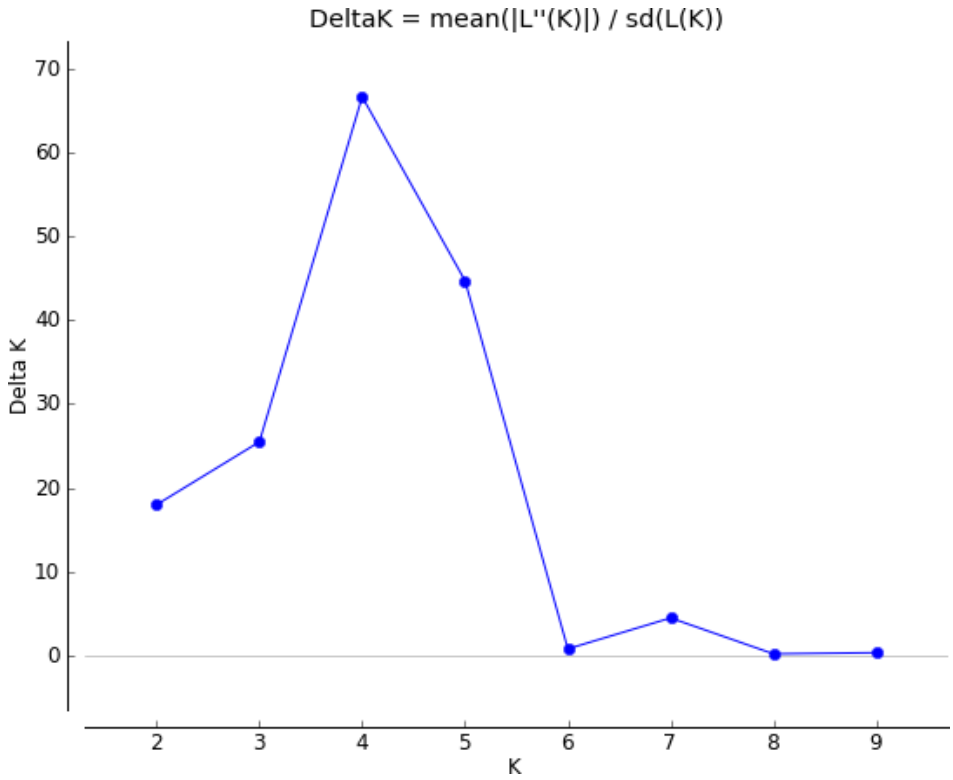

Figure 3. Structure Harvester was used to create the population structure and grouping of 30 mulberry accessions, and Delta K is the potential number of genetic clusters that express 30 mulberry accessions in cluster form.

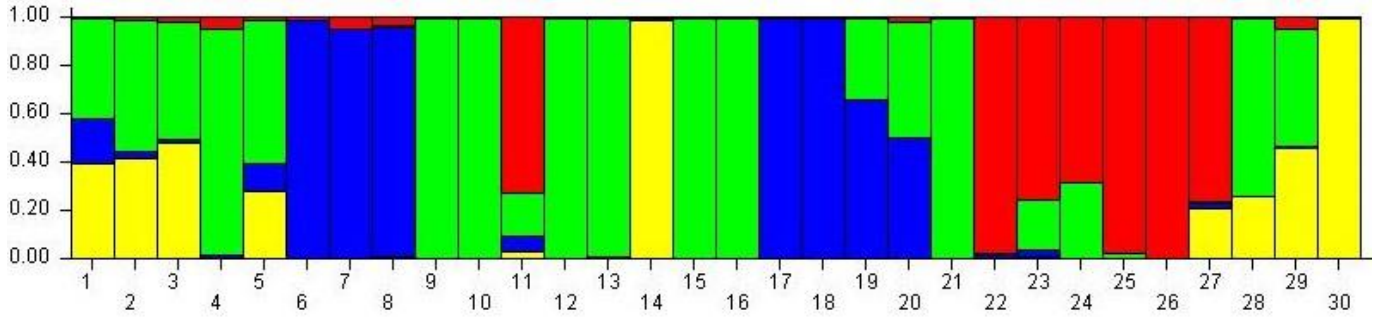

Figure 4. Allelic variation of nine iPBS loci is used in a Bayesian study of 30 mulberry accessions. The colored lines represent the accession groupings of 30 mulberry accessions, showing the ancestor of the cluster. The number of different colors shows the number of populations identified by Structure Harvester software v. 2.3.4 (yellow, blue, green, and red depict the four groups).

4. Discussion

Bottlenecks characterized by the establishment of new populations with a limited number of progeny plants, result in a reduction of genetic variation that corresponds to the severity of the bottleneck. Smaller populations, especially those persisting over the long term, undergo a more substantial loss of genetic variation over time [31]. Accurate quantification of genetic variation within local varietal germplasm is essential for enhancing polymorphism and genetic diversity among plant varieties [32,33]. This process is pivotal for analyzing the distribution of biodiversity with respect to geographical locations and for informing strategies related to breeding and conservation methods. According to previous studies, retrotransposons comprise half of the plant genome's repetitive DNA and play a vital role in evolution and genetic diversity. Mulberry has been reported to contain 357 Mb, 2n = 14 of LTR-transposons comprising 47% of total genome size [34].

When endeavoring to characterize Pakistani mulberry accessions encompassing *M. alba*, *M. nigra*, and *M. rubra*, the iPBS analysis effectively distinguished between the accessions, confirming the robustness of the iPBS marker system. In our study, we observed polymorphic bands ranging from 21 to 75 of nine retrotransposon-based markers used for 30 mulberry accessions. A previous study reported on a genetic diversity analysis conducted on 43 mulberry accessions obtained from the National Institute of Sericultural and Entomological Science (NISES) in Tsukuba, Japan [35]. In the investigation, each of the five AFLP primer combinations utilized produced an average of 110 amplification products, and the percentage of polymorphic bands observed varied between 70 and 82 [35]. The genetic diversity analysis of 48 accessions from three species *M. alba*, *M. latifolia* Poir., and *M. bombycis* Koidz utilized six AFLP primer combinations. Each combination yielded an average of 110 scored bands, with 72.2% of them exhibiting polymorphism [36]. iPBS primers demonstrate greater reproducibility compared to AFLP markers due to their precise targeting of specific regions within the genome. This specificity reduces the likelihood of nonspecific amplification, resulting in more consistent and reliable results across experiments. Additionally, iPBS primers typically generate fewer artifacts and background noise, further enhancing their reproducibility compared to AFLP [33,37–40]. Even though the accessions gathered from Sahiwal and Faisalabad exhibited a level of polymorphism similar to that observed in previous studies, this suggests a notable degree of polymorphism within the accessions examined in the current study.

In our study, the number of alleles observed is compared with the findings of Pinto et al. [41], where the allelic number was reported to be within 3 to 17 per locus. Similarly, 83 alleles were obtained from 16 SSR loci analyzed in 73 mulberry accessions in Turkey [42]. Among 15 SSR primers utilized for genetic diversity of mulberry, the range of observed alleles per locus across ten mulberry accessions varied from two to 15, with an average of 6.3. [43]. Among these, the SS09 primer yielded the highest number of alleles at 15, whereas SS19, SS20, MulSTR2, MulSTR6, and MulSTR5 primers produced the lowest number of alleles, each generating only two [43]. SSR analysis was conducted on 37 mulberry

accessions sourced from nine distinct countries: Cuba, Costa Rica, Brazil, South Korea, China, Japan, Italy, Ethiopia, and Spain [44]. The number of alleles obtained ranged from two (SS19) to 19 (MulSTR3), resulting in a total of 68 alleles across the nine loci; on average, there were eight bands per locus [44]. Aggarwal et al. [45] reported a mean number of 19 alleles in mulberries, while in the study conducted by Wani et al. [46], 17 mulberry accessions were characterized using six SSR markers. The molecular analysis revealed substantial variability among these accessions. A total of 17 alleles were identified across the 17 mulberry accessions using the six SSR markers. The number of alleles per locus, as determined by these markers, ranged from two (MulSTR3) to four (MulSTR4), with an average of three per locus. This average indicated lower values compared to our study. In previous research, iPBS had not been utilized to assess the genetic diversity of mulberries. Earlier studies frequently depended on SSR primers to evaluate mulberry genetic diversity. In our investigation, we employed iPBS, unveiling its capacity to differentiate mulberry accessions into distinct groups. This observation underscores the substantial polymorphism inherent in iPBS. Discrepancies in the reported number of alleles in other studies may also be attributed to variations in accession and primer selection.

