



Article

Inter Simple Sequence Repeat-Based Genetic Divergence and Varietal Identification of Banana in Pakistan

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Abstract: Banana is one of the major cash and fruit crops of Pakistan. The lack of information concerning genetic diversity and purity within locally cultivated banana varieties is a major bottleneck in improving its genetics. Due to the existence of a narrow genetic background, it's quite important to find genomic variations in banana varieties. DNA marker-based techniques have been used to effectively characterize banana varieties. In the current study, Inter Simple Sequence Repeat (ISSR) markers were used to characterize banana cultivars and to assess the genetic diversity of 14 local banana varieties grown in Pakistan. Out of the 45 primers used, 40 primers revealed reproducible results and produced 121 polymorphic bands, which contributed a ratio of 47.87 polymorphism. The ISSR UBC-835 and UBC-834 possessed the highest PIC ranged between (86–88%) in banana varieties, while the lowest PIC (46%) was detected in the case of UBC-857 marker with (100–1500 bp) PCR product size. Pairwise Jaccard's similarity coefficient values were also calculated, and these were ranged from 0.56–0.88. Multivariate analysis divided 14 banana varieties into two distinct groups—A and B respectively—and furthermore into subgroups, clusters, and sub-clusters. Our results indicated that at the molecular level, the banana varieties in group—A were found to be 66% similar whereas in group B were 88% similar. Nei's genetic diversity, PCA analysis, and a minimum spanning tree depicted Fenjiao, Dajiao, and NIGAB-2 as the most diverse members as compared to all other varieties of the three populations. Out of 14 varieties used, 11 varieties were uniquely identified by 54 polymorphic ISSR bands of different sizes. Some varieties like NIGAB-2 and NIGAB-3 were uniquely identified only with one band while others were tagged by multiple unique bands. In future, this study will be utilized to establish a molecular-based protocol for the identification of banana varieties.

Keywords: genetic diversity; ISSR; varieties identification; cluster analysis



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1. Introduction

Banana belongs to the *Musaceae* family and order *Zingiberales* [1], and its genus, *Musa*, is one of the major cash crops in tropical and subtropical regions. Most banana germplasm is found in the Asian continent [2]. Bananas are distributed over more than a hundred countries worldwide, and are the fourth most popular world crop after wheat, maize, and rice [3]. In Pakistan, bananas are cultivated on 0.034 million hectares, and their annual production is 0.155 million tons. Sindh province of Pakistan has favorable conditions for banana cultivation. Suitable climatic conditions and fertility enable Sindh province to produce an 87% share of the country's banana production [3].

The world population is expected to be over 9 billion near the year 2050 [4]. To fulfill the requirements of this rapidly growing population, agricultural crop production

has become an increasing challenge [5]. For future aspects of these needs, it is necessary to investigate the genetic diversity and varietal identification of various closely related genotypes of banana varieties to increase the productivity of banana for increased resistance/tolerance to different environmental biotic and abiotic stress to meet present and future challenges of food security. Any given germplasm should be characterized on a morphological and molecular basis [6]. Morphological-based approaches are not always easy, because of the long-time lapse to attain plant maturity for the morphological characterization. Also, morphological characteristics are influenced by the environment, so they can be altered by changing environmental conditions, whereas molecular characterization cannot be influenced by the environment [7].

The diversity of bananas plays a vital role in the development of *Musa* breeding programs, but climate change has become a major global issue that is decreasing crop yields and productivity. Partly as a response, plant scientists have used various DNA fingerprinting methods to classify plant species and varietal identification. Molecular characterization in a banana is used to determine the genotypes, their relationship, and genetic diversity among banana varieties. Based on molecular markers new germplasm classifications have gained importance due to the reliability and quality of data generated [8].

In bananas, several DNA markers-based methods have been investigated to identify genetic diversity in the *Musa* species. Diversity studies in *Musa* have been carried out using various DNA based marker methods such as Simple Sequence Repeat (SSR) [8], Directed Amplified Mini Satellite DNA (DAMD) [9], NGS-based methods [10], Random Amplified Polymorphic DNA (RAPD) [11], Sequence-Related Amplified Polymorphism (SRAP) [12], Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), and Inter-Simple Sequence Repeats (ISSR) [13]. Using various DNA marker techniques to investigate diversity within plant species has provided a complete and more useful characterization for the scientist than a single marker technique [10]. ISSR has been used in different regions for the genetic diversity and stability analysis in different sets of banana germplasm from Brazil [14], regions of Karnataka, India [15], and Gujarat, India [8]. To date, cultivar identification has been effectively used in various fruit species, such as flowering mei, fruiting mei, grape, lemon, loose-skin mandarin, pear, pomegranate, and sweet orange [16]. Along with other techniques, ISSR has been effectively used to analyze the genetic variations in the wild *Musa* germplasm and in *Musa acuminata* Colla. In previous studies, Inter Simple Sequence Repeats (ISSR) system rapidly produced many vastly replicable DNA markers, therefore allowing high-resolution genotyping [17].

The present study was undertaken to explore the potential ISSR markers for breeding of genetic resources, explore genetic relationships, distinguishing individual accessions, and estimating genetic diversity among different banana cultivars and varietal identification. Although numerous worldwide studies have already reported on banana DNA-based fingerprinting, no such data is available in Pakistan to date. The main objective of this research was to estimate genetic relationships in the banana gene pool available in Pakistan, providing a systematic reference based on DNA fingerprints, thereby establishing unique genotypic identification.

2. Materials and Methods

2.1. Plant Material and DNA Markers

Commercially grown 14 banana varieties—viz., NIGAB-1, NIGAB-2, NIGAB-3, Grand Naine, Adi, Gayl, Jafa, Red Banana, Dhaka, Dajiao, Fenjiao, Basrai, Sprouts-1, and Sprouts-2—were collected from Agriculture Research Institute (ARI), Tandojam, Sindh, and grown at the screen house of the National Institute of Genomics and Advanced Biotechnology (NIGAB), National Agriculture Research Centre (NARC). After 6 weeks, three replicates of each variety from three different plants were taken for DNA extraction and stored in the refrigerator. Forty-five previously reported ISSR markers were synthesized and used to characterize the diversity and variety identification between 14 local banana varieties (Synbio Technologies LLC, Monmouth Junction, NJ, USA). Forty primers out of 45 ISSR

markers were successfully used to differentiate the understudied local banana varieties, whereas five ISSR markers—namely UBC-891, UBC-825, UBC-855, VHV, and IG-06—did not amplify any allele in 14 local banana varieties (Supplementary Table S1).

2.2. Genomic DNA Isolation and Quantification

For genetic diversity, samples from the young leaves of 14 banana varieties were extracted by using the 2% Cetyl-trimethyl ammonium bromide (CTAB) technique [18]. We added 600 µL 2% Cetyl-trimethyl Ammonium Bromide Buffer (CTAB) (10 g (g)/L CTAB salt (SERVA Electrophoresis GmbH D-69115 Heidelberg Carl-Benz-Str. 7 Telefon 06221/138400), (SIGMA, Lot. No NDT093648), 20 g/L PVP (Phytotech Labs, Lenexa, KS, USA, Lot No HYY0728019A)), 0.5 Molar (M) (pH: 8) EDTA (Bio Basic Inc. is a life science research-support company founded in Toronto, Canada—Headquartered in Markham, Ontario and Amherst, New York, Cat. No. 2616B024), 1 M Tris HCL (pH: 8) (Invitrogen, Carlsbad, CA, USA, REF, No. 15506-017, REF, No. 15506-017), 5 M NaCl (pH: 8) (Scharlab S.L. Gato Perez, 33-P. I, Spain, Scharlau, cat no. 17137702, cat No. 17137702) dissolved in 1000 mL of distilled water (pH: 8) and then autoclaved. 600 µL Chloroform (Scharlab S.L. Gato Perez, 33-P. I, Spain, Scharlau, Cat. No 02102500, Cat. No. 02102500): Isoamyl alcohol (Bio Chemo pharma, ZA Conse Sur Loire, 58200 France, cat. No 209012500, Cat. No. 209012500) (24:1) was added and the tubes were centrifuged at 13,000 revolution per minute (rpm) (Backman Coulter, Germany, cat. No. A46473, Cat. No. A46473) for 10 min. 80 µL of 3 M Sodium Acetate (Applichem, GmbH Darmstadt, Germany, Lot. No. 0E003463) and 520 µL of 99.5% Chilled Isopropanol (Sigma Aldrich, St Louis, MO, USA, Cat. No ZBJ7838, Cat. No. ZBJ7838) was added to extracted solution for rich precipitation of DNA. The DNA pellet was washed with 70% ethanol (Merck KGaA, Darmstadt, Germany, Lot. No K47072383, Lot. No. K47072383) and dissolved in Tris EDTA (Bio Basic Inc., Cat. No. 2616B024). Furthermore, the quality of the DNA was assessed with Nanodrop and Qubit (Biospec-nano (230 V), serial No., A11645200561, P/N, 206-26300-48, Shimadzu, Japan, Takase Rivers).

2.3. Polymerase Chain Reaction (PCR)

The PCR mixture of each sample was prepared of 50 µL reaction volume. PCR amplification was carried out in a 4.8 µL MgCl₂ (25 mM), (Thermo Scientific, Vihius, Lithuania), 5 µL PCR buffer (NH₄)₂SO₄ (10X (Thermo Scientific, Vihius, Lithuania)), 0.6 µL Taq DNA polymerase (5 U/µL) (Ferment Life Science, Vihius, Lithuania Cat no. EP0402, Cat No. EP0402), 1.0 µL dNTPs (25 mM) (Fermentas life Science, Hoffman-La Roche, U.S, Cat. No. 00056721), 4 µL (20 pmol/µL) of ISSR Primer (Synbio Technologies LLC, Monmouth Junction, NJ, USA)), and 4 µL banana DNA template (50 ng/µL), and the total volume was maintained with 26.6 µL nuclease-free water (Invitrogen Thermo Fisher Scientific, Austin, TX, USA, Cat. No. 2005355), Cat. No. 2005355). Amplification was performed in 96 well plates (0.2 mL) (Thermo Scientific, Mexico City, MX, USA, Cat. No. AB-0600), Cat. No. AB-0600). ISSR-PCR amplification was performed at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 40 s, annealing at 52–60 °C for 40 s, and extension at 72 °C for 60 s. The final extension was carried out at 72 °C for 10 min in PCR machine (Bio-Rad, Bio-Rad Laboratories, Inc., Singapore, Cat. No. 621BR62612), -, Cat. No. 621BR62612).

2.4. Gel Electrophoresis

The PCR products were checked on a 3% agarose gel (Thermo Scientific, Vihius, Lithuania, Cat. No. 01200415, Cat. No. 01200415) dissolved in 1x TBE buffer (10.9 g Tris Base (Invitrogen, Bio World, Genelix International Inc, dba bioWORLD Dublin, OH, USA, Cat. No. 42020236-2, Cat. No. 42020236-2), 5.5 g Boric Acid (Daejung, Siheung-si, Korea Lot. No. B0089PG2, Lot. No. B0089PG2), and 0.745 g EDTA (Bio Basic Inc. company, U.S., Cat. No 2616B024, Cat. No. 2616B024) dissolved in 1000 mL of distilled water with adjusting pH 8.00), with 7.5 µL ethidium bromide (Thermo Scientific, Scharlau Chemie S.A. Gato Perez, 33-P-08181, Sentmenat Spain, European Union, CE Label, 10 mg/mL, 10 mg/mL) in agarose gel added for staining. The detected bands were visualized by ultraviolet light

through the Gel Documentation System (Cleaver Scientific manufactured by Synoptics Ltd. Cambridge, UK). The presence and absence of bands were counted and compared with 100 bp and 1 kb DNA Ladder Plus (Thermo Fisher Scientific, Vilnius, Lithuania, Cat. No. SM0323, and Cat. No. EP0402 respectively).

2.5. Scoring and Data Analysis

The amplification product of 14 banana varieties from 40 amplified Inter Simple Sequence Repeats (ISSR) Markers were scored based on absence (0) and presence (1). The molecular size of each fragment was counted by matching with 1 kb DNA Ladder Plus (Thermo Fisher Scientific). The experiment was repeated with biological replicates and technical replicates thrice for each cultivar to provide reliable and repeatable results. Then, the genetic parameters were assessed, such as the percentage of the polymorphic locus (P) [19] and polymorphic information content (PIC), using formulas $p = (k/n) \times 100\%$, and $(PIC) = 1 - \sum (P_i)^2$ respectively [20]. Pair-wise genetic distance matrix among banana varieties was determined according to Jaccard's similarity coefficient. Unweighted pair-group methods with arithmetic mean (UPGMA) were used to construct the tree/dendrogram. Cluster analysis was performed by using the SAHN (Sequential Hierarchical and Nested Clustering) program of NTSYS-PC (Numerical Taxonomy System version 2.1) [21]. Principal component analysis (PCA) and Nei's genetic distance parameters of the banana varieties were computed using POPPR Software version 2.9.2 [22].

3. Results

3.1. Gene Diversity and Polymorphic Information Content (PIC)

To identify the genetic variation and varietal identification among 14 local banana varieties, 40 out of the 45 ISSR primers were used for PCR based amplification of respective loci, and 12 ISSR markers (namely UBC-815, UBC-811, UBC-864, IG-19, ISSR-4, UBC-835, ISSR-3, UBC-827, UBC-808, UBC-822, and UBC-817) uniquely identified 11 varieties of banana. ISSR amplified primers were able to amplify a total of 260 scorable bands and 121 bands were polymorphic which contributed the ratio of polymorphic polymorphism loci (PPL) 46.53 of 40 amplified ISSR markers. The total ratio of polymorphism of (47.87) was observed from 40 ISSR markers and the percentage of PIC value range was between (46–88%) from the 40 amplified ISSR markers. Based on genetic diversity richness, 12 polymorphic ISSR markers exhibited PIC ranged between 80–88%. UBC-834 and UBC-835 detected a higher PIC value range (88–86%) in banana cultivars and UBC-857 detected the lowest PIC value (46%). The maximum numbers of bands were obtained from UBC-840 (300–1500 bp), UBC-834 (200–1400 bp), UBC-835 (200–1300), UBC-857 (500–1100), IG-02 (400–1000), UBC-815 (300–1000), ISSR-3 (300–1000), and UBC-827 (200–1000), while the lowest number of bands were obtained from UBC-809 (100–450) and UBC-ISSR-1 (130–600). The Bi-plot of Principal Component Analysis of three groups of banana varieties shows PC1 contributing 22.14% variation and PC2 13.66% variation. Fenjiao and Dajiao are the most diverse lines as compared with the other two groups. It is suggested that the polymorphic bands observed for different varieties can be used for the identification of 11 banana varieties (Table 1).

A total of 40 ISSR markers detected a total number of alleles = 260 and a total number of polymorphic bands = 121 ($121/260 \times 100 = 46.53$). The detected total number of polymorphisms of the 40 ISSR markers was counted by the sum of 40 individual ISSR markers divided by total number of markers used = 1915.11 ($1915.11/40 = 47.87$).

Table 1. Features of ISSR primers used for ISSR–PCR of 14 banana varieties.

Primer	Scorable Bands	Product Size bp	Polymorphic Bands	Ratio of Polymorphism	PIC
UBC-815	6	300–1000	4	66.66	0.72
UBC-811	10	150–650	5	50	0.83
UBC-864	7	250–650	5	71.42	0.75
UBC-840	8	300–1500	4	50	0.85
ISSR-2	9	200–650	4	44.44	0.85
UBC-862	5	200–500	4	80	0.75
ISSR-4	9	200–850	4	44.44	0.83
IG-03	9	180–700	3	33.33	0.84
IG-23	5	200–600	5	100	0.74
UBC-834	12	200–1400	3	25	0.88
UBC-835	9	200–1300	3	33.33	0.86
UBC-807	10	200–600	3	30	0.84
UBC-818	8	300–900	3	37.5	0.84
UBC-826	7	400–630	3	42.85	0.77
UBC-880	6	300–650	4	66.66	0.78
ISSR-3	5	300–1000	3	60	0.63
UBC-827	8	200–1000	4	50	0.74
IG-02	7	400–1000	3	42.85	0.84
UBC-868	4	250–400	2	50	0.67
IG-19	5	250–900	2	40	0.74
UBC-808	6	100–850	3	50	0.81
UBC-844	6	400–850	2	33.33	0.80
UBC-822	5	300–850	3	60	0.65
IG-05	3	200–400	2	66.66	0.65
UBC-823	6	400–850	2	33.32	0.81
UBC-861	5	380–600	2	40	0.76
UBC-847	3	250–350	1	33.33	0.53
UBC-817	5	200–600	2	40	0.68
UBC-813	5	300–800	2	40	0.68
DBD	2	450–480	1	50	0.58
UBC-857	2	500–1100	1	50	0.46
ISSR-1	10	130–600	5	50	0.81
UBC-812	6	200–600	3	50	0.82
UBC-809	6	100–450	2	33.33	0.78
UBC-810	6	300–650	3	50	0.80
UBC-854	3	150–400	1	33.33	0.56
UBC-842	10	150–650	5	50	0.83
UBC-836	6	200–500	3	50	0.74
UBC-841	6	150–480	2	33.33	0.82
UBC-848	10	200–850	5	50	0.85
Total	260		121	1915.11	
			46.53	47.87	

3.2. Cluster Analysis

Genetic similarity between pair-wise comparisons was considered according to Jaccard's similarity co-efficient, followed by cultivars analysis by UPGMA in the SAHN program of NTSYS-PC version 2.1. The dendrogram separated 14 banana varieties from Pakistan into two distinct groups—i.e., Groups A and B—based on a similarity matrix using the UPGMA method. Both the groups were further divided into subgroups, clusters, and sub-clusters. The similarity coefficient ranged from 0.56 to 0.88. Based on the 40 ISSR markers the cultivars in group A were 66% similar, indicating the level of diversity in that germplasm. The cultivars in group B were 88% similar to each other.

Locally grown 14 banana varieties were clearly differentiated by ISSR markers and further grouped into several sub-clusters. The cultivars in group 'A' Fenjiao (AAB) and Dajiao (ABB) were the most diverging, having a similarity of 66% at the molecular level

with the other ten banana varieties grown in Pakistan. The majority of 10 banana varieties presented high similarity at the molecular level, and were located in group B. The group B was found to be divided into sub-groups, namely B-1 and B-2. Sub-group B-1 consisted of Red Banana which was 65.2% similar at the molecular level to the other eleven varieties.

Sub-group B-2 is divided into clusters, namely B-2-2 and B-2-1. Cluster B-2-1 consisted of 1 banana variety, namely Dhaka, which was 68% similar at the molecular level to the other 10 cultivars. Cluster B-2-2 is further divided into sub-clusters, namely B-2-2-1 and B-2-2-2. Sub-cluster B-2-2-1 consisted of four banana varieties and was distributed into more sub-sub-clusters, namely B-2-2-1A and B-2-2-1B. Sub-sub-cluster B-2-2-1A contained two cultivars, Sprout-1 and Sprout-2, which were 83% similar to the other 8 banana varieties. Sub-sub-cluster B-2-2-1B consisted of two cultivars, Grand Naine and Gayl, which were 80% similar to the other six banana varieties. Sub-cluster B-2-2-2 contained 6 banana varieties, and this was further allocated into sub-sub clusters, namely B-2-2-2A and B-2-2-2B. Sub-sub-cluster B-2-2-2A comprised 3 banana varieties and was categorized into sub-sub-sub-clusters (A) B-2-2-2AA and B-2-2-2AB. B-2-2-2AA comprised Basrai which was 85% similar to the other five banana varieties, and B-2-2-2AB contained 2 banana varieties, ADI and Jafa, with 86% similarity to the other three banana cultivars. Sub-sub cluster B-2-2-2B consisted of three banana varieties and was further divided into sub-sub-sub-clusters (B) B-2-2-2BA and B-2-2-2BB. B-2-2-2BA consisted of three cultivars, including NIGAB-3 and NIGAB-2, with 87.5% similarity. The dendrogram showed that NIGAB-3 and NIGAB-2 are closely related to each other. B-2-2-2BB contained one banana variety, NIGAB-1, and showed 86.5% similarity. The resultant analysis showed that the maximum genetic distance was 34% in group A (Dajiao and Fenjiao). The minimum genetic distance of 12% was observed in group B between two local varieties, NIGAB-2 and NIGAB-3. (Figure 1).

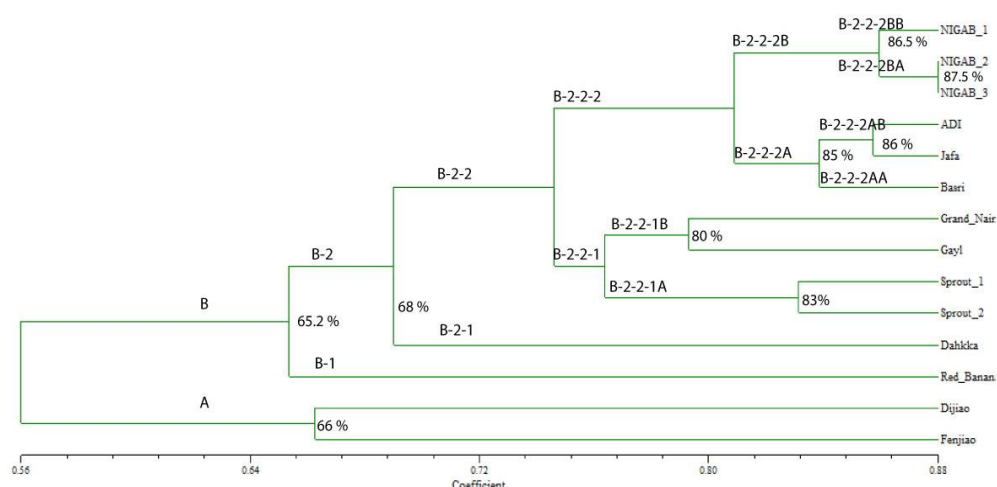


Figure 1. Dendrogram showing the relationships among 14 banana varieties from Pakistan. The similarity coefficient ranged from 0.56–0.88. Multivariate analysis divided 14 banana varieties into two distinct groups, A and B, indicating the varieties in group A were 66% and group B 88% similar to the rest of the population at the molecular level. The result of this analysis showed that the maximum genetic distance was 34% in group A (Dajiao and Fenjiao). A minimum genetic distance of 12% was observed in group B between two local varieties NIGAB-2 and NIGAB-3.

3.3. Principal Component Analysis (PCA) of Three Genomic Groups of 14 Banana Varieties

PCA is a helpful method for exploratory data analysis, showing a better picture of the variation present in 14 locally grown banana cultivars in Pakistan. The PCA bi-plot of the banana data showed diversity among each variety (dots) from the eigenvalue. Based on PCA analysis, principal component 1 contributed 22.14% of the variation, whilst principal component 2 contributed 13.66% of variation concerning PC1. The variation in a variety depends upon its horizontal distance covered in PCA plot X-axis. As far as the eigenvalue is concerned, the Fenjiao and Dajiao varieties can be seen as the most

diverse lines as compared to other cultivars, using data from 40 ISSR markers as depicted in (Figure 2). Principal Components 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13 contributed 10.61, 9.565, 8.494, 8.18, 6.47, 4.975, 4.079, 3.724, 2.855, 2.71, and 2.546 percent (Table 2), respectively. Population Genetics parameters consisted of three measures used to detect the variation in the difference between the varieties used in this study. In this study, a comparison between the varieties present in group 1 and group 2 shows that the Shannon-Wiener index *H* is greater for group 1 (1.79 vs. 1.39), but Stoddard and Taylor's index *G* is also greater (6 vs. 4). This shows that the diversity in the group 1 is lower than that of varieties in group 2. This may be due to the sensitivity of the *H* to the genotype richness in the even sample sizes. Where the *H* is equal for group 2 (1.39 vs. 1.39), *G* is also equal (4 vs. 4) resulting in the divergence among the varieties of groups 2 and 3.

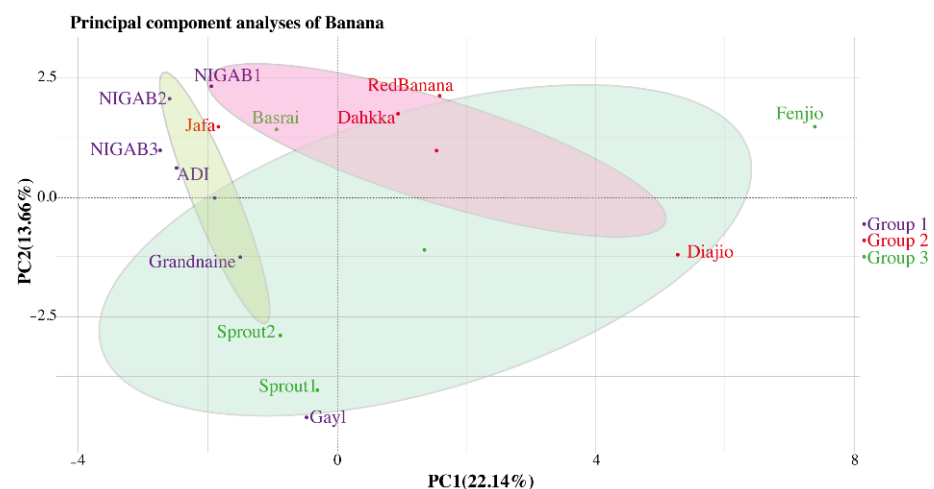


Figure 2. Bi-plot of Principal Component Analysis of three groups of banana varieties showing PC1 contributing 22.14% variation and PC2 13.66% variation. Fenjiao and Dajiao are the most diverse line as compared with the other two groups.

Table 2. Principal Component Analysis (PCA), bi-plot, and varieties of the structure of three genomic constitutions of Banana varieties as revealed by ISSR markers.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC 10	PC 11	PC 12	PC 13	PC 14
Standard deviation	3.0212	2.3728	2.0913	1.98585	1.87144	1.8365	1.6333	1.43221	1.29681	1.2319	1.08501	1.057	1.02461	2.2×10^{-15}
Proportion of Variance	0.2214	0.1366	0.1061	0.09565	0.08494	0.0818	0.0647	0.04975	0.04079	0.03724	0.02855	0.0271	0.02546	0
Cumulative proportion	0.2214	0.3579	0.464	0.55966	0.64461	0.7264	0.7911	0.84086	0.88164	0.91889	0.94744	0.9745	1	1

3.4. Genetic Distance within Conserved DNA-Derived Polymorphism Used in Assessing the Genetic Diversity of Three Genomic Groups of 14 Banana Varieties

Based on Nei's genetic distance, the minimal spanning tree was also generated for three groups of 14 banana cultivars. The tree showed the maximum genetic distance between the varieties, namely Fenjiao and Sprout 2. As far as the node length is concerned, Dajiao and Fenjiao are far apart from each other depicting that these two varieties are more diverse. Likewise, Red Banana and Jafa are more diverse banana varieties, as shown in (Figure 3).

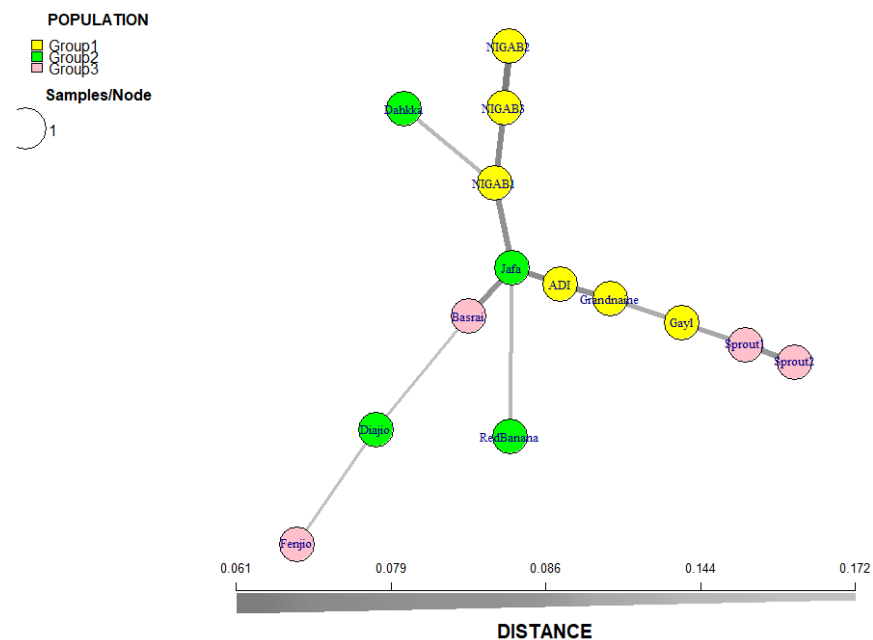


Figure 3. Minimal spanning tree based on Hemming Nei's genetic distance for three groups of banana varieties.

3.5. Varietal Identification Using ISSR Markers

Twelve ISSR markers with several polymorphic bands were amplified and used for varietal identification of locally-grown bananas. The varietal identification approach can be an efficient option in the practical utilization of ISSR markers in the complete and preferable identification of plant varieties. For identification of the 14 local banana varieties, the electro-photogram of 40 reproducible ISSR markers was carefully studied to look for unique reproducible bands which could identify banana varieties under study. Unique PCR bands with 12 ISSR markers were found to identify the 11 local banana varieties successfully except ADI, GAYL, and Jafa. The various PCR patterns of the 12 ISSR markers are shown in (Figures 4 and 5) with indicated unique bands used to identify the 11 banana varieties. NIGAB 1 was identified by unique bands UBC-835⁶⁷⁰, UBC-827²¹⁰, UBC-827³¹⁰, and UBC-808⁸⁵⁰. NIGAB-2 and NIGAB-3 were identified by UBC-827⁵⁰⁰ and UBC-835²²⁰ markers, respectively. Grand-Naine was uniquely identified with eight markers of different band size, i.e., UBC-835⁶⁹⁰, UBC-815⁶⁰⁰, UBC-815⁷⁰⁰, UBC-815⁸⁵⁰, IG-19¹⁰⁵⁰, ISSR-3⁸⁵⁰, UBC-808⁴⁵⁰, UBC-822⁷⁰⁰. Nine markers, namely UBC-811⁶⁸⁰, UBC-815⁶⁰⁰, UBC-815⁷⁰⁰, UBC-815⁸⁵⁰, IG-19¹⁰⁵⁰, ISSR-3⁸⁵⁰, UBC-808⁴⁵⁰, and UBC-822⁷⁰⁰, clearly identified the banana variety Dhaka. Red Banana was uniquely identified using UBC-811⁶⁰⁰, UBC-864⁴⁵⁰, UBC-835¹⁷⁰, ISSR-4³⁵⁰, and UBC-827⁶⁰⁰ markers. Nine ISSR markers identified Dajiao uniquely, namely, UBC-811 at 250 bp, UBC-864 at 220 bp, UBC-864 at 660 bp, IG-19 at 530 bp, UBC-835 at 1150 bp, ISSR-3 at 550 bp, ISSR-3 at 630 bp, UBC-822 at 600 bp, and UBC-826 at 600 bp. Fenjiao was uniquely identified using UBC-811⁵⁰⁰, UBC-864³⁰⁰, UBC-864⁸⁴⁰, UBC-864⁸⁵⁰, UBC-864⁹⁵⁰, IG-19⁸⁵⁰, UBC-835¹⁰⁰⁰, ISSR-41⁴⁰⁰, ISSR-3³²⁰, ISSR-3⁶⁰⁰, UBC-822³¹⁰, UBC-826⁵⁸⁰, and UBC-817²⁵⁰ markers with a unique band size. Basrai was identified by two ISSR primers, namely UBC-827⁵⁵⁰, and UBC-826⁶⁵⁰. Sprout 1 and 2 were uniquely identified using UBC-811²⁹⁰, UBC-815⁶⁹⁰, ISSR-4⁹⁰⁰, UBC-808¹⁷⁰, and UBC-817⁵⁰⁰ respectively. We employed a cultivar identification strategy and successfully identified 11 local banana cultivars using 12 amplified polymorphic ISSR markers (Table 3).

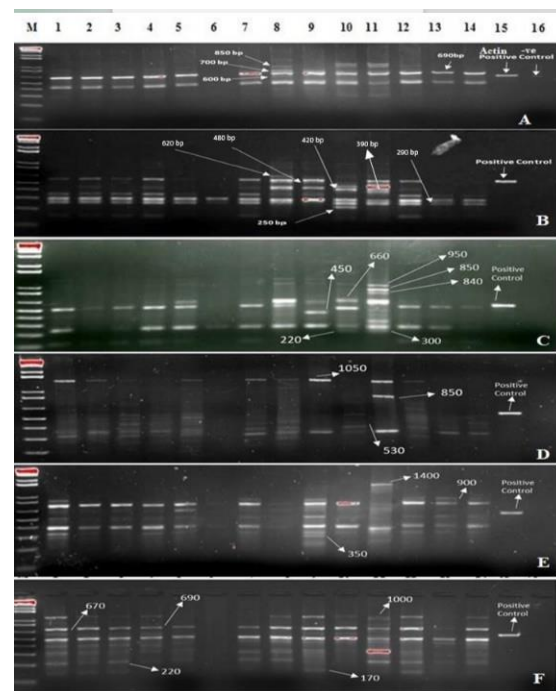


Figure 4. Electropherogram of 14 banana varieties amplified by ISSR markers—M = 1 kb plus DNA ladder, Variety order (1 to 14 from left to right), 1 = NIGAB-1, NIGAB-2, NIGAB-3, Grand Naine, Adi, Gayl, Jafa, Dhaka Red Banana Dajiao, Basrai, Sprout-1 and Sprout-2, 15 = Actin (positive control gene present in a banana), 16 = negative control. White-colored arrows indicate uniquely identified polymorphic bands in eleven banana varieties. (A) ISSR marker UBC-815 uniquely identified Grand Naine, Dhaka, and Sprout-1 with band sizes 600, 690, 700, and 850 bp. (B) ISSR marker UBC-811 uniquely identified Dhaka, Red banana, Fenjiao, Dajiao, and Sprout-1 with band sizes 250, 390, 290, 420, 480, and 620 bp. (C) ISSR marker UBC-864 uniquely identified Red banana, Fenjiao, and Dajiao with band sizes 220, 300, 450, 660, 840, 850, and 950 bp. (D) ISSR marker IG-19 uniquely identified Grand-Naine, Dhaka and Fenjiao with band sizes 530, 850, and 1050 bp. (E) ISSR-4 markers uniquely identified Red Banana, Fenjiao, and Sprout-1 with band sizes 350, 900 and 1400 bp. (F) ISSR marker UBC-835 uniquely identified NIGAB-1, Grand-Naine, Red banana, Dajiao, and Fenjiao with band sizes 170, 220, 670, 690, and 1000 bp.

Table 3. Summary of ISSR markers uniquely identifying the banana varieties of Pakistan.

Variety Name	Markers Uniquely Identifying Varieties
NIGAB-1	UBC-835 ⁶⁷⁰ *, UBC-827 ²¹⁰ , UBC-827 ³¹⁰ , UBC- 808 ⁸⁵⁰
NIGAB-2	UBC-827 ⁵⁰⁰
NIGAB-3	UBC-835 ²²⁰
GRAND-NAINE	UBC-835 ⁶⁹⁰ , UBC-815 ⁶⁰⁰ , UBC-815 ⁷⁰⁰ , UBC-815 ⁸⁵⁰ , IG-19 ¹⁰⁵⁰ , ISSR-3 ⁸⁵⁰ , UBC-808 ⁴⁵⁰ , UBC-822 ⁷⁰⁰
ADI **	Did not produce a unique allele
GAYL **	Did not produce a unique allele
JAFa **	Did not produce a unique allele
DHAKA	UBC-811 ⁶⁸⁰ , UBC-815 ⁶⁰⁰ , UBC-815 ⁷⁰⁰ , UBC-815 ⁸⁵⁰ , IG-19 ¹⁰⁵⁰ , ISSR-3 ⁸⁵⁰ , UBC-808 ⁴⁵⁰ , UBC-822 ⁷⁰⁰ ,
RED BANANA	UBC-811 ⁶⁰⁰ , UBC-864 ⁴⁵⁰ , UBC-835 ¹⁷⁰ , ISSR-4 ³⁵⁰ , UBC-827 ⁶⁰⁰
DAJIAO	UBC-811 ²⁵⁰ , UBC-864 ²²⁰ , UB-864 ⁶⁶⁰ , IG-19 ⁵³⁰ , UBC-835 ¹¹⁵⁰ , ISSR-3 ⁵⁵⁰ , ISSR-3 ⁶³⁰ , UBC-822 ⁶⁰⁰ , UBC-826 ⁶⁰⁰ , UBC-817 ²⁵⁰

Table 3. Cont.

Variety Name	Markers Uniquely Identifying Varieties
FENJIAO	UBC-811 ⁵⁰⁰ , UBC-864 ³⁰⁰ , UBC-864 ⁸⁴⁰ , UBC-864 ⁸⁵⁰ , UBC-864 ⁹⁵⁰ , IG-19 ⁸⁵⁰ , UBC-835 ¹⁰⁰⁰ , ISSR-4 ⁴⁰⁰ , ISSR-3 ³²⁰ , ISSR-3 ⁶⁰⁰ , UBC-822 ³¹⁰ , UBC-826 ⁵⁸⁰
BASRAI	UBC-827 ⁵⁵⁰ , UBC-826 ⁶⁵⁰
SPROUT-1	UBC-811 ²⁹⁰ , UBC-815 ⁶⁹⁰ , ISSR-4 ⁹⁰⁰
SPROUT-2	UBC-808 ¹⁷⁰ , UBC-817 ⁵⁰⁰

* Marker name superscripted by the band size of the marker which uniquely identifies banana variety. ** Varieties Adi, Gayl, and Jafa were not uniquely identified by any marker.

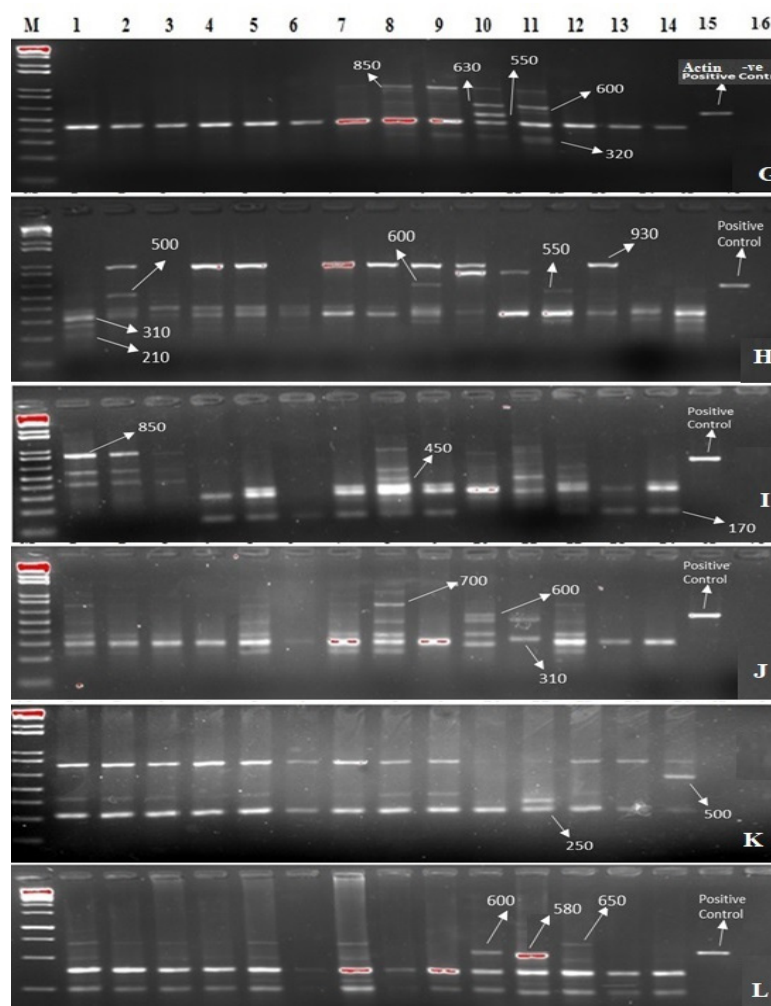


Figure 5. (G) ISSR-3 marker UBC-815 uniquely identified Grand-Naine, Dhaka, and Fenjiao with band sizes 320, 600, 550, 630, and 850 bp. (H) ISSR marker UBC-827 uniquely identified NIGAB-1, NIGAB-2, Red banana, Fenjiao, and Basrai with band sizes 210, 310, 500, 600, 550, and 930 bp. (I) ISSR marker UBC-808 uniquely identified NIGAB-1, Grand-Naine, Dhaka, and Sprout-2 with band sizes 170, 450, and 850 bp. (J) ISSR marker UBC-822 uniquely identified Grand-Naine, Dhaka, Dajiao, and Fenjiao with band sizes 310, 600, and 700 bp, (K) ISSR marker UBC-817 uniquely identified Dajiao and Fenjiao with band sizes 250 and 500 bp. (L) ISSR marker UBC-826 uniquely identified Dajiao, Fenjiao, and Basrai with band sizes 580, 600, and 650 bp.

4. Discussion

In recent years, various target-oriented scientific approaches have been devised to overcome the yield loss inflicted by various conditions for crops, which has huge global impact. A long-term effective technique with fewer side-effects is required, with a combined methodology instead of a short-range approach, and a targeted way out of conventional approaches for the exploration of new variety with increased yield. SSR markers-based technique have been used to investigate the allelic variation of loci controlling rust resistance genes in wheat [23], and have been used to characterize commercially grown banana cultivars based on RAPD, ISSR, and SSR markers [24]. These studies are important because low yields affect the global economy, especially developing countries [25].

ISSR markers are useful in various plant species like peanut, banana, wheat, garlic, and many other crops due to their high efficiency and reproducibility for investigating genetic diversity and variety identification [26]. Present findings in our study indicated a high potential for investigating and characterizing the diversity, as well as varietal identification of *Musa* cultivars at the genomic level [27]. These molecular markers influenced strong discriminatory power; they may be helpful in the validation of uniformity among the plant population [28].

In our study, 40 ISSR markers showed DNA polymorphisms among the 14 banana varieties following banding patterns. ISSR marker techniques in the current study showed that 47.87 polymorphisms revealed by 40 ISSR markers were sufficient to differentiate the banana cultivars in line with previous studies [28]. The genetic distance among the varieties is also related to the nature and the sequence repeat of the motif of the primer used [29]. ISSR marker systems are more effective and efficient, revealing a higher number of alleles, polymorphic bands, and their percentage of polymorphic bands when compared with other polymorphic markers such as SSR, RAPD, and RFLP [13]. We detected higher number of alleles with ISSR primers, i.e., ISSR-2(77), UBC-807(71), IG-02(69), UBC-835(70), UBC-841(65), UBC-848(60), UBC-840(57) and UBC-811(57) compared with the number of alleles reported using UBC-808(57), UBC-827(48), UBC-811 (48), UBC-835(32), and UBC-840(19) [30]. Our results also showed that the total number ($n = 260$) of scorable bands was higher than in the previous studies ($n = 139$ and 125). However, the present study showed a lower number of polymorphisms (47.87) detected after comparison with 21 and 25 accessions (67% and 98%) of *Musa* species [14]. The differences shown in the number of loci and the percentage of polymorphism arise due to the number of markers used and of varieties studied. The number of polymorphic loci is used to investigate the effectiveness of markers in assessing the genetic diversity among various plant species [31]. The range of polymorphic polymorphism loci (PPL) (46.53) generated from 40 ISSR markers is highest when compared to the ones obtained from RAPD (44.44–100%) and lower than those obtained from DAMD (66.66–100%) and ISSR (66.66–100%). A higher ratio of polymorphism (47.87) identifiable by molecular markers has been shown to rely on the presence of repeated sequences of AC, CA, AG, and GA [32]. Interestingly, in our results, 40 ISSR markers produced a lower percentage of PIC value (46.46–88%) compared to the previous studies, which detected a higher PIC value range (91.21–100%) based on 13 ISSR markers [33]. A similar finding was also found in durum wheat where high (100%) polymorphisms were recorded using ISSR markers [26]. Earlier studies were also used to differentiate the *Musa* species and detected a higher 85% polymorphism among the genotypes [34], considerably in contrast with 97% in [14].

Cluster analysis revealed two major groups (A and B) based on 40 ISSR markers data, ordered into different sub-clusters categorized in 14 varieties. The result of these ISSR markers showed genetic variations in several sub-clusters, which resulted in the categorization of some varieties in a separate group because of efficient targeting at different loci. Group B illustrated exclusive topology, due to which they were further divided into 10 sub-clusters. Ref. [32] reported the results of several marker systems like RAPD, AFLP, and SSR, showing five clusters, depending on the number of accessions and the nature of marker used. Based on genetic resemblance inherited from their progenitors, some of

the genomic groups with mixed ploidy were grouped and other groups were resolved separately. Our results showed that “Dajiao” and “Fenjiao” were found in the same group (A), which is in accordance with previous studies in which Pisang Klutuk Wulung and Tani were placed in the same group because of polygenetic earlier findings [35]. High divergence among the various genotypes was also reported by previous studies that emphasized the broad genetic diversity, which also categorizes their place in clusters [36].

The ISSR marker showed polymorphism among groups, subgroups, and clusters, though some could not be differentiated because of their common inheritance [37]. However, similar findings are shown in the present study. Group B (sub-sub-sub-cluster B-2-2-2BA) comprises the three local banana varieties, namely NIGAB-1, NIGAB-2 and NIGAB-3, clustered closely related in the same cluster. This type of relatedness is not unusual between the cultivars due to the cultivars and markers' nature, but these three varieties reported in earlier studies were characterized on the bases of morphological parameters and were more diverse from each other [38]. Two of the banana varieties grown in Pakistan, namely NIGAB-1 and NIGAB-2, were reported as distinct varieties based on morphological characterization, whereas similar studies were conducted and published in Singapore on various banana varieties, namely Grand Nain and Raja Udang (Dwarf Cavendish), which were designated as distinct cultivars on basis of morphological characterization cultivars [39]. After that study, when the research work was instead conducted based on molecular markers, these two varieties clustered together [40]. The variation in morphological traits could be due to euploidy divergence but allelic differences in one or several genes [41]. This type of close relationship has been shown between *M. Acuminate* (A genome) [37].

The principal components analysis (PCA), Nei genetic distance, and spinning tree analysis were performed for the relationship among the 14 closely related local banana varieties. PCA analysis has shown that the variations present at priming sites among the varieties of banana are strongly related to the variance in the data [41]. The PCA results show that PCA-1 and PCA-2 represented 22.14% and 13.66% of the variation in the binary data matrix in banana varieties, respectively. They indicate that a total 35.80% variation present in the 14 locally grown banana varieties could be represented by just two dimensions defined by the first two PCs. Two-dimensional dispersion showed that two, Dajiao and Fenjiao, were distinct from other genotypes. Most of the accessions had low genetic variation. This shows that Dajiao, Fenjiao, and NIGAB-2 have the best potential to adapt themselves according to the changing environment.

DNA-based molecular markers are playing a major role in the variety identification of field-crop and horticulture species [42]. The present investigation confirmed that the 14 local banana varieties could be classified through ISSR primers. We employed a varieties identification strategy and successfully identified 11 local banana varieties by 12 polymorphic ISSR markers. Our studies are in line with the results of [43], which differentiated six cultivars of red fresh Loquat by 12 EST-SSR. Twelve primers uniquely identified a banana variety with different sizes of polymorphic bands, namely Fenjiao. Dajiao was uniquely identified by a maximum of 10 ISSR primers. Grand Nain and Dhaka varieties were uniquely identified by eight primers with different sizes of bands. Four ISSR markers identified NIGAB-1 by giving specific different sizes of polymorphic bands. Three primers differentiate Sprout-1 from the other varieties. Similarly, NIGAB-1 and NIGAB-2 were uniquely identified by one ISSR marker. Basrai and Sprout-2 were uniquely identified by two different ISSR markers.

In conclusion, the ISSR markers study is a helpful technique in characterizing the genetic variation in banana cultivars grown in Pakistan. The present study results were similar to earlier reported studies that have used ISSR markers effectively to assess the genetic diversity information of banana [44] and other economically important plants. In the present investigation, some varieties of banana closely related at the molecular level to other varieties are a part of this study based on ISSR markers. This type of relatedness was also reported in previous studies in which cultivars were distinguished based on morphological characters [45]. The current study has distinguished the ISSR technique

from analyses such as the phylogenetic, with the present study showing that each ISSR primer could show DNA polymorphisms among the 14 banana varieties. We differentiated cultivars according to banding patterns with the help of ISSR markers. Earlier studies reported that these results correlated with the morphological classification of selected banana varieties grown in Pakistan [38]. In our case, however, the results showed that they were significantly different based on ISSR marker and morphological classifications. The dendrogram analysis indicated that the 12 varieties in Group B might have a common ancestor and the other two varieties in group A were diverse from these 12 varieties.

5. Conclusions

ISSR can be successfully employed to characterize the diversity and varietal identification in 14 local banana varieties. The above results obtained by ISSR markers of banana varieties provide useful information for genetic diversity and varietal identification. The phylogenetic data and PCA analysis obtained from the ISSR markers showed a significant variation among the 14 banana cultivars. Finally, we selected 12 markers from the ISSR results that can easily identify 11 varieties within the banana cultivars using a simple PCR technique. Our study after a series of analyses workflow suggested that Dajiao, Fenjiao, and NIGAB-2 can perform very well in a changing environment as compared to all other members of the three populations. This is the first report based on ISSR markers in Pakistan investigating the diversity and identification of varieties of *Musa* species. The primers can be useful in the unequivocal identification of the related *Musa* species.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy12122932/s1>, Table S1. List of ISSR primers used for Genetic Diversity and varietal identification among 14 banana varieties from Pakistan.

Author Contributions: S.N. and A.M. developed the research concept, research work, and data analysis, and wrote the original draft of the manuscript; A.S., K.A., I.H. and M.Z. assisted in the literature search and revision; S.B., M.A., R.M.M.N., H.H. and S.S. assisted in the LAB work; M.N. and I.U.Z., assisted in data compilation and formatting. G.M.A. supervised the research work. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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