ASSESSMENT OF GENETIC DIVERSITY IN BANANA CULTIVARS FROM PAKISTAN BASED ON ISSR MARKERS

BY

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2019

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A Thesis
Submitted to Quaid-i-Azam University, Islamabad in the Partial fulfillment of the requirements for the degree of

MASTER OF PHILOSOPHY
IN
PLANT GENOMICS AND BIOTECHNOLOGY

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2019

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DEDICATION

I sincerely dedicate this life time achievement to my loving and very caring parents whose prays and support made my path smooth and comfortable to my goals. They are my mentor and I am proud of them.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my advisor **Dr. Aish Muhammad** for his sincere guidance and motivation, which helped me in completing my research. I owe my deep and sincere gratitude to my co-advisor **Dr. Armaghan Shehzad** for his guidance and generous supervision throughout my research.

Besides my advisor, I am also grateful to Chairman, DG NARC and all staff members of Plant Genomics and Advanced Biotechnology Department, for facilitating the quality of work in our department.

The road to my degree has been long and windy but I was lucky enough to have the support of my professors and friends especially **Dr. kazim Ali, MuhammadZeeshan, Dr. Iqbal Hussain, Dr. SabahatNoor, Iftikhar Ahmed Tabbasum, Sahir Hameed Khattak, Sania Begum, Aliya Iram, Shehla Shoukat, Shagufta Parveen, Farruhk Ishaq** and **Raja Sheraz Rafique** for helping and supporting me in every vital task.

I am highly obliged to express my sincere feelings for the moral and emotional support and encouragement by my beloved parents, brothers and family members making me strong in reaching my destination successfully.

Saima Noor

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LIST OF ABBREVIATIONS

Words	**Abbreviations**
ISSR	Inter – simple sequence repeats
DNA	Deoxyribonucleic Acid
RFLP	Restriction Fragment Length Polymorphism
RAPD	Random Applied Polymorphism DNA
NIGAB	National Institute of Genomics and Advanced Biotechnology
ARI	Agriculture Research Institute
FAOSTAT	Food and Agricultural Organization of the United Nations Statistical Databases
CTAB	Cetyl-trimethyl Ammonium Bromide
$NaCl_2$	Sodium Chloride
HCl	Hydrochloric Acid
TAE	Tris Acetyl Ethylene
EtBr	Ethidium Bromide
EDTA	Ethylene Diamine Tetra Acetic Acid
PCR	Polymerase Chain Reaction
PVP	Polyvinyl Pyrrolidone
ddH_2O	Double Distilled Water
$(NH_4)_2SO_4$	Ammonium Sulphate

$MgCl_2$	Magnesium Chloride
dNTP	Deoxyribo Nucleotide Triphosphate
PIC	Polymorphic Information Content
A	Actin
UPGMA	Unweighted Pair-group Method using Arithmetic averages
SAHN	Sequential Heirarchial and Nested Clustering
NTSYS-PC	Numerical Taxonomy System version
SNPs	Single Nucleotide Polymorphism
MW	Molecular Weight
BP	Base Pair

ABSTRACT

Banana is a major crop grown in Pakistan but there is a lack of information on genetic diversity and purity in local cultivars, It is quite important in assisting duplication in banana cultivars. Since 1947 till 2016 banana clones were introduced, Basrai was the first clone in Sindh province due to its favorable climatic condition and fertility, later on NIGAB-1, Dhaka, Grand Naine, Fenjiao, NIGAB-3, NIGAB-2 and three cultivars of Costa Rica were successfully adapted. Diversity of banana is very important because so far it is recorded by focusing on morphological parameters rather than genomic approaches. Morphologically, sometimes it becomes very difficult to differentiate among banana cultivars due to influence of environmental factors, time consuming and expensive. So environment greatly effects phenotypic expression make it difficult to differentiate cultivars morphologically. Alternatively, DNA markers based techniques have been employed to rapidly measure the community composition based on the genetic identification of species. Present study employed inter_simple sequence repeats (ISSR) to investigate the genetic variation in local banana cultivars from Pakistan. Out of forty-five primers used, forty primers were reproducible and produced total of 260 scorable bands and 109 were polymorphic, which account to 44.57% per cent polymorphism. UBC-835 and UBC-834 detected a higher level of polymorphism in banana cultivars (86% -88%) and UBC-857 detected lowest level of polymorphism (46%). The size of DNA amplified bands ranged between (100-1500-bp). Pair-wise was calculated Jaccard's similarity co-efficient through unweighted pair group of arithmetic mean (UPGMA) in the SAHN program of NTSYS-PC version 2.1. UPGMA identified and divided fourteen banana cultivars into two prominent and distinct groups A and B and further divided into subgroups, clusters and sub-clusters. The similarity co-efficient ranged between 0.56 - 0.88. This study indicated that at molecular level, the cultivars in group A 66% were similar with other cultivars and 34% genetically differed from other cultivars proving the broad base of germplasm. The cultivars in group B 88% similar with other cultivars and 12% variation existed among the genotype with other cultivars based on ISSR markers used. Analysis clearly revealed that ISSR can be used by the scientist to authenticate cultivar identification for domestication of banana.

Key words: Banana, Genetic diversity, Pakistan, ISSR, Cultivars, Dendrogram Analysis

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Banana and Plantains belong to *Musaceae* family in the order *Zingiberales* (Manzo-Sanchez *et al.,* 2015). The Musa genus is one of the major cash crop in tropical and sub-tropical (Lescot, 2014). Its domestican process started in the region now called South East Asia, 7,000 years ago (D'Hont *et al.,* 2012). Mostly banana germplasm is found in the Asian continent. Apart from this it is also found in Eastern Africa, in some islands of the Pacific Ocean and in Western Africa (Champion, 1967). Banana is forth world crop after wheat, maize and rice (Tripathi *et al*., 2007). Banana is known for its high nutrient profile as it is a rich source of vitamin A, phosphorus, magnesium and potassium. The banana fruit is free from fats, cholesterol and easy to digest. Banana is beneficial by-products, like as fiber, wine, beer, vinegar and vegetables (Aurore *et al.,* 2009). In Pakistan, banana is cultivated on 0.03 million hectares with the annual production of 0.135 million tons as compare to world's cropped area of 5.64 million hectares and yield of 114 million tons. (FAOSTAT, 2018)

Cultivated bananas are basically hybrid of two wild diploid species *Musa acuminate Colla* (AA genome) and *M. balbisiana Colla* (BB genome) (Schwarzacher and Heslop-Harrison, 2007). Banana is parthenocarpic, vegetative hybrid and seedless (Mckey *et al.,* 2010). Inter-interaspecific hybridizations within *M. Acuminata* and *M. Balbisiana* through numerous combinations of A (genome) and B (genome). Genome A and B further categorized into more six groups AA, AAA, AB, AAB, ABB, ABBB (Simmonds and Shepherd, 1955). Most popular cultivated bananas are diploids ($2n=2\times=22$), triploids ($2n=3\times=33$) and tetraploids ($2n=2\times=44$) (Simmonds and Shepherd, 1955). The chromosome number of haploid banana is 11, whose genes number is estimated to be 36,542 (Davey *et al*., 2013). The most of the edible cultivars are allopolyploid triploids with AAA genome whereas plantains have genome AAB and cooking bananas have ABB genome (Jeridi *et al*., 2011; D'Hont, 2012).

Pakistan's province, Sindh is the area where climatic conditions are favorable for banana cultivation. The best climatic conditions and fertility enables Sindh province to produce 87% share of the country. Till day, the most famous banana cultivar in Pakistan "Basrai" first time adopted, cultivated and successfully distributed throughout the Sindh province. Total cultivation land for banana in Sindh province is about 98%

(Junejo, 2014). The first clone i.e. Basrai was introduced before partition. The cultivar William was introduced from Australia in the early nineties, after that Bangladesh introduced the cultivar Dhaka. The cultivar Grand Naine was brought to Pakistan during 2007-09 by the progressive farmers. During 2009-2010, Fenjiao, Pisang, Brazilian and William-8818 and some other Chinese cultivars were introduced. During 2015-2016, three cultivars of Costa Rica were introduced in Pakistan (Junejo, 2014).

In Pakistan, diversity of banana is quite important for future studies on banana improvement there is a need to study the genetic diversity of the local cultivars of bananas and identification of duplication in banana cultivars, furthermore, it is very important to highlight and emphasize the need to ensure that each particular accessions cultivated by farmers. Estimation of genetic diversity in almost every crops is of great importance. The ambiguous names, chances of the cultivation of varietal clones, cultivation of local varieties, uneven distribution, genetic material change within varieties and/or farmers of different countries and regions, uncertainty of varietal certification in nurseries and have complex the identification of genotypes (Chandra *et al.,* 2014). On the other hand, day by day population is increasing and to meet food requirements it is of dire importance to ensure the food demands. One of the way to improve is by growing high yielding cultivars or to grow early maturing varieties in order to save time and resources. But to fulfil such requirements, one must not compromise the nutritional value of the food. The only way to do it, is by finding genetic diversity that will help in meeting such demands. Due to urbanization agricultural land is losing its worth for crop production. Apart from this deforestation, land degradation and climatic stresses leads to extinction of plant species including important food crops therefore modern techniques helps to overcome such issues (Turrent and Serratos, 2004).

To effectively preserve diversity measures of all biological organization, the first fundamental step is observing and second step is assessment (Patrício *et al.,* 2016). In past, cultivars have been broadly categorized by help of morphological traits, such as, plant height, leaf, fruit, and seed characteristics. To assess the genetic diversity, breeders mostly focus on morphological parameters rather than genomic approaches. (Muñoz-Falcón *et al.,* 2011). Morphological studies in *Musa* have been carried out by using different molecular techniques, like morphological characters by (Simmonds,

1962 ; Simmonds and Weatherup, 1990), cytogenetics (Cheesman, 1948 and Osuji *et al,*. 1997), isozymes by (Bhat *et al.,* 1992) ,molecular cytogenetics by (Osuji *et al,*. 1998), cryptic developmental stages (Trontelj and Fišer, 2009) and intergenic spacers evaluated by (Lanaud *et al.,* 1992). These methods in *Musa* species is required specific expertise, costly for routine monitoring especially at large scales and too time consuming, which the results are obtain based on morphological parameters are not broad (Carugati *et al*., 2015). By the use of morphological technique, sometimes differentiating among banana cultivars becomes very difficult. The identification and diversity analysis of cultivars according to morphological approaches is not easy because of influence of environmental factors (Lopez-Vizcón and Ortega, 2012).

On the other hand, molecular characterization has been suggested as a favorable technique to decipher genetic make-up of the plants. Molecular characterization in banana is used to determine the genotypes, their relationship and genetic diversity among banana cultivars. At molecular level the genetic difference present in germplasm molecular technology helpful for determining as compared to morphologically based taxonomic categorizations reveal patterns of genomic differentiation. Molecular technology provide helpful information for cultivar improvement and development which help to breeders to use genetic resources on the population structure, diversity parameters of germplasm and allelic richness with less pre breeding activities. Based on molecular markers new germplasm classifications effects has gained importance due to quick and quality of data generated (Govindaraj *et al*., 2015). Molecular data is able to identify cultivars, otherwise based on morphology would be impossible to identify (Kostamo *et al*., 2013).

In banana, several DNA markers based methods have been used to carry out the genetic diversity in the *Musa* species (Manzo-Sanchez *et al*., 2015), such as , restriction fragment length polymorphism (RFLP) (Gawel *et al.,* 1992), random amplified polymorphism DNA (RAPD) (Bhat *et al*., 1995), inter_ simple sequence repeats (ISSR) (Godwin *et al*., 1997), microsatellites (Grapin *et al*., 1998) and NGS based method (Heslop-Harrison and Schwarzacher, 2007. Molecular technology in banana used to investigate the genotype, genetic diversity, and genetic relationship among banana cultivars. Out of the available techniques, inter_simple sequence repeats (ISSR) markers have been demonstrated to be a consistent, easy to produce,

reproducible, less expensive and multipurpose set of markers. Inter_ simple sequence repeats (ISSR) markers identified repeatable amplification of DNA sequences by using single set of primers (Lu *et al*., 2011). ISSR has been effectively used to analyze the genetic variation in the wild *Musa* germplasm and *Musa acuminate Colla* (Lamare and Rao, 2015). ISSR has been used for the genetic diversity and stability analysis in different sets of banana germplasm of Brazil (Silva *et al.,* 2017), Odisha (Swain *et al.,* 2016), Gujrat region of India (Kharadiet *et al*., 2009), Guangxi, China (Long *et al*., 2009).

Keeping in view the significance of banana crop in Pakistan present research was under taken to investigate the genetic relationship and genetic diversity between different banana cultivars grown in Pakistan.

1.1 Objective

1. To access the genetic diversity amongst banana cultivars grown in Pakistan.

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CHAPTER 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Chandra *et al.,* (2014) conducted research work in some banana hybrids to check the molecular diversity by using ten ISSR primers. Out of six primers amplified scorable markers, 54 bands amplified, the average number of bands per primer was 9. UBC 811 produced highest DNA bands 18 and UBC 812 produced maximum number of polymorphic (100.00%) bands. 30 were polymorphic and 24 were monomorphic. Pairwise genetic distances ranged from 0.31 to 1.00. The dendrogram separated the eighteen hybrids and parents into two major clusters. The first cluster comprised Zonly hybrid H 504. All the other hybrids and parents were grouped into one major cluster two.

Silva *et al.,* (2017) studied and worked on genetic stability and diversity among twenty one banana accessions by using ISSR markers. Twelve primers was used to check the molecular diversity of banana accessions. Twelve primers were amplified and produced 71 fragments. UBC-810 and UBC-864 amplified highest number of bands was 8. Which of them 68 were polymorphic and showed a 97.5% polymorphism at molecular level. Genetic distance matrix was identified by the total twelve primers and data analysis was performed by Jaccard’s similarity coefficient by using UPGMA Method. Two main clusters A and B were categorized and identified in the UPGMA analysis.

Swain *et al.,* (2016) studied and worked to detect the molecular characterization of the twenty two dessert banana cultivars from Odisha was using by ISSR markers. Ten primers out of forty primers were amplified. Total of 76 bands produced, forty were monomorphic and 36 were polymorphic. USB-835 and USB-841 produced the highest number of polymorphic bands. USB-836 produced only one polymorphic band. The lowest band size 100 base pair was produced by the ISSR markers UBC-818 and UBC-864 detected highest band size 3000 base pair. The genetic distance values ranged between 0.71 to 0.96 .The lowest genetic similarity was observed 0.7. Dendrogram constructed by using UPGMA method in the NTSYS-PC version 2.1. Data analysis showed that at molecular level the cultivars were 75% similar to each other and only 25% variation was existed among the genotypes based on the ISSR markers used.