In our examination of diversity statistics, we noted that the average and expected heterozygosity were determined to be 0.23 and 0.24, respectively. Garcia-Gomez et al. [44] employed thirty-seven mulberry accessions from nine different countries in their SSR analysis. They observed that SSR markers' heterozygosity ranged from 0 (SS19) to 0.9 (MulSTR3), with a mean value of 0.5. Wangari et al. [43], using 15 SSR primers, reported a mean observed heterozygosity (Ho) of 0.37 and He of 0.36. Additionally, Aggarwal et al. [45] found a higher observed Ho value of 0.6 across 45 mulberry accessions from diverse species origins using SSR markers. Our findings generally align with the mentioned studies, although they indicate lower levels of both observed and expected heterozygosity. Our results signify a reduced genetic variability within the mulberry accessions under investigation. This disparity could potentially be attributed to variations in the choice of primers and the specific mulberry accessions utilized in the studies.

The assessment of polymorphism among mulberry accessions involved the examination of the number of alleles and PIC values for each of the nine iPBS primers. A locus is deemed to exhibit significantly lower levels of polymorphism if the PIC mean value falls below 0.25 [47]. PIC values serve as indicators of allelic diversity and frequency among the accessions and ranged from 0.29 to 0.47 for primers 2230 and 2257, respectively. These findings suggest that all markers have the potential to provide substantial information for mulberry genetics and breeding research. Wani et al. [46] conducted an analysis of 17 mulberry accessions using six SSR markers, obtaining PIC values ranging from 0.26 (MulSTR3) to 0.62 (MulSTR4), with an average of 0.44 per locus indicating lower values compared to our study. In another study, Zhao et al. [48] utilized 27 mulberry accessions representing 11 species and 3 varieties within the *Morus* genus, revealing PIC values ranging from 0.20 to 0.68, with an average of 0.45. The substantial diversity observed is likely attributed to the primary propagation system of mulberries through seeds [42]. The elevated level of polymorphism, especially in cultivated mulberry, reflects the outcrossing nature of the species [49].

In our investigation, the AMOVA results indicated notable variation, with 96% occurring within populations and 5% between populations. This aligns with the findings of Wangari et al. [43] in their examination of mulberry species, wherein high variation was observed within species (*M. alba* and *M. indica*), while there was comparatively lower variation between species. This pattern could be attributed to the substantial outcrossing observed in woody crops, as suggested by Hamrick and Godt [50]. The analyses indicate that populations composed of different ecotypes exhibit high genetic diversity, yet within each population originating from the same ecotypes, there is a low level of genetic variation. Notably, mulberry primarily disperses through clonal growth, and local varieties have been domesticated through long-term cultivation. We propose that these characteristics,

coupled with the founder effect, account for the low diversity within populations and the substantial genetic differentiation among populations of this species.

In the dendrogram, varieties from the same ecotype primarily clustered together, suggesting an association with their geographic origin. However, some variations were noted, including instances where populations from the same districts formed clusters that were more distant from each other. Earlier investigations by Sharma et al. [35] utilizing AFLP and Vijayan et al. [51] employing ISSR on mulberry accessions revealed an absence of distinct relationships with geographical locations. This lack of a clear correlation can be attributed to the introduction and subsequent naturalization of mulberry accessions in regions distant from their original source. Consequently, the discrepancies observed in our study, both in terms of the grouping of populations based on genetic similarity and their geographical origin, can be attributed to human activities, propagation methods, and ecological conditions [51]. The analysis of population structure uncovered the existence of three genetic clusters characterized by a significant degree of admixture. This suggests that trait plasticity is influenced by genetic variation rather than ploidy. This phenomenon may be attributed to the substantial heterozygosity resulting from natural cross-pollination [52].

5. Conclusions

The clustering pattern of accessions, as determined through iPBS analysis, demonstrates high robustness and reliability in illustrating genetic similarities and dissimilarities. The findings from this study highlight the utility of iPBS markers for taxonomists, enabling them to verify genetic relatedness among accessions, eliminate duplication, and identify informative markers for species fingerprinting. The results presented herein underscore the necessity of comprehensive investigations utilizing a larger number of primers, including microsatellites, in order to mitigate ambiguity regarding the origin and species identity of the accessions. The outcomes of this study suggest that an effective breeding/hybridization program can be initiated by leveraging molecular and morphological/agronomical data to identify diverse accessions, ultimately leading to the development of new varieties with enhanced agronomic traits.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture14030400/s1, Figure S1: Banding pattern of PCR products of 30 mulberry accessions detailed in Table 1 using 2230 iPBS primers. Well 1 includes a 1 kb size DNA ladder; red-colored arrows point to polymorphic bands, while the blue arrows point to monomorphic bands.

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