Yuyu *et al.,* (2013) done studies to estimate the variation present at molecular level analyses of twenty five accessions of *Musa Balbisiana Colla* based on ISSR markers in Indonesian. Six ISSR markers was amplified to detect the molecular characterization of twenty five accessions of *Musa Balbisiana Colla* which is originated in Asia, and distributed from India to Papua New Guinea. ISSR primers amplified 61 fragments ranged from 250-2200-bp in size. 29.30 % were polymorphic. The results showed low genetic variation of twenty five accessions of Indonesian *M. balbisiana* with coefficient of similarity distance ranged between 0.81-0.99.

Lu *et al.,* (2011) evaluated a study to access the diversity and genetic stability in thirty banana cultivars from China by using forty five ISSR markers. Forty five inter_simple sequence repeats (ISSR) markers produced bands ranged between 5-9. Total polymorphism was 85.1%. Dendrogram was constructed by using similarity coefficient matrix by using UPGMA method. Data analysis showed that the thirty cultivars divided them into major four clusters. This studies provided at a large genetic diversity present among the selected cultivars. Majority of cultivars bananas present in China are taken from Brazil. Micropragagated plants from Brazil when compared to their mother plants.

Long *et al.,* (2009) studied to investigate the classification and genetic diversity of twenty seven wild banana genotype collected in Guangxi, China. Collected two unique wild species of *Musa* A and B genome. Classified twenty seven genotypes of banana cultivars with the help of sixteen primers out of hundred primers. Total of 168 scorable bands were amplified, which 142 bands were polymorphic. Cluster analyses was performed Jaccard's coefficient similarity index by using UPGMA method. Genetic distance ranged 0.67. Data analysis indicated preset high diversity in the studied materials. This studies showed all selected banana genotypes differentiate to each and other. Previous studies showed that cultivated genotypes genome A and B genotypes could be differentiated from each other. 'Dajiao' and 'Fenjiao' both genotypes were different to each other although they have the same genome.

Kharadi *et al*., (2009) identified the genetic diversity amongst banana cultivars in Gujarat region of India using ISSR markers. This study was to identify genetic variation amongst local hybrids to main banana cultivars by means of ISSR-based genetic analysis. Seven primers were selected. Total of 56 scorable fragments were amplified, 39 primers were polymorphic and 17 were monomorphic. Maximum genetic similarity 94.64% was observed between Mahalaxmi and Robusta. Minimum genetic similarity 50% was observed between Hill banana and Sona. Mahalaxmi, Robusta, Williams, Sona and Grand Naine cultivars as all belongs to same genome group AAA and Hill banana was placed in a separate group, as it belongs to genome group AAB.

Rout *et al.,* (2009) evaluated studies on genetic fidelity and genetic identification of selected micro propagated genotypes of banana by using ISSR markers. Fifteen ISSR primers were used to investigate the four cultivated banana. Distance at molecular level between micro propagated genotypes was estimated and their genetic relationship to each other was found out. The banana cultivars having same genome constitution AAA were grouped together and other cultivars genome constitution BBB was out grouped. Primer IG-13 showed genetic variation at the molecular level in case of Grand Naine. On the other hand, morphologically studies showed they were similar when compared to their mother plant.

(Racharak and Eiadthong. 2007) carried out the hereditary relationship amongst banana cultivars of *Musa acuminata Colla* and edible cultivated bananas examined by using ISSR markers. For this study twelve samples of wild type bananas, thirty-three samples of edible cultivated bananas and *M. balbisiana* were used as plant materials. Six primers were amplified out of 36 primers. Total of 128 bands produced, band size range from 200 - 3,000 base pair. Cluster analyses were carried out on similarity index using UPGMA method. Similarity coefficient ranged value was 0.18. Cluster distributed into main two sub-groups. The first cluster combined two cultivars and second cluster comprised of four banana cultivars including the plantains and edible cultivated bananas.

Godwin *et al.,* (1997) defined applications of ISSR markers for mapping, fingerprinting and genetic diversity for two popular selected crops Sorghum bicolor, and the diploid *Musa Acuminata* (AA genome). Detection methods carried out by polyacrylamide, agarose gel staining with ethidium bromide, silver staining and radioactive. A total of eighty seven primers were used for amplification. Fifty one identified out of eighty seven. Ten primers were amplified which produced the clearest banding patterns. Total of 940 scorable bands, of which 123 were polymorphic and only six primers gave monomorphic bands. Results showed inter _ simple sequence repeats (ISSR), markers are effective and multi-locus, used for finger printing, genetic diversity and for genome mapping. Previous research have exhibited that ISSR detected maximum polymorphism as compared with other molecular techniques.

CHAPTER 3

MATERIALS AND METHOD

3. MATERIALS AND METHODS

The research work was at "National Institute of Genomics and Advanced Biotechnology" (NIGAB), National Agricultural Research Centre, Islamabad. The main aim of this research work was to assess the genetic diversity among banana cultivars grown in Pakistan. The fourteen Cavendish banana cultivars that were used in the proposed study included NIGAB-1, NIGAB-2, NIGAB-3, Grand Naine, ADI, Gayl, Jafa, Red Banana, Dhaka, Dajiao , Fenjiao, Basrai ,Sprout-1 and Sprouts-2. (Table 3.1)

3.1 Plant Material:

Fourteen commercially grown banana cultivars mentioned above from germplasm maintained at Agriculture Research Institute (ARI), Sindh were used for this study (Table 3.1). Fresh and tender leaves were collected in different labeled sealed envelopes for DNA extraction. Three replicates of each cultivar of banana were taken and then stored in refrigerator.

3.2 DNA Markers:

Previously reported forty-five inter_ simple sequence repeats (ISSR) markers, were selected from literature, on the basis of polymorphism for inter _ simple sequence repeats (ISSR), markers and synthesized from Macrogen (Table 3.2).

Table 3.1. List of banana cultivars used for Genetic Diversity from Pakistan

S. No.	Cultivars	Sub-group
1.	NIGAB-1	Cavendish
2.	NIGAB-2	Cavendish
3.	NIGAB-3	Cavendish
4.	Grand-Naine	Cavendish
5.	ADI	Cavendish
6.	Gayl	Cavendish
7.	Jafa	Cavendish
8.	Dhaka	Cavendish
9.	Red Banana	Cavendish
10.	Dajiao	Cavendish
11.	Fenjiao	Cavendish
12.	Basrai	Cavendish
13.	Sprout-1	Cavendish
14.	Sprout-2	Cavendish

Table3.2. ISSR Primers used for Genetic Diversity of fourteen Banana cultivars from Pakistan.

Sr. No	Primer Code	Primer Sequence 5' – 3'	Reference Paper / Journal Name
1	UBC-823	TCT CTC TCT CTC TCT CC	Chandra *et al.,* (2018)
2	UBC-808	AGA GAG AGA GAG AGA GC	Chandra *et al.,* (2018)
3	UBC-812	GAG AGA GAG AGA GAG AA	Chandra *et al.,* (2018)
4	UBC-809	AGA GAG AGA GAG AGA GG	Chandra *et al.,* (2018)
5	UBC-864	ATG ATG ATG ATG ATG ATG	Silva et al., (2017)
6	UBC-834	AGA GAG AGA GAG AGA GTT	Silva *et al.,* (2017)
7	UBC-842	GAG AGA GAG AGA GAG ATG	Swain *et al.,* (2016)
8	UBC-841	GAG AGA GAG AGA GAG ATC	Swain *et al.,* 2016)
9	UBC-840	GAG AGA GAG AGA GAG ATT	Swain *et al.,* (2016)
10	UBC-826	ACA CAC ACA CAC ACA CC	Swain *et al.,* (2016)
11	UBC-811	GAG AGA GAG AGA GAG AC	Swain *et al.,* (2016)
12	UBC-835	AGA GAG AGA GAG AGA GCC	Swain et al. (2016)
13	UBC-836	AGA GAG AGA GAG AGA GCA	Swain *et al.,* (2016)
14	UBC-815	CTC TCT CTC TCT CTC TG	Swain *et al.,* (2016)
15	UBC-818	CAC ACA CAC ACA CAC AG	Swain *et al.,* (2016)
16	UBC-807	AGA GAG AGA GAG AGA GT	Swain *et al.,* (2016)
17	UBC-891	TGT GTG TGT GTG TG	Swain *et al.,* (2016)
18	UBC-880	GGA GAG GAG AGG AGA	Swain *et al.,* (2016)
19	UBC-825	ACA CAC ACA CAC ACA CT	Swain *et al.,* (2016)
20	UBC-810	GAG AGA GAG AGA GAG AT	Swain *et al.,* (2016)
21	UBC-827	ACA CAC ACA CAC ACA CG	Swain *et al.,* (2016)
22	UBC-857	ACA CAC ACA CAC ACA CTG	Lamare and Rao, (2015)
23	UBC-854	TCT CTC TCT CTC TCT CAG	Lamare and Rao, (2015)
24	UBC-813	CTC TCT CTC TCT CTC TT	Lu *et al.,* (2011)
25	UBC-847	CAC ACA CAC ACA CAC AAC	Lu *et al.,* (2011)

26	UBC-855	ACA CAC ACA CAC ACA CCT	Lu *et al.,* (2011)
27	UBC-848	CAC ACA CAC ACA CAC AGG	Lu *et al.,* (2011)
28	UBC-817	CAC ACA CAC ACA CAC AA	Lu *et al.,* (2011)
29	DBD	ACA CAC ACA CAC AC	Thangavelu *et al.,* (2012)
30	VHV	GTG TGT GTG TGT GT	Thangavelu *et al.,* (2012)
31	IG-O5	GAC AGA CAG ACA GAC A	Rout *et al.,* (2009)
32	IG-O6	GAC AGA TAG ACA GAT A	Rout *et al.,* (2009)
33	IG-O3	GAG GGT GGA GGA TCT	Rout *et al.,* (2009)
34	IG-O2	AGA GGT GGG CAG GTG G	Rout et al, (2009)
35	IG-19	A C A C A C A C A C A C A C T G G	Rout *et al.,* (2009)
36	IG-23	GTG TGT GTG TGT GTA CG	Rout *et al.,* (2009)
37	UBC-844	CTC TCT CTC TCT CTC TGC	Racharak *et al.,* (2007)
38	ISSR- 1	GAG AGA GAG AGA GAG AGA C	Godwin *et al.,* (1997)
38	UBC-822	TCT CTC TCT CTC TCT CTC G	Godwin *et al.,* (1997)
40	ISSR- 2	GAG AGA GAG AGA GAG AGA AC	Godwin *et al.,* (1997)
41	UBC-861	ACC ACC ACC ACC ACC ACC T	Godwin *et al.,* (1997)
42	ISSR- 3	CCT CCT CCT CCT CCT CCT T	Godwin *et al.,* (1997)
43	UBC-868	AAG AGG AGG AGG AGG AGG G	Godwin *et al.,* (1997)
44	ISSR -4	AAC AAC AAC AAC AAC ACC C	Godwin *et al.,* (1997)
45	UBC-862	AGC AGC AGC AGC AGC AGC T	Godwin *et al.,* (1997)

3.3 Genomic DNA isolation and quantification:

For genetic diversity, DNA of fourteen Pakistan banana cultivars were extracted by using modified cetyl-trimethyl ammonium bromide (CTAB) (Table 3.5) extraction technique (Doyle and Doyle, 1990). Almost 3g of fresh leaf was grounded in pestle mortar with added 2-3ml extraction buffer (2% CTAB, 100mM Tris HCl, 20mM EDTA, 1.4M NaCl, 0.2% B-merceptoetanol (pH=8). Pipette out 700µl extracted solution and transferred in eppendorf tube and incubated at 65°C for 10 minutes. 700µl of Chloroform: Isoamylalcohol (24:1) was added to purify DNA from other cell constituents. Then the tube was centrifuged for 10min at 12000rpm and the 600µl supernatant was shifted in to a new eppendorf tube and 520µl chilled isopropanol was added and placed at -20°C for 30 mints for DNA precipitation. Added 80ul sodium acetate and centrifuged 10min at 12000rpm, supernatant discarded and pallet was saved. Pellet was raised with 70% ethanol and centrifuged for 3min at 12000rpm. (X2 times repeated this procedure). Dried the DNA pallet for 2hrs and re-suspended in 50µl sterile Milli-Q water. 1µl of RNase A (10 mg/ml) was added and incubated at 37ºC for 45 min to remove RNA, and placed -20° C for further experiment. DNA quantification was determined using Biospec-nano (Shimadzu Corporation Japan) as well as Agarose gel. 1µl of each DNA sample were used to quantify in comparison with elution buffer as blank on Biospec-nano (Fig 3.3). After quantification (Table 3.2), DNA samples were diluted to 50 ng/ul (Fig 3.2)

Table 3.3. Results of absorbance during quantification of DNA Samples on Biospec-nano

Sample	Analyte	Nucleic Acid Conc(ng/µl)	OD260/280	OD260/230
NIGAB-1 (R1)	dsDNA	1350.03	2.41	0.59
NIGAB-1 (R2)	dsDNA	938.84	2.43	0.57
NIGAB-1 (R3)	dsDNA	1350.03	2.41	0.59
NIGAB-2 (R1)	dsDNA	1086.57	2.34	0.64
NIGAB-2 (R2)	dsDNA	1054.82	2.36	0.63
NIGAB-2 (R3)	dsDNA	1054.82	2.36	0.63
NIGAB-3 (R1)	dsDNA	1536.41	2.21	0.64
NIGAB-3 (R2)	dsDNA	1548.3	2.12	0.85
NIGAB-3 (R3)	dsDNA	1536.41	2.21	0.64
Grand Naine (R1)	dsDNA	1135.79	2.38	0.6
Grand Naine (R2)	dsDNA	947.05	2.33	0.64
Grand Naine (R3)	dsDNA	947.05	2.33	0.64
ADI (R1)	dsDNA	902.38	2.48	0.54
ADI (R2)	dsDNA	1684.03	2.62	0.5
ADI (R3)	dsDNA	1684.03	2.62	0.5
Gayl (R1)	dsDNA	3137.87	2.37	0.71
Gayl (R2)	dsDNA	3137.87	2.37	0.71
Gayl (R3)	dsDNA	3137.87	2.37	0.71
Jafa (R1)	dsDNA	2170.55	2.18	0.89
Jafa (R2)	dsDNA	1071.48	2.14	0.83
Jafa (R3)	dsDNA	2170.55	2.18	0.89
Dhaka (R1)	dsDNA	1099.37	2.53	0.52
Dhaka (R2)	dsDNA	1500.13	2.51	0.75
Dhaka (R3)	dsDNA	1099.37	2.53	0.52
Dajiao (R1)	dsDNA	1621.87	2.31	0.8
Dajiao (R2)	dsDNA	1500.13	2.25	0.8
Dajiao (R3)	dsDNA	1621.87	2.31	0.8
Fenjiao (R1)	dsDNA	971.66	2.24	0.8

Fenjiao (R2)	dsDNA	976.65	2.25	0.69
Fenjiao (R3)	dsDNA	976.65	2.25	0.69
Basrai (R1)	dsDNA	1260.59	2.36	0.64
Basrai (R2)	dsDNA	1260.59	2.36	0.64
Basrai (R3)	dsDNA	1260.59	2.36	0.64
Sprout-1 (R1)	dsDNA	678.44	2.01	0.61
Sprout-1 (R)	dsDNA	678.44	2.01	0.61
Sprout-1 (R3)	dsDNA	678.44	2.01	0.61
Sprout-2 (R1)	dsDNA	356.34	1.92	0.45
Sprout-2 (R2)	dsDNA	356.34	1.92	0.45
Sprout-2 (R3)	dsDNA	356.34	1.92	0.45

3.4 Gel electrophoresis and DNA quantification:

First quantified DNA samples on Biospec-nano (Table 3.4). After that, DNA samples diluted at 50 ng/ul. Dilution of DNA was done by using this standard equation:

$$C_1V_1=C_2V_2$$

For DNA quantification, diluted DNA samples were quantified 1% agarose were added 100ml of 1x TAE buffer (Table 3.6) stained with ethidium bromide (400ng/ml) for 20min at 100V. Equal volumes of lambda DNA standard 50ng/ul (well 1) and 100 ng/ul (well 2) and 3ul of DNA samples (well 3-well 4) was loaded in the wells of the gel (Fig 3.1). The second gel was run with same procedure with 3 population of each cultivar (Fig 3.3). DNA Samples were visualized under UV by using Gel Documentation System (Alpha Innotech). Concentration of DNA samples was assessed by comparing with Lambda DNA standard Biospec-nano and agarose gel

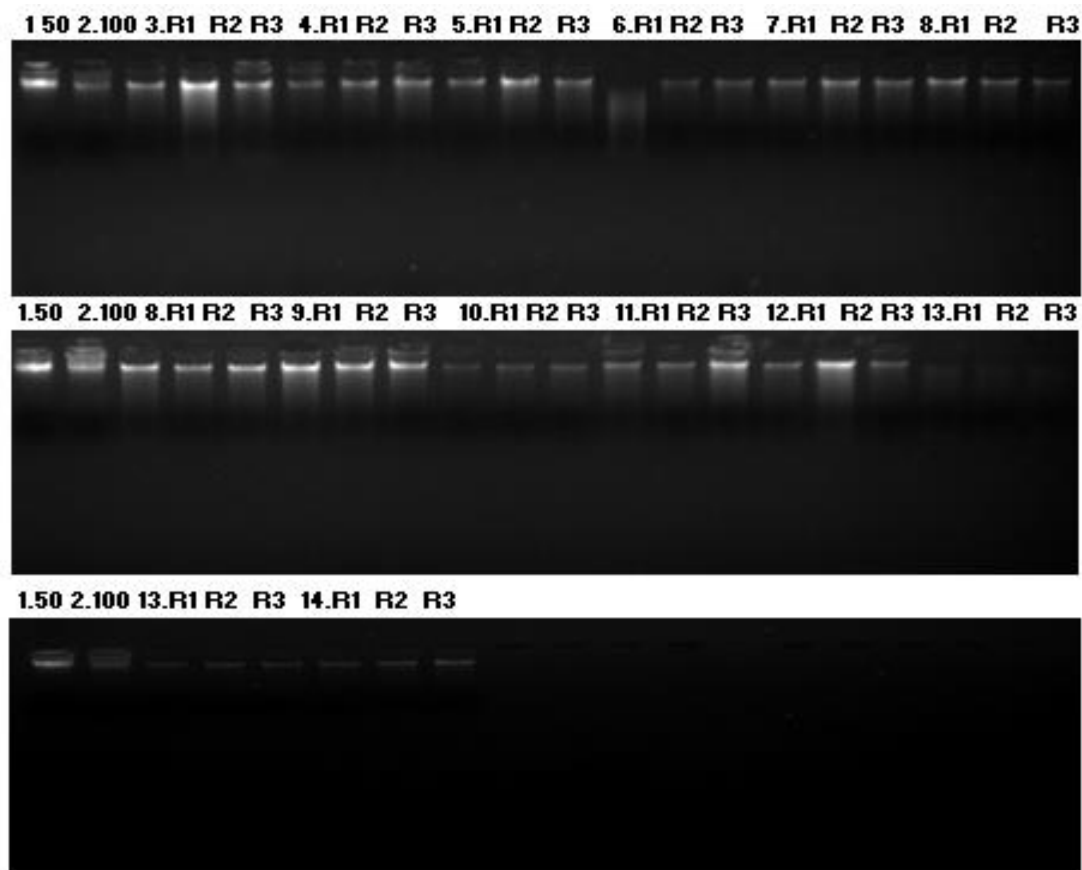


Figure 3.1: Gel electrophoresis for detection of DNA samples (three replicates) of each fourteen Banana cultivars from Pakistan 1. 50ng/ul Lambda DNA, 2. 100ng/ul Lambda DNA, 3. NIGAB-1, 4. NIGAB-2, 5. NIGAB-3, 6. Grand Naine, 7. ADI, 8. Gayl, 9. Jafa, 10. Dhaka, 11. Red banana, 12. Dajiao, 13. Fenjiao, 14. Basrai, 15. Sprout-1, 16. Sprout-2

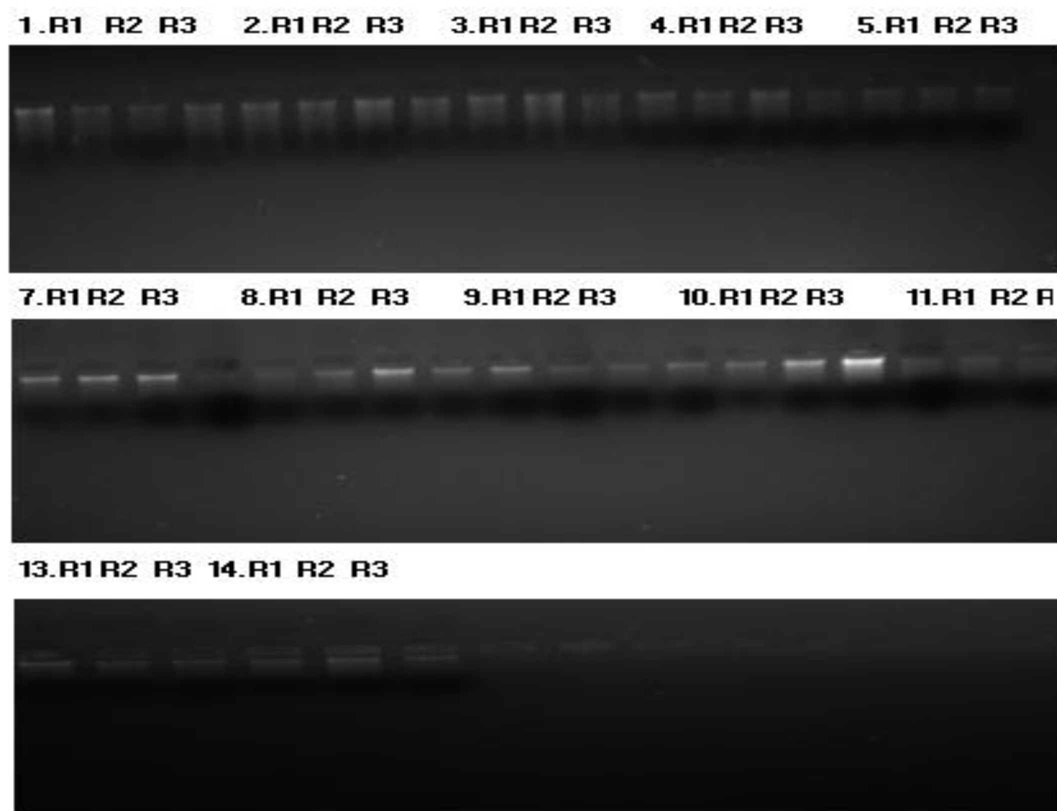


Figure 3.2: Gel electrophoresis of diluted DNA samples (three replicates) of each fourteen cultivars from Pakistan (agarose 1%) 1. NIGAB-1, 2. NIGAB-2, 3. NIGAB-3, 4. Grand Naine, 5. ADI, 6. Gayl, 7. Jafa, 8. Dhaka, 9. Red banana, 10. Dajiao, 11. Fenjiao, 12. Basrai, 13. Sprout-1, 14. Sprout-2

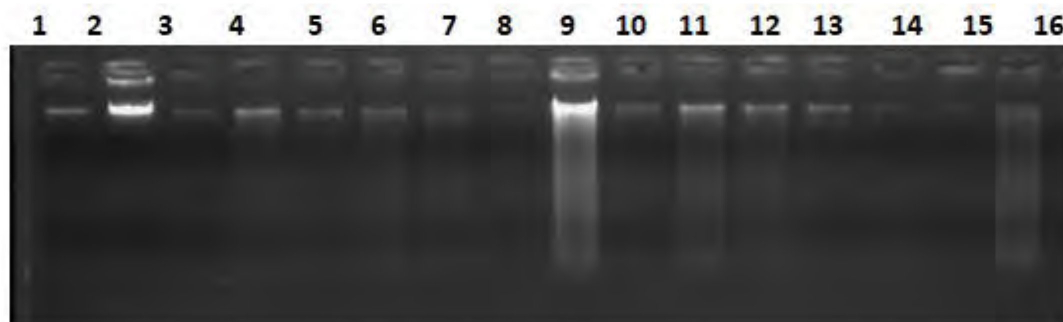


Figure 3.3: Gel electrophoresis of 50ng/ul diluted DNA samples of fourteen banana cultivars used for PCR amplification. 1. 50ng/ul Lambda DNA, 2. 100ng/ul Lambda DNA, 3. NIGAB-1, 4. NIGAB-2, 5. NIGAB-3, 6. Grand Naine, 7. ADI, 8. Gayl, 9. Jafa, 10. Dhaka, 11. Red banana, 12. Dajiao, 13. Fenjiao, 14. Basrai, 15. Sprout-1, 16. Sprout-2

Table 3.4. Absorption values of DNA samples of Banana cultivars used for PCR amplification.

Sample	Analyte	Nucleic Acid Conc (ng/µl)	OD260/280	OD260/230
NIGAB-1	dsDNA	404.90	1.98	0.53
NIGAB-2	dsDNA	294.73	2.00	0.43
NIGAB-3	dsDNA	418.82	1.95	0.45
Grand Naine	dsDNA	404.49	2.13	0.46
ADI	dsDNA	252.47	1.92	0.44
Gayl	dsDNA	251.02	1.42	0.52
Jafa	dsDNA	798.42	1.95	0.60
Dhaka	dsDNA	216.48	1.51	0.30
Red Banana	dsDNA	931.76	1.84	0.73
Dajiao	dsDNA	282.05	2.16	0.43
Fenjiao	dsDNA	407.96	1.76	0.72
Basrai	dsDNA	282.39	1.69	0.37
Sprout-1	dsDNA	678.44	2.01	0.61
Sprout-2	dsDNA	356.34	1.92	0.45

Table 3.5. Composition of 2% CTAB

Chemicals	MW	Final Concentration	100 ml
Tris Base (pH 8.0)	121.11 g/mole	100mM	10ml
0.5M Na_2EDTA (pH 8.0)	372.23 g/mole	20mM	4mM
CTAB	364.4 g/mole	2%	2g
$NaCl_2$	58.44 g/mole	1.4M	8.18g
PVP	40,000 g/mole	1%	1g
ddH_2O	18		100ml

Table 3.6. Preparation of 50X Stock of TAE (Tris Acetyl EDTA) 1L Buffer.

Chemicals	MW	Quantity
Tris Base	121.11g/mole	242g
0.5M EDTA	372.23g/mole	100ml
Glacial Acetic Acid	60.052g/mole	57.1ml
ddH_20	18	600ml

Bring volume to 1000ml with ddH20

3.5 Dilution of the Primers:

Forty-five ISSR primers sequence are given (Table 3.2). ISSR primers were commercially synthesized from Macrogen. The primers stock (100pmol/ul) was prepared by added sterile Milli-Q water i.e. nmoles = ul of sterile Milli-Q water. Primers were diluted from stock (100pmol/ul) to a working concentration of 30pmol/ul primers from stock by adding 70pmol/ul of sterile Milli-Q water

3.6 Polymerase chain reaction:

Polymerase chain reaction (PCR) mixture was prepared in volume 25µl reaction. Each cultivar was separated three times for PCR analysis. The (1X) reaction mixture, contained 2.4 µl (10X) PCR buffer $(NH_4)_2SO_4$, 2.4 µl (25mM) $MgCl_2$, 0.3 µl (5U/ µl) Taq DNA polymerase (Ferment Life Science), 0.5 µl (25mM) dNTPs, 2 µl (20pmol) Primer, 25ng/µl DNA template from Banana and total volume was maintained with sterile Milii-Q water. PCR based amplification was done in an applied Biosystems Thermal Cycler (Veriti 96 wells). Amplification was performed in PCR plates. ISSR-.PCR amplification was performed in a applied Biosystems Thermal Cycler (Veriti 96 wells) at 94°C for 4mins, followed by 35 cycles, each cycle consisting of 3 steps, first step denaturation at 94°C: 40sec, in second step annealing temperature 52°C - 60°C: 40secs and third step extension at 72°C: 1mins final extension was achieved out at 72° C: 10mins and stored at 4°C. (Table 3.8).

Table 3.7. Readiness of ISSR-PCR reaction mix 25ul.

S. No.	Components	Stock	Final Concentration	1(ul) Reaction mix	25 (ul) Reaction Mix
1	10x-PCR buffer	10X	1x	2.4	60
2	$MgCl_2$	25mM	3.0mM	2.4	60
3	dNTP MIX	10mM	0.2mM	0.5	12.5
4	Primer	10pmol	10pmol	2	50
5	Taq DNA polymerase	5U/ ul	1U/25ul	0.3	7.5
6	Template DNA	25ng/ ul	25ng/rxn	2	2
7	H_2O(Nuclease Free)			15.4	385

Table 3.8. ISSR Primers and their ISSR-PCR Profiles used for Genetic Diversity in Banana Cultivars from Pakistan.

Sr. No	Primer Name	Sequence	Annealing Tmp	ISSR-PCR Profile
1.	UBC-815	CTC TCT CTC TCT CTC TG	55°C	94°C : 3min (1) cycle 94°C : 40s, 55°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)
2.	UCB-811	GAG AGA GAG AGA GAG AC	55°C	94°C : 3min (1) cycle 94°C : 40s, 55°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)
3.	UBC-864	ATG ATG ATG ATG ATG ATG	55°C	94°C : 3min (1) cycle 94°C : 40s, 55°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)
4.	UBC-840	GAG AGA GAG AGA GAG ATT	55°C	94°C : 3min (1) cycle 94°C : 40s, 55°C : 40s,72°C:1min (35cycles) 72°C : 10min (1 cycle)
5.	ISSR-2	GAG AGA GAG AGA GAG AGA AC	55°C	94°C : 3min (1) cycle 94°C : 40s, 55°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)
6.	UBC-862	AGC AGC AGC AGC AGC AGC T	53°C	94°C : 3min (1) cycle 94°C : 40s, 53°C : 40s,72°C:1min (35cycles) 72°C : 10min (1 cycle)
7.	ISSR-4	AAC AAC AAC AAC AAC ACC C	53°C	94°C : 3min (1) cycle 94°C : 40s, 53°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)

8.	IG-03	GAG GGT GGA GGA TCT	55°C	94°C : 3min (1) cycle 94°C : 40s, 55°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)
9.	IG-23	GTG TGT GTG TGT GTA CG	58°C	94°C : 3min (1) cycle 94°C : 40s, 58°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)
10.	UBC-834	AGA GAG AGA GAG AGA GTT	55°C	94°C : 3min (1) cycle 94°C: 40s, 55°C: 40s,72°C:1min (35cycles) 72°C: 10min (1 cycle)
11.	UBC-835	AGA GAG AGA GAG AGA GCC	56°C	94°C : 3min (1) cycle 94°C : 40s, 56°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)
12.	UBC-807	AGA GAG AGA GAG AGA GT	55°C	94°C : 3min (1) cycle 94°C : 40s, 55°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)
13.	UBC-818	CAC ACA CAC ACA CAC AG	55°C	94°C : 3min (1) cycle 94°C : 40s, 55°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)
14.	UBC-826	ACA CAC ACA CAC ACA CC	56°C	94°C : 3min (1) cycle 94°C : 40s, 56°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)
15.	UBC-880	GGA GAG GAG AGG AGA	55°C	94°C : 3min (1) cycle 94°C : 40s, 55°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)
16.	ISSR-3	CCT CCT CCT CCT CCT CCT T	55°C	94°C : 3min (1) cycle 94°C : 40s, 55°C : 40s,72°C:1min (35cycles) 72°C : 10min (1 cycle)

17.	UBC-827	ACA CAC ACA CAC ACA CG	55°C	94°C : 3min (1) cycle 94°C : 40s, 55°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)
18.	IG-02	AGA GGT GGG CAG GTG G	55°C	94°C : 3min (1) cycle 94°C : 40s, 55°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)
19.	UBC-868	AAG AGG AGG AGG AGG AGG G	52°C	94°C : 3min (1) cycle 94°C : 40s, 52°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)
20.	IG-19	ACA CAC ACA CAC ACT GG	55°C	94°C : 3min (1) cycle 94°C : 40s, 55°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)
21.	UBC-808	AGA GAG AGA GAG AGA GC	55°C	94°C : 3min (1) cycle 94°C : 40s, 55°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)
22.	UBC-844	CTC TCT CTC TCT CTC TGC	55°C	94°C : 3min (1) cycle 94°C : 40s, 55°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)
23.	UBC-822	TCT CTC TCT CTC TCT CTC G	55°C	94°C : 3min (1) cycle 94°C : 40s, 55°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)
24.	IG-05	GAC AGA CAG ACA GAC A	55°C	94°C : 3min (1) cycle 94°C: 40s, 55°C: 40s,72°C:1min (35cycles) 72°C: 10min (1 cycle)
25.	UBC-823	TCT CTC TCT CTC TCT CC	55°C	94°C : 3min (1) cycle 94°C : 40s, 55°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)

26.	UBC-861	ACC ACC ACC ACC ACC ACC T	55°C	94°C : 3min (1) cycle 94°C : 40s, 55°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)
27.	UBC-847	CAC ACA CAC ACA CAC AAC	55°C	94°C : 3min (1) cycle 94°C : 40s, 55°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)
28.	UBC-817	CAC ACA CAC ACA CAC AA	55°C	94°C : 3min (1) cycle 94°C : 40s, 55°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)
29.	UBC-813	CTC TCT CTC TCT CTC TT	55°C	94°C : 3min (1) cycle 94°C : 40s, 55°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)
30.	DBD	ACA CAC ACA CAC AC	55°C	94°C : 3min (1) cycle 94°C : 40s, 55°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)
31.	UBC-857	ACA CAC ACA CAC ACA CTG	55°C	94°C : 3min (1) cycle 94°C: 40s, 55°C: 40s,72°C:1min (35cycles) 72°C: 10min (1 cycle)
32.	ISSR-1	GAG AGA GAG AGA GAG AGA C	58°C	94°C : 3min (1) cycle 94°C : 40s, 58°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)
33.	UBC812	GAG AGA GAG AGA GAG AA	56°C	94°C : 3min (1) cycle 94°C : 40s, 56°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)
34.	UBC809	AGA GAG AGA GAG AGA GG	55°C	94°C : 3min (1) cycle 94°C : 40s, 55°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)

35.	UBC810	GAG AGA GAG AGA GAG AT	58°C	94°C : 3min (1) cycle 94°C : 40s, 58°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)
36.	UBC854	TCT CTC TCT CTC TCT CAG	58°C	94°C : 3min (1) cycle 94°C : 40s, 58°C: 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)
37.	UBC842	GAG AGA GAG AGA GAG ATG	56°C	94°C : 3min (1) cycle 94°C : 40s, 56°C: 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)
38.	UBC-836	AGA GAG AGA GAG AGA GCA	56°C	94°C : 3min (1) cycle 94°C : 40s, 56°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)
39.	UBC-841	GAG AGA GAG AGA GAG ATC	56°C	94°C : 3min (1) cycle 94°C : 40s, 56°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)
40.	UBC-848	CAC ACA CAC ACA CAC AGG	55°C	94°C : 3min (1) cycle 94°C : 40s, 55°C : 40s,72°C :1min (35cycles) 72°C : 10min (1 cycle)

3.7 Gel electrophoresis:

PCR amplified product was dissolved in 3% of agarose gel, added (0.001g/ml) concentration of ethidium bromide for staining the gel. Gel run for 1hr and 30 min at 100V. The amplified bands were visualized under ultraviolet light using Gel Documentation System (Alpha Innotech). Presence and absence of bands were counted and compared with 1kb plus DNA Ladder Plus (Invitrogen) with well-known band sizes (100-15000-bp).

3.8 PIC (Polymorphic Information Content) value for count of forty markers:

To find PIC values of all markers "total number of counts 1 (heterozygosity) divided by total number of alleles count" i.e. Pi 2÷36 = 0.055556, square the Pi^2 = (0.055556) ^2 and addition of ΣPi^2 and finally subtraction from (1- PΣi^2) by using the following standard equation (Botstein *et al.,* 1980).

$$PIC= 1- P\Sigma i^2$$

Like example of one marker UBC815

Table No.3.9: PIC value (Example)

Count 0	**12**	**1**	**1**	**12**	**10**	**12**
Count 1	**2**	**13**	**13**	**2**	**4**	**2**
Total allele count	36	36	36	36	36	36
Pi	0.055556	0.361111	0.361111	0.055556	0.111111	0.055556
pi^2	0.003086	0.130401	0.130401	0.003086	0.012346	0.003086
Σpi^2						0.282407
1-pΣi^2						0.717593

CHAPTER 4

RESULTS

4. RESULT

UBC 815

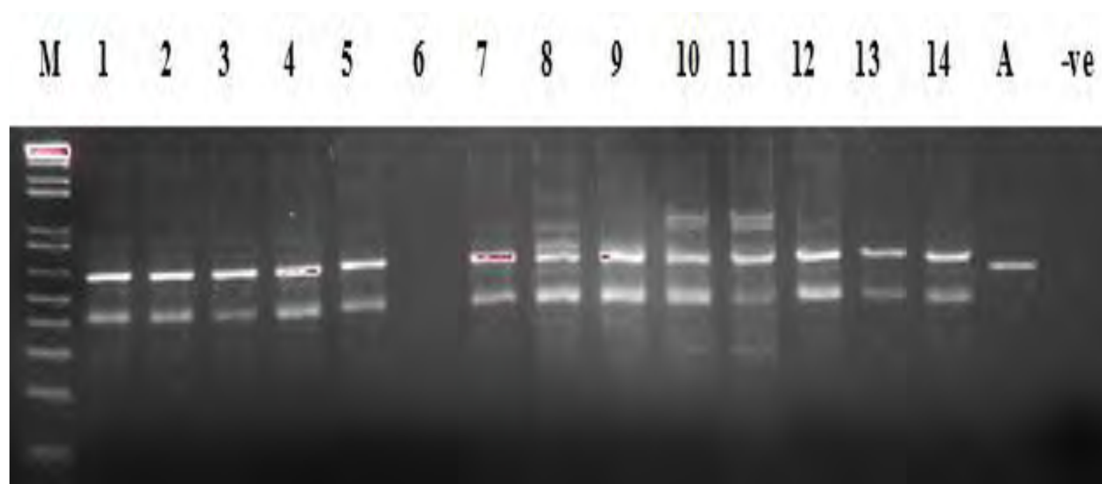


Figure 4.1: PCR amplification pattern of ISSR UBC-815 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

UBC 811

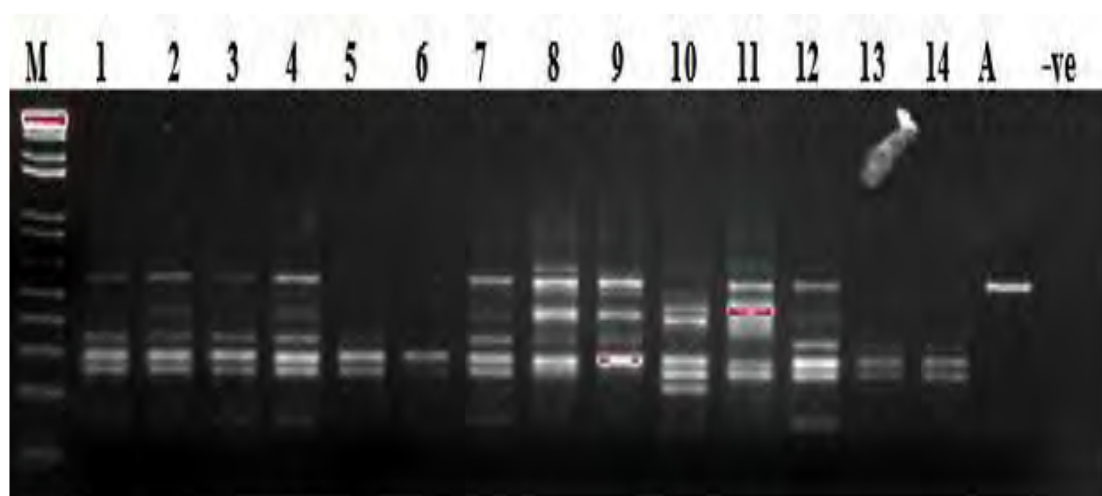


Figure 4.2: PCR amplification pattern of ISSR UBC-811 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

UBC864

Figure 4.3: PCR amplification pattern of ISSR UBC-864 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

UBC 840

Figure 4.4: PCR amplification pattern of ISSR UBC-840 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

ISSR-2

Figure 4.5: PCR amplification pattern of ISSR-2 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

UBC862

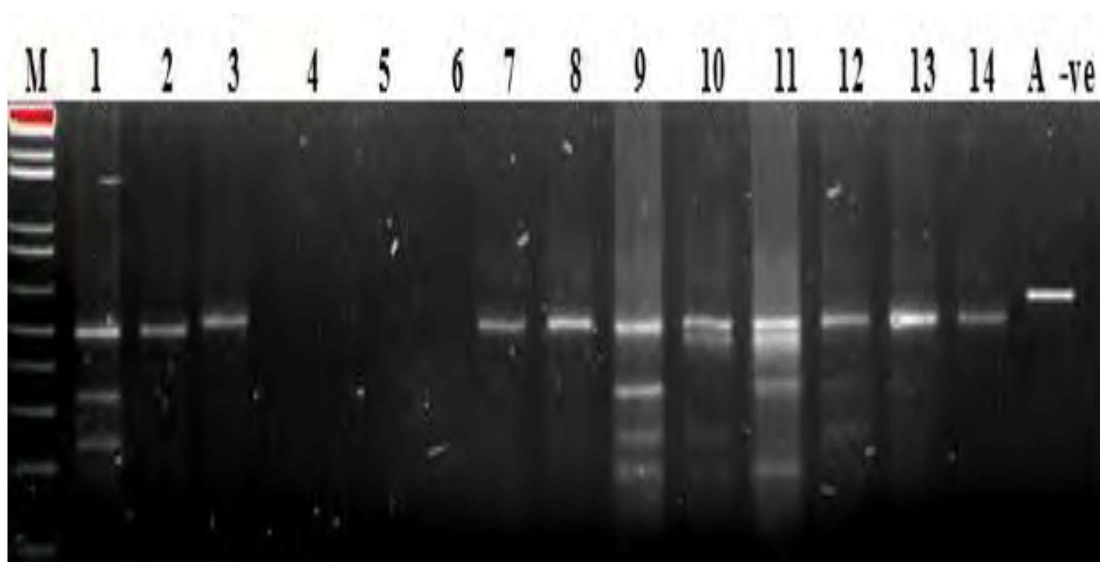


Figure 4.6: PCR amplification pattern of ISSR UBC-862 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

ISSR-4

Figure 4.7: PCR amplification pattern of ISSR-4 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

IG-03

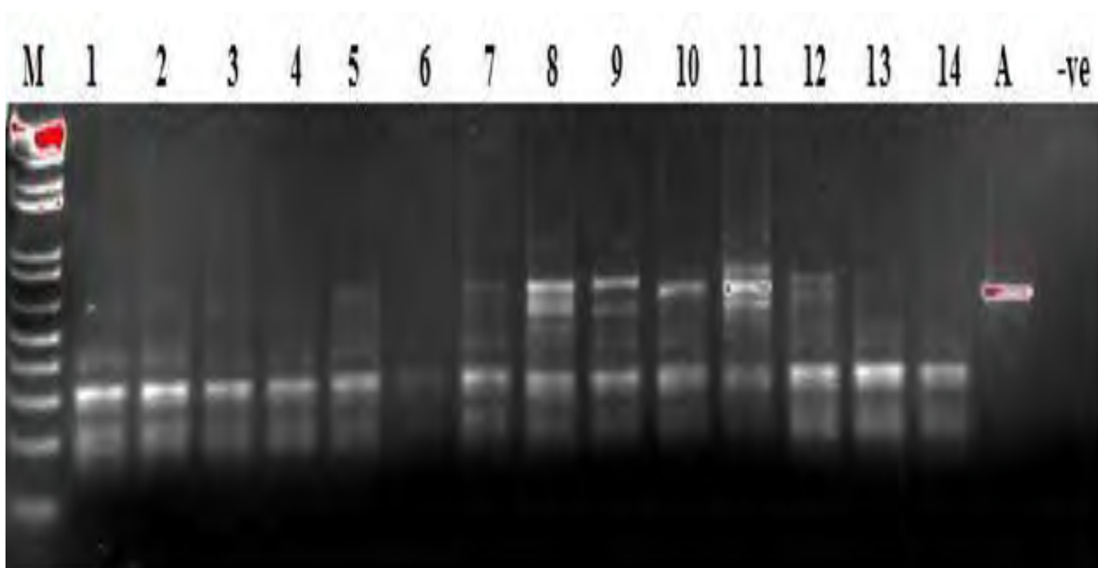


Figure 4.8: PCR amplification pattern of ISSR IG-03 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

IG-23

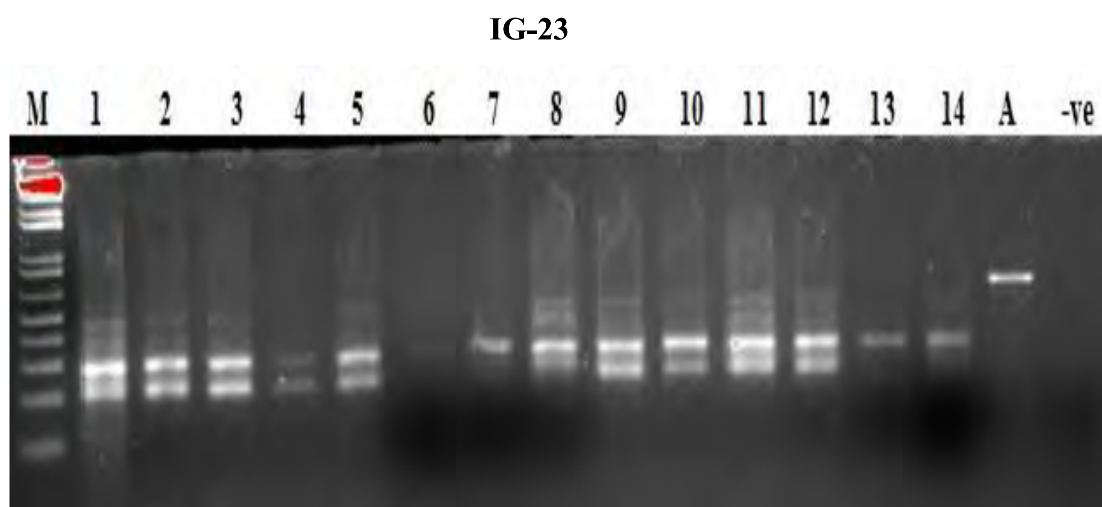


Figure 4.9: PCR amplification pattern of ISSR IG-23 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

UBC 834

Figure 4.10: PCR amplification pattern of ISSR UBC-834 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

UBC 835

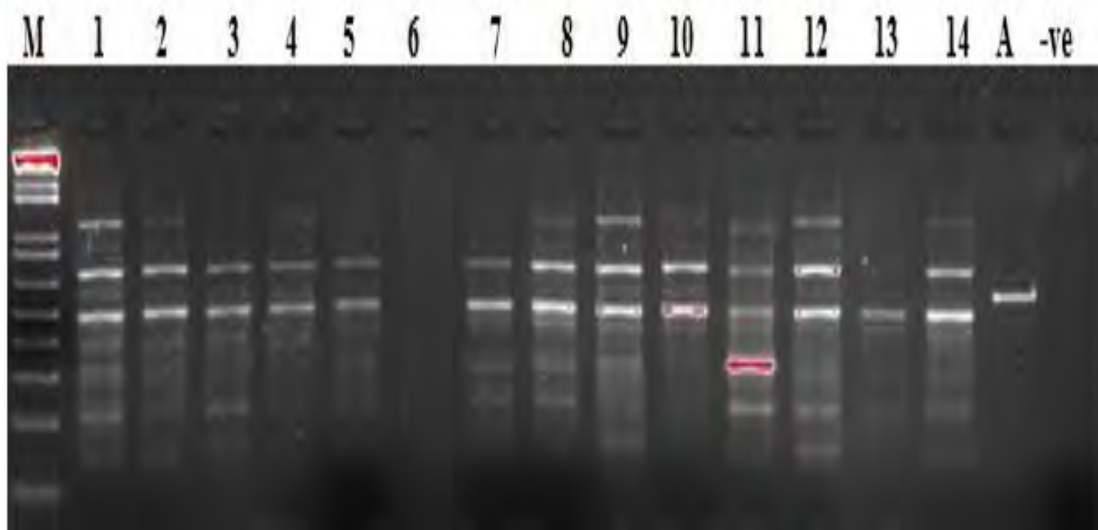


Figure 4.11: PCR amplification pattern of ISSR UBC-835 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

UBC 807

Figure 4.12: PCR amplification pattern of ISSR UBC-807 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

UBC 818

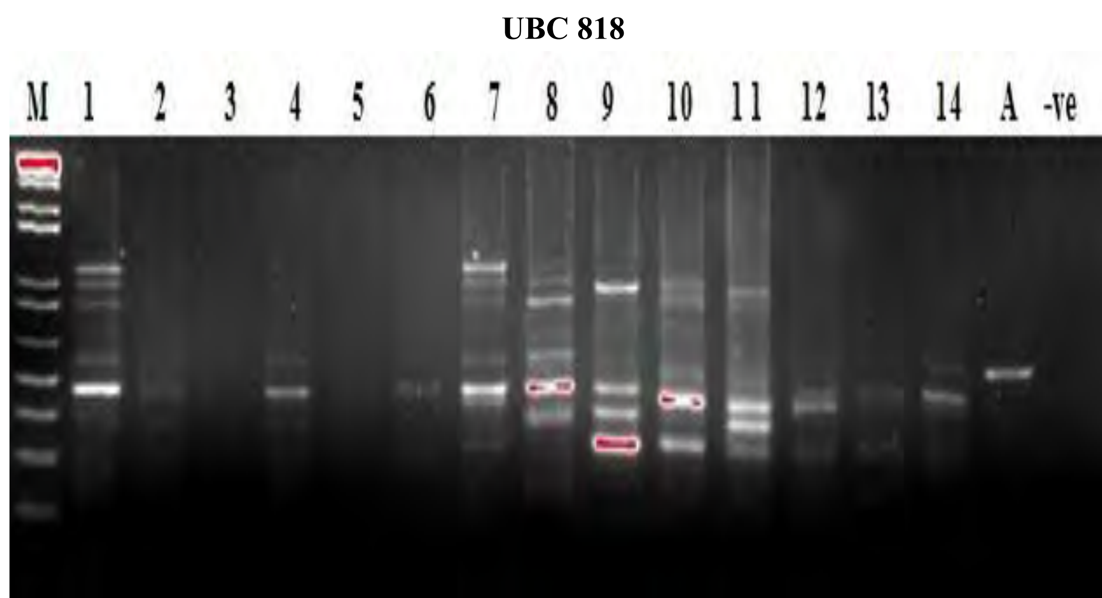


Figure 4.13: PCR amplification pattern of ISSR UBC-818 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

ISSR-1

Figure 4.14: PCR amplification pattern of ISSR-1 indicating diversity of fourteen banana from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1, 2. NIGAB-2, 3. NIGAB-3, 4. Grand Naine, 5. ADI, 6. Gayl, 7. Jafa, 8. Dhaka, 9. Red banana, 10. Dajiao, 11. Fenjiao, 12. Basrai, 13. Sprout-1, 14. Sprout-2, 15. Actin, 16. –ve Control

UBC 812

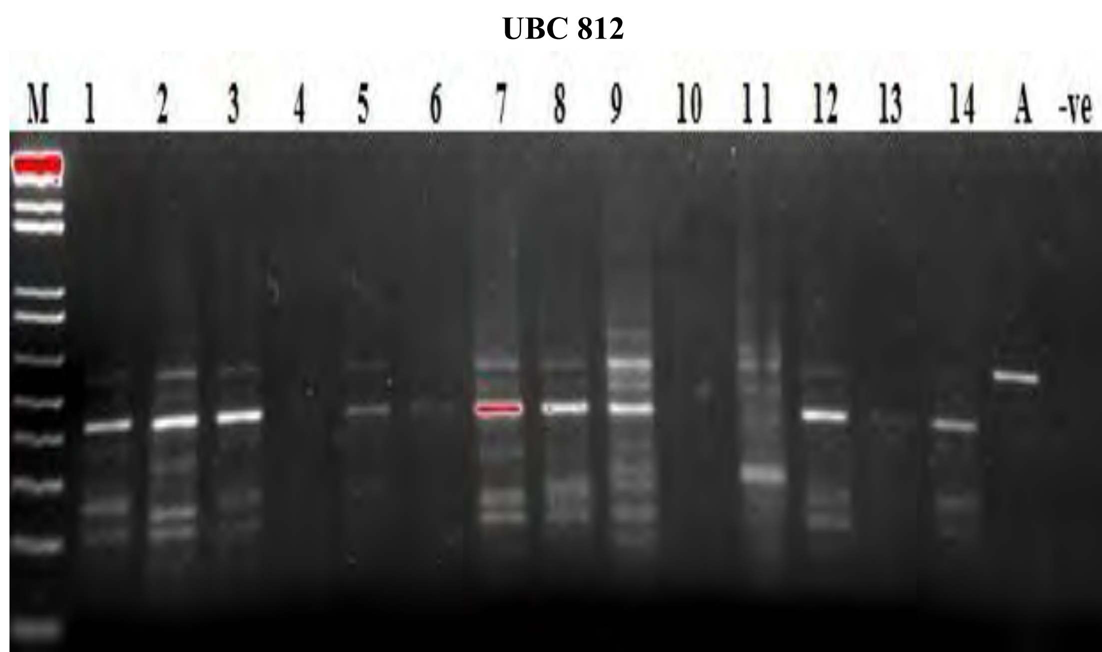


Figure 4.15: PCR amplification pattern of ISSR UBC-812 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

UBC 809

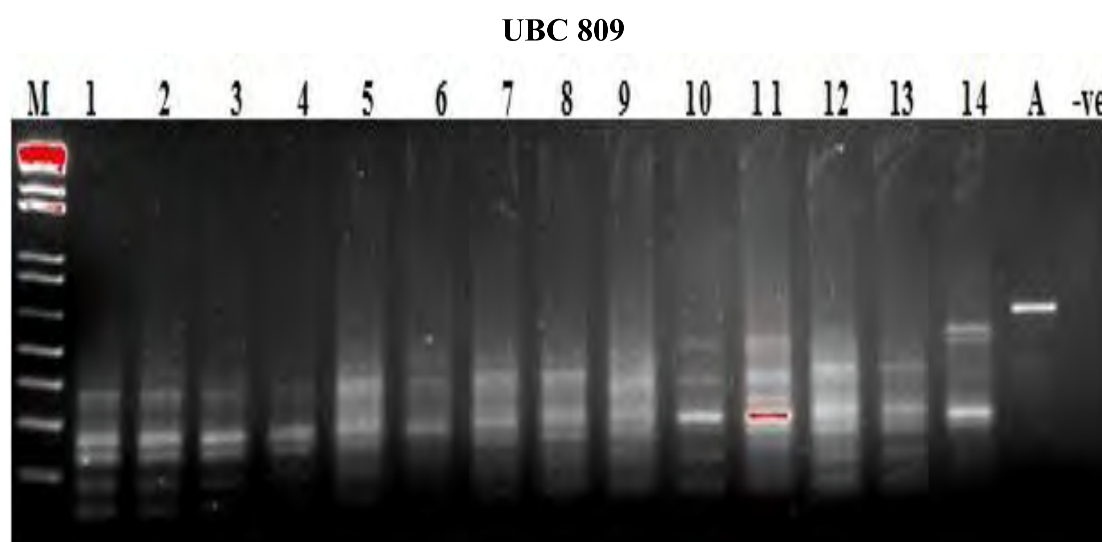


Figure 4.16: PCR amplification pattern of ISSR UBC-809 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

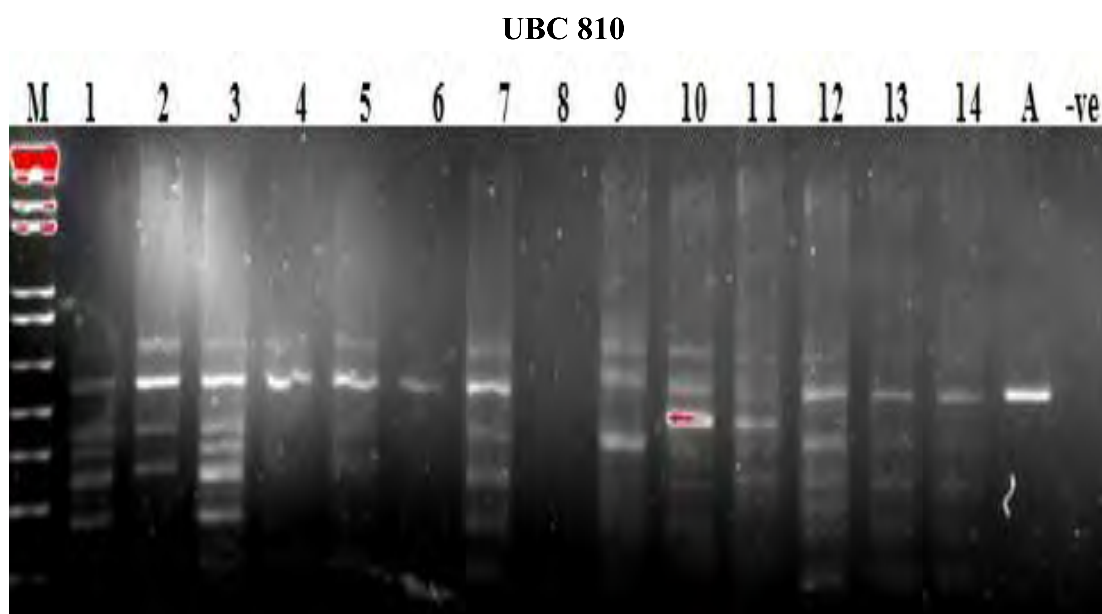


Figure 4.17: PCR amplification pattern of ISSR UBC-810 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

Figure 4.18: PCR amplification pattern of ISSR UBC-854 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

UBC 842

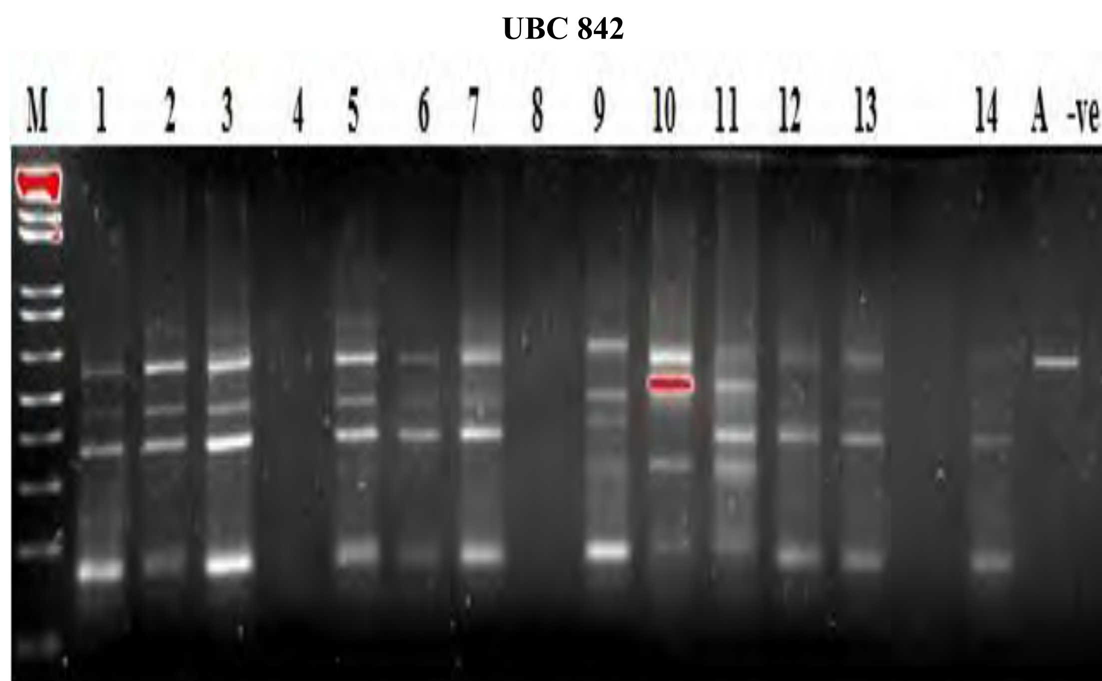


Figure 4.19: PCR amplification pattern of ISSR UBC-842 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

UBC 836

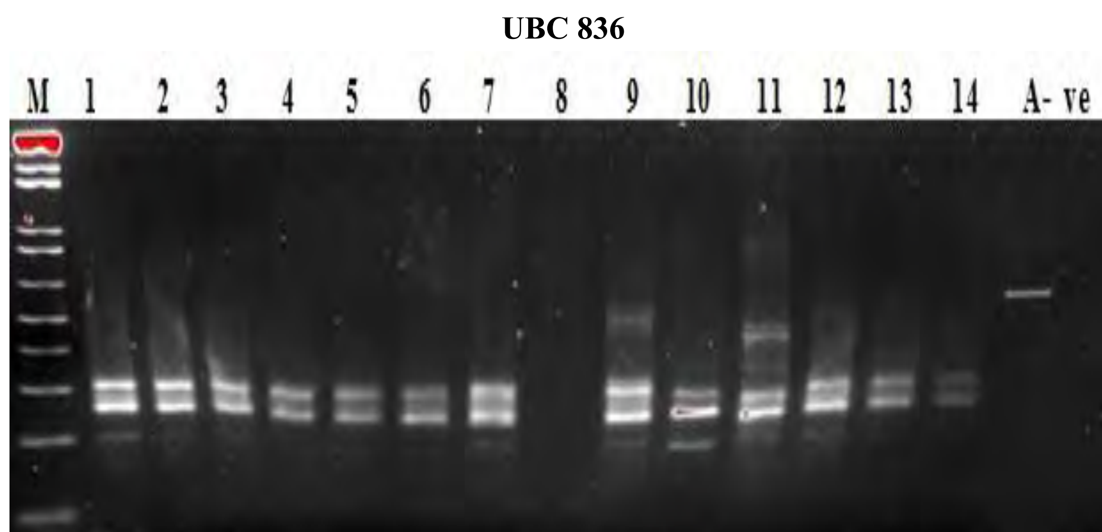


Figure 4.20: PCR amplification pattern of ISSR UBC-836 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

UBC 826

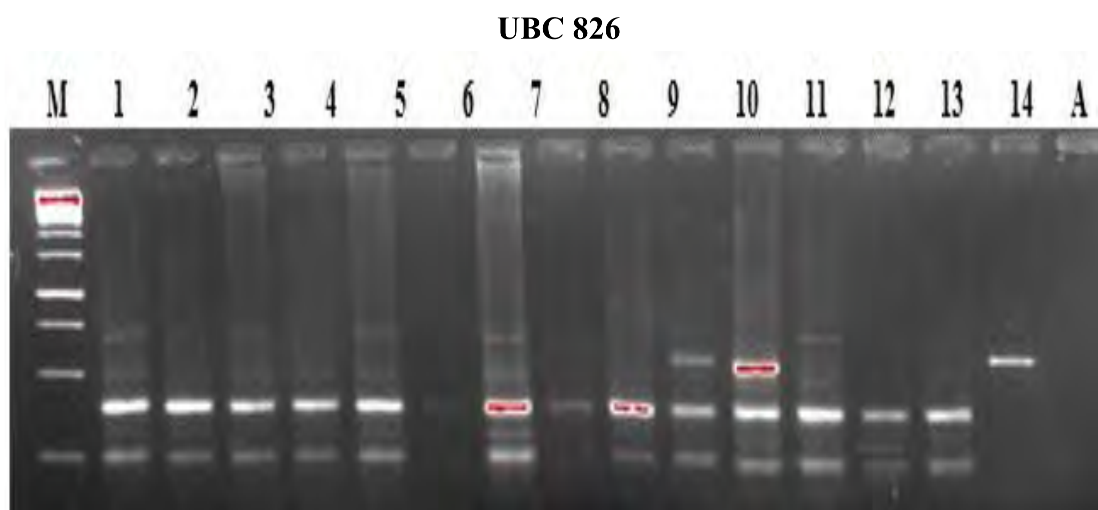


Figure 4.21: PCR amplification pattern of ISSR UBC-826 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

UBC 880

Figure 4.21: PCR amplification pattern of ISSR UBC-880 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

ISSR-3

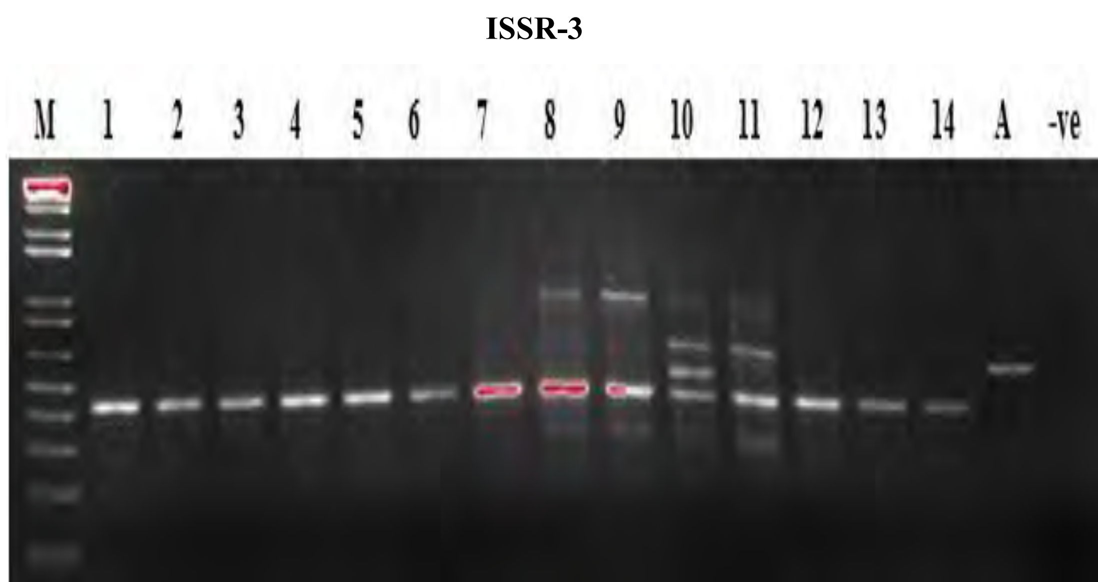


Figure 4.23: PCR amplification pattern of ISSR-3 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. –ve Control.

UBC 827

Figure 4.24: PCR amplification pattern of ISSR UBC-827 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

IG-02

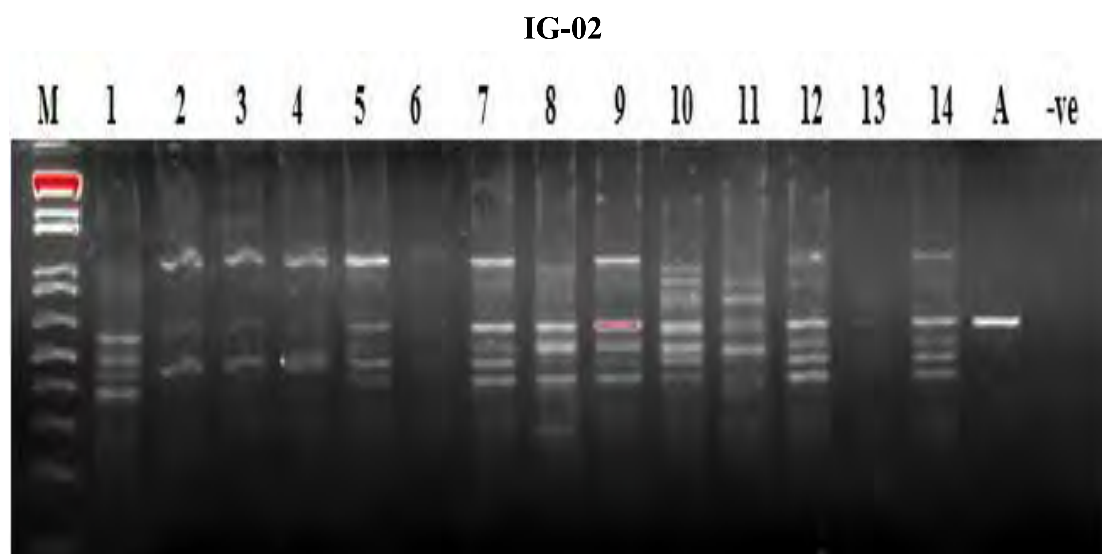


Figure 4.25: PCR amplification pattern of ISSR IG-02 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. –ve Control.

UBC 868

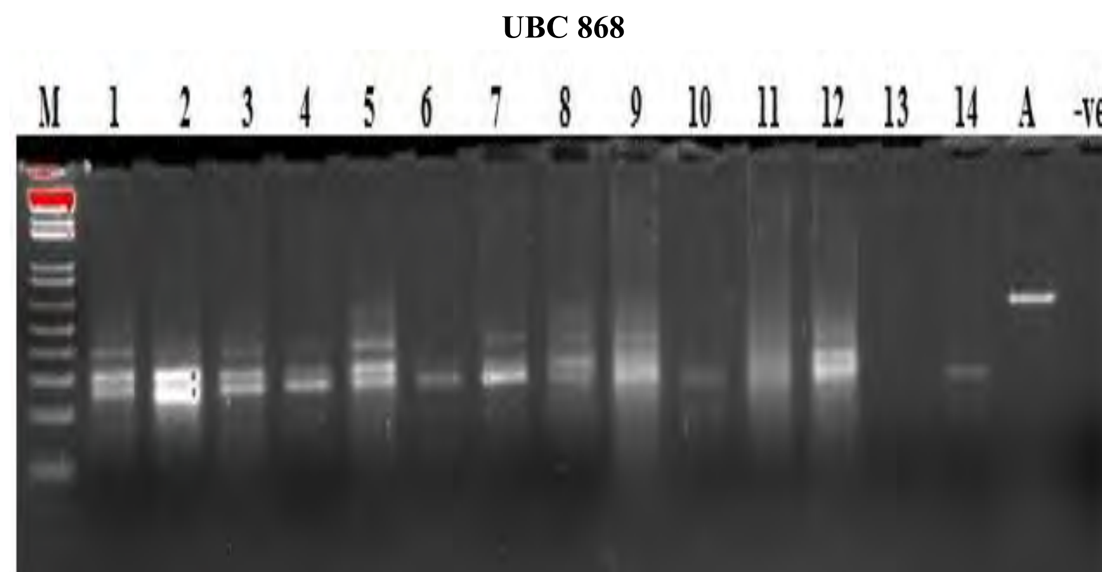


Figure 4.26: PCR amplification pattern of ISSR UBC-868 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

IG-19

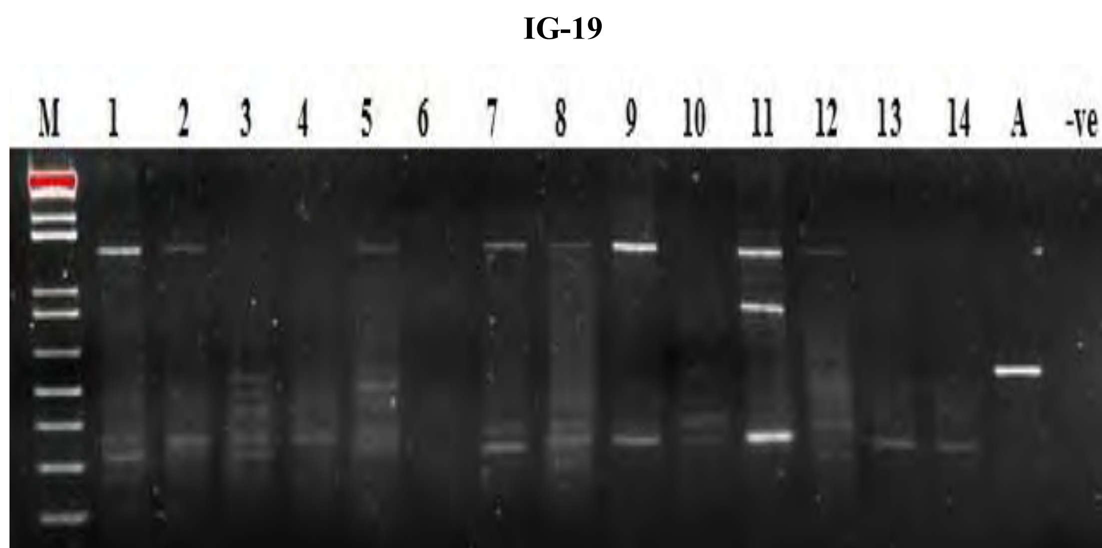


Figure 4.27: PCR amplification pattern of ISSR IG-19 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

UBC 808

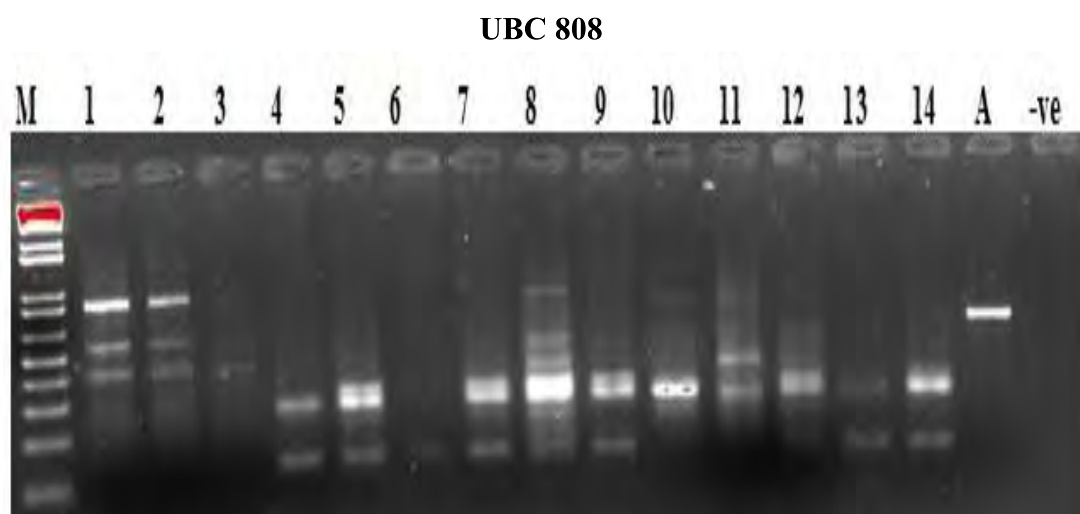


Figure 4.28: PCR amplification pattern of ISSR UBC- indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

UBC 844

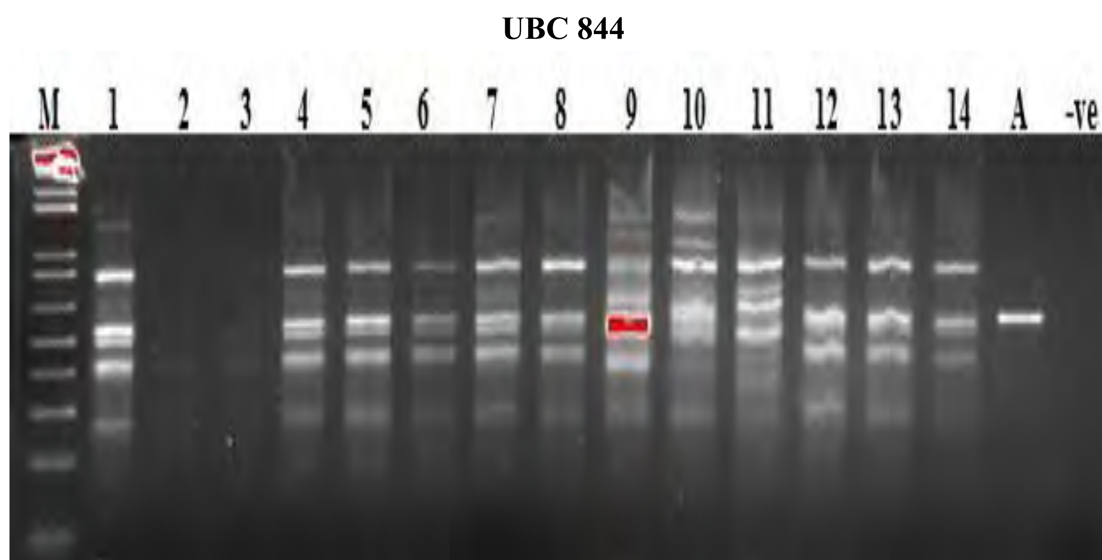


Figure 4.29: PCR amplification pattern of ISSR UBC-844 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

UBC 822

Figure 4.30: PCR amplification pattern of ISSR UBC-822 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

IG-05

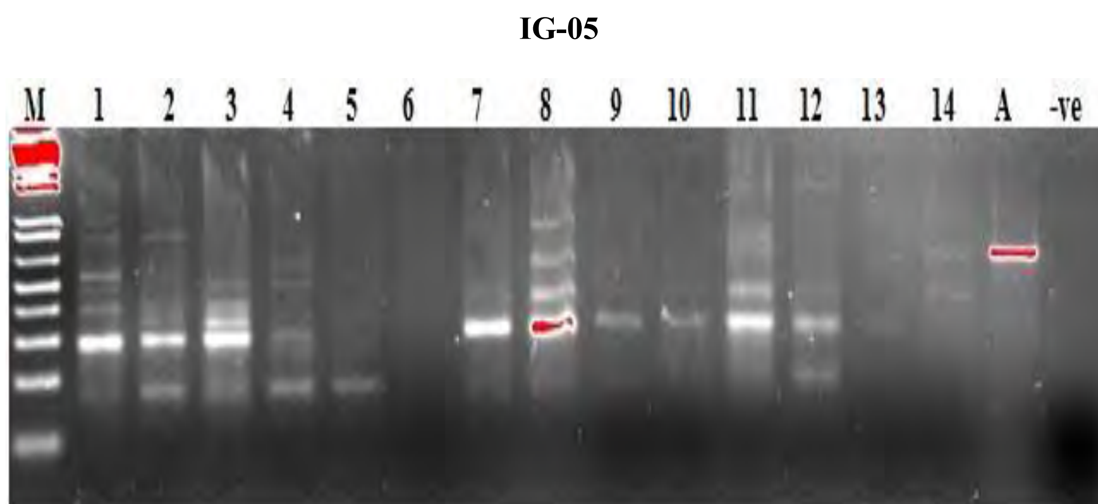
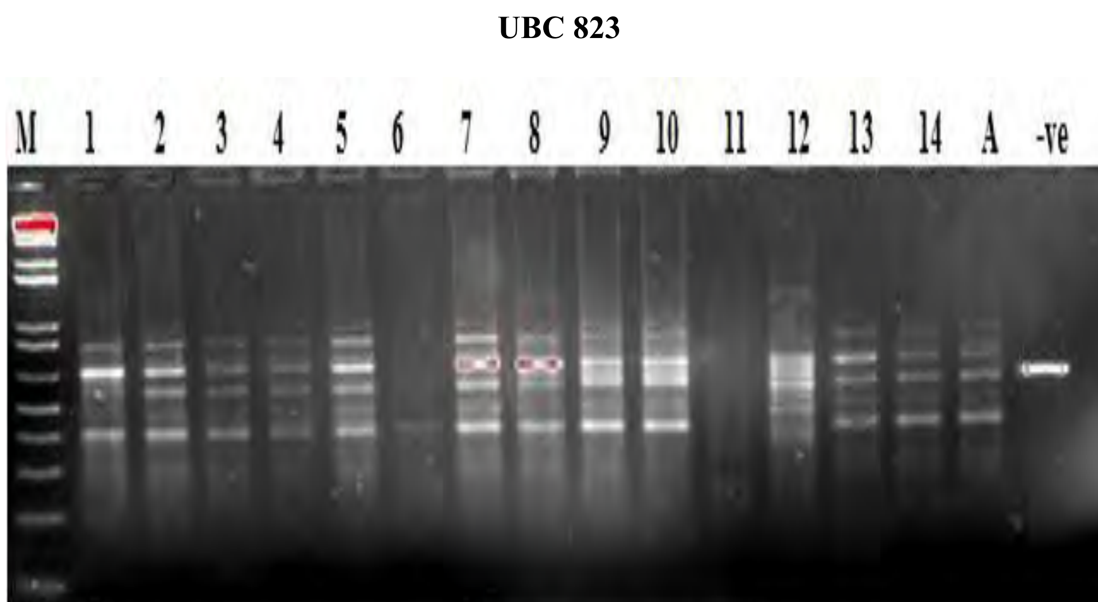


Figure 4.31: PCR amplification pattern of ISSR IG-05 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

UBC 823



Figur 4.32: PCR amplification pattern of ISSR UBC-823 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

UBC 861

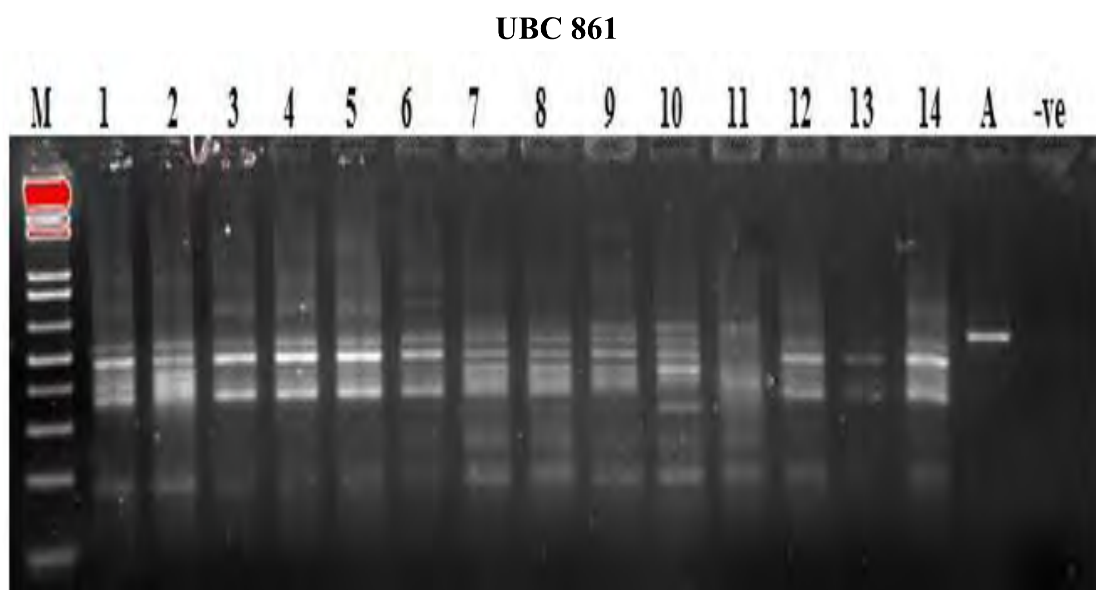


Figure 4.33: PCR amplification pattern of ISSR UBC-861 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

UBC 847

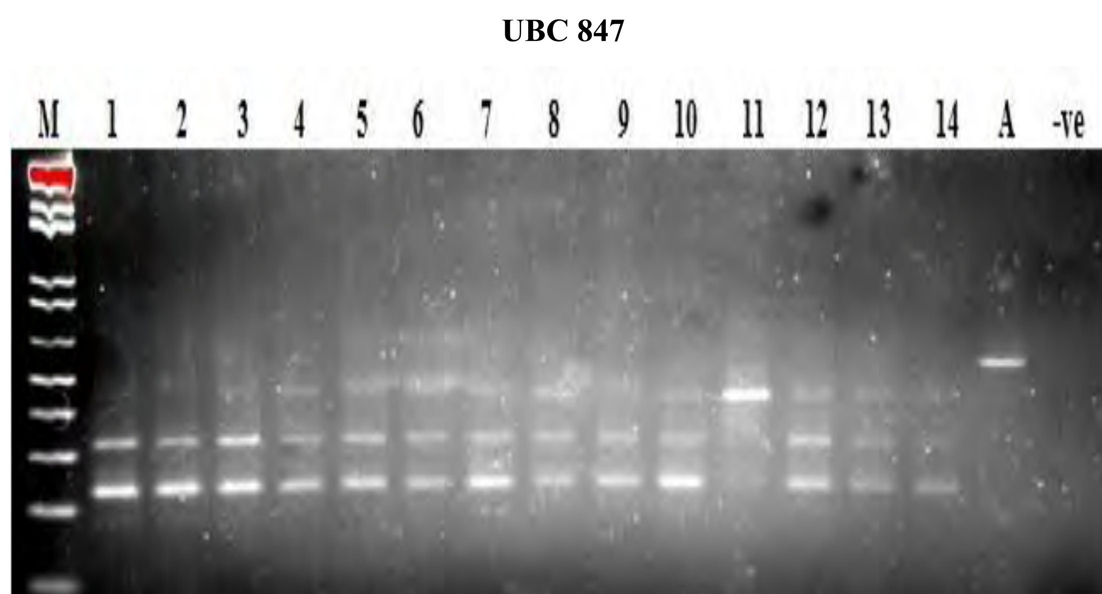


Figure 4.34: PCR amplification pattern of ISSR UBC-847 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

UBC 817

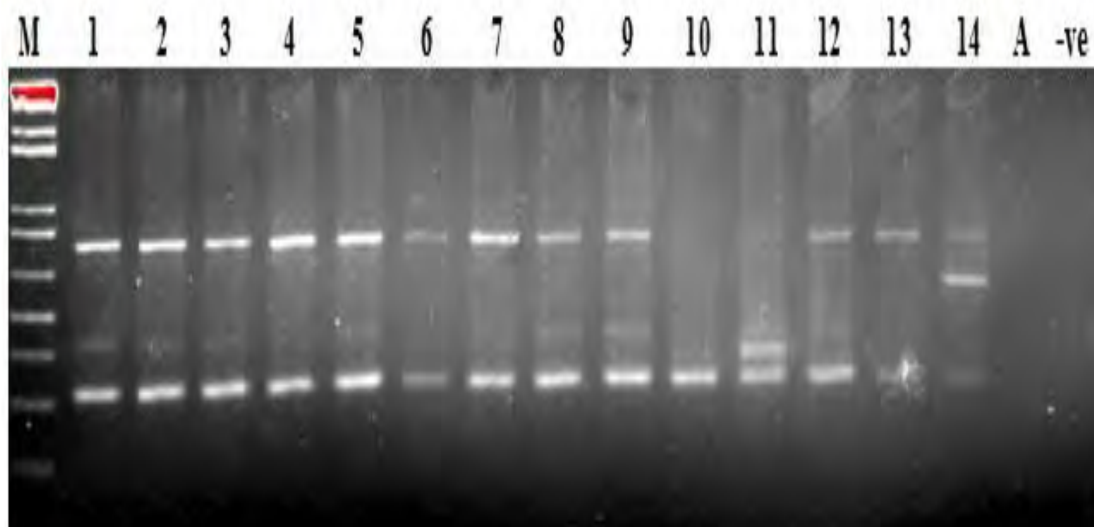


Figure 4.35: PCR amplification pattern of ISSR UBC-817 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

UBC 813

Figure 4.36: PCR amplification pattern of ISSR UBC-813 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

DBD

Figure 4.37: PCR amplification pattern of ISSR DBD indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

UBC 857

Figure 4.38: PCR amplification pattern of ISSR UBC-857 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control.

UBC 841

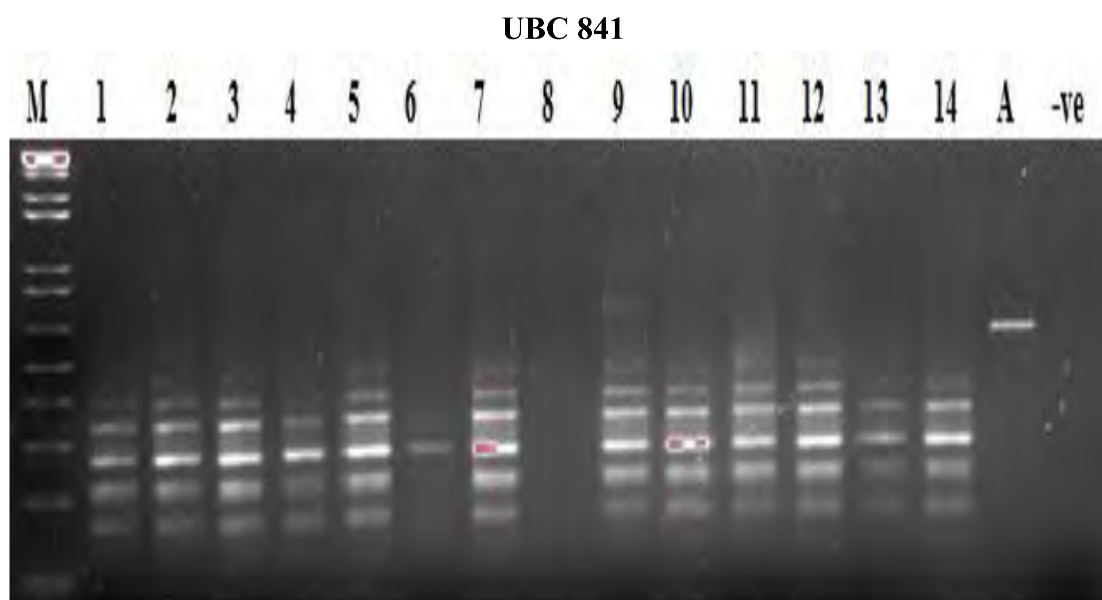


Figure 4.39: PCR amplification pattern of ISSR UBC 841 indicating genetic diversity of fourteen banana from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1, 2. NIGAB-2, 3. NIGAB-3, 4. Grand Naine, 5. ADI, 6. Gayl, 7. Jafa, 8. Dhaka, 9. Red banana, 10. Dajiao, 11. Fenjiao, 12. Basrai, 13. Sprout-1, 14. Sprout-2, 15. Actin, 16. –ve Control

UBC 848

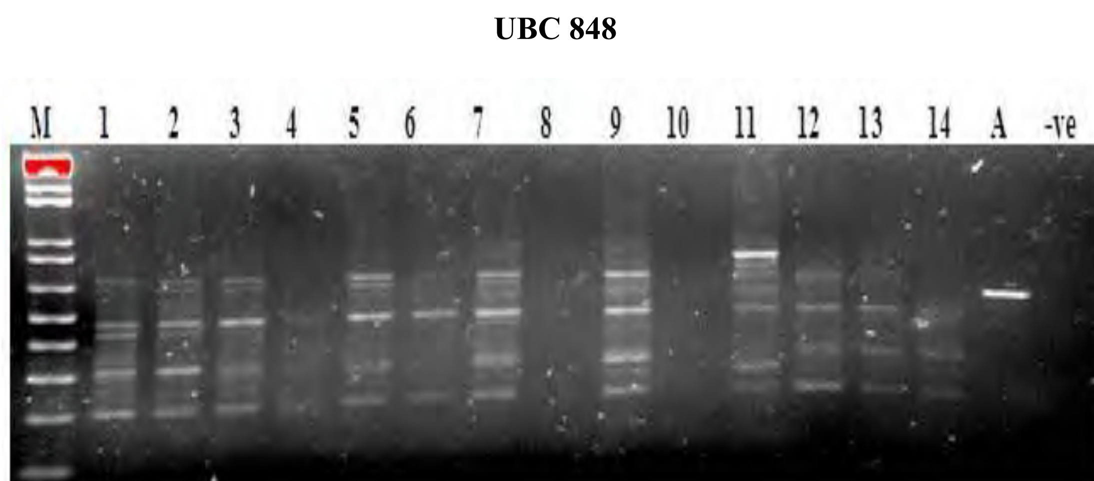


Figure 4.40: PCR amplification pattern of ISSR UBC-848 indicating genetic diversity of fourteen banana cultivars from Pakistan (M=100-15000-bp, agarose 3%) 1. NIGAB-1; 2. NIGAB-2; 3. NIGAB-3; 4. Grand Naine; 5. ADI; 6. Gayl; 7. Jafa; 8. Dhaka; 9. Red banana; 10. Dajiao; 11. Fenjiao; 12 Basrai; 13. Sprout-1; 14. Sprout-2; 15. Actin; 16. –ve Control

Results

Forty-five ISSR reported primers were used to access the genetic relationship and diversity of fourteen banana cultivars from Pakistan. Out of these selected ISSR primers, forty primers were reproducible and have amplified total of 260 scorable bands, of which 109 were polymorphic, which account to 44.57% per cent polymorphism (Table 4.1). Whereas, rest of five ISSR primers UBC891, UBC825, UBC855, VHV, IG-06 were not amplified.

Table 4.1. Features of ISSR primers used for ISSR-PCR of 14 banana cultivars

Primer	Sequence	Scorable bands	Product size base pair	Amplified Bands		
				Polymorphic Bands	polymorphism	% PIC
UBC-815	CTC TCT CTC TCT CTC TG	6	300-1000	4	66.66	72
UCB-811	GAG AGA GAG AGA GAG AC	10	150-650	5	50	83
UBC-864	ATG ATG ATG ATG ATG ATG	7	250-650	5	71.42	75
UBC-840	GAG AGA GAG AGA GAG ATT	8	300-1500	4	50	85
ISSR-2	GAG AGA GAG AGA GAG AGA AC	9	200-650	4	44.44	85
UBC-862	AGC AGC AGC AGC AGC AGC T	5	200-500	4	80	75
ISSR-4	AAC AAC AAC AAC AAC ACC C	9	200-850	4	44.44	83
IG-03	GAG GGT GGA GGA TCT	9	180-700	3	33.33	84
IG-23	GTG TGT GTG TGT GTA CG	5	200-600	5	100	74
UBC-834	AGA GAG AGA GAG AGA GTT	12	200-1400	3	25	88

UBC-835	AGA GAG AGA GAG AGA GCC	9	200-1300	3	33.33	86
UBC-807	AGA GAG AGA GAG AGA GT	10	200-600	3	30	84
UBC-818	CAC ACA CAC ACA CAC AG	8	300-900	3	73.5	84
UBC-826	ACA CAC ACA CAC ACA CC	7	400-630	3	42.85	77
UBC-880	GGA GAG GAG AGG AGA	6	300-650	4	66.66	78
ISSR-3	CCT CCT CCT CCT CCT CCT T	5	300-1000	3	60	63
UBC-827	ACA CAC ACA CAC ACA CG	8	200-1000	4	50	74
IG-02	AGA GGT GGG CAG GTG G	7	400-1000	3	42.85	84
UBC-868	AAG AGG AGG AGG AGG AGG G	4	250-400	2	50	67
IG-19	ACA CAC ACA CAC ACT GG	5	250-900	2	40	74
UBC-808	AGA GAG AGA GAG AGA GC	6	100-850	3	50	81
UBC-844	CTC TCT CTC TCT CTC TGC	6	400-850	2	33.33	80
UBC-822	TCT CTC TCT CTC TCT CTC G	5	300-850	3	60	65
IG-05	GAC AGA CAG ACA GAC A	3	200-400	2	66.66	65
UBC-823	TCT CTC TCT CTC TCT CC	6	400-850	2	33.32	81
UBC-861	ACC ACC ACC ACC ACC ACC T	5	380-600	2	40	76
UBC-847	CAC ACA CAC ACA CAC AAC	3	250-350	1	33.33	53
UBC-817	CAC ACA CAC ACA CAC AA	5	200-600	2	40	68

UBC-813	CTC TCT CTC TCT CTC TT	5	300-800	2	40	75
DBD	ACA CAC ACA CAC AC	2	450-480	1	50	58
UBC-857	ACA CAC ACA CAC ACA CTG	2	500-1100	1	50	46
ISSR-1	GAG AGA GAG AGA GAG AGA C	10	130-600	5	50	81
UBC-812	GAG AGA GAG AGA GAG AA	6	200-600	3	50	82
UBC-809	AGA GAG AGA GAG AGA GG	6	100-450	2	33.33	78
UBC-810	GAG AGA GAG AGA GAG AT	6	300-650	3	50	80
UBC-854	TCT CTC TCT CTC TCT CAG	3	150-400	1	33.33	56
UBC-842	GAG AGA GAG AGA GAG ATG	10	150-650	5	50	83
UBC-836	AGA GAG AGA GAG AGA GCA	6	200-500	3	50	74
UBC-841	GAG AGA GAG AGA GAG ATC	6	150-480	2	33.33	82
UBC-848	CAC ACA CAC ACA CAC AGG	10	200-850	5	50	85

To Find total number of polymorphism of each markers “total number of polymorphic bands divided by total number of scorable bands and multiply with 100” i-e UBC815 Polymorphism = 4/6X100 = 66.66 and to find total polymorphism of forty markers “addition of all markers polymorphism divided by total number of forty markers” i-e (66.66+ 50 +…………. n)/40 = 44.57% and PIC values find out by giving in (Table 3.9), (Botstein *et al.,* 1980

4.1 Scoring and Data Analysis

The amplification product of fourteen banana cultivars from forty selected ISSR primers were scored as a 1 (presence) or 0 (absence). Molecular size of each fragment was estimated with a reference to a size marker of (100-15000-bp) DNA Ladder Plus (Invitrogen). The experiment were repeated thrice for each cultivars to conform only reliable and repeatable bands. Pair-wise genetic distance matrix among

banana cultivars was determined according to Jaccard's similarity co-efficient and data analysis by the unweighted pair-group method with athematic average (UPGMA). Cluster analysis were performed by using SAHN (Sequential Heirarchial and Nested Clustering) program of NTSYS-PC (Numerical Taxonomy System version 2.1) (Rohlf, 1998).

4.2 Banana polymorphism

Out of these selected forty five ISSR primers, forty ISSR primers have given amplification, whereas, rest of five ISSR primers UBC891, UBC825, UBC855, VHV, IG-06 were not amplified. Forty ISSR primers percentage of total polymorphism showed about 44.57%. UBC-835 and UBC-834 detected a higher level of polymorphism in banana cultivars (86% -88%) and UBC-857 detected lowest level of polymorphism (46%) given in (Table 4.1). The number of DNA amplified bands ranged between (100-1500-bp). The maximum number of bands was obtained by using primers UBC-840, UBC-834, UBC827 and UBC-835 minimum number of bands obtained by using primers UBC-847 and UBC-854 range (100-350-bp). UBC-835 and UBC-834 is useful for the genetic diversity of fourteen banana cultivars from Pakistan (Table 4.1).

4.3 Cluster Analysis

Genetic similarity between pairwise comparisons was calculated 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 fourteen banana cultivars from Pakistan into two distinct groups i.e. A and group B based on similarity matrix using UPGMA method. Both the groups are further divided into subgroups, clusters and sub-clusters. The similarity co-efficient ranged 0.56 to 0.88. This study indicated that at molecular level, the cultivars in group A were 66% similar with other cultivars indicating the level of diversity in germplasm. The cultivars in group B were 88% similar to other cultivars. All fourteen cultivars were clearly differentiated by ISSR markers but grouped into several sub-clusters. The cultivars in group B 88% similar to other cultivars and 12% variation existed among the genotype, which differentiate from other cultivars. (Fig 4.41). All fourteen cultivars separated from each other but grouped into several clusters were clearly differentiated by ISSR markers. The dendrogram separated fourteen banana cultivars into group A and group B (Fig 4.41). The cultivars

in group 'A' Fenjiao (AAB) and Dajiao (ABB) both of the cultivars were the most diverging having similarity 66% at molecular level and 34% genetic distance with other ten banana cultivars grown in Pakistan. Majority of ten banana cultivars presents higher similarity at molecular level were located in group B. The group B found to be divided into sub-group namely, (B -1 and B-2). The sub - group B - 1 consisted of Red Banana 62.2% similar at molecular level with other eleven cultivars.

The sub-group B-2 divided into clusters namely, (B-2-1 and B-2-2). Cluster B-2-1 consisted of one banana cultivars namely, Dhaka which 68% similar at molecular level with other ten cultivars. And cluster B-2-2 further divided into sub-clusters namely, (B-2-2-1 and B-2-2-2). Sub-clusters namely B-2-2-1 consisted four banana cultivars and distributed into more sub - sub clusters namely, (B-2-2-1A and B-2-2-2B). Sub-sub clusters B-2-2-1A contained two cultivars Sprout-1 and Sprout-2, which 83% similar with other eight banana cultivars. And sub-sub clusters B-2-2-1B having two cultivars Grand Naine and Gayl at 80% similarity at molecular level with other seven banana cultivars. Sub cluster B-2-2-2 contained six banana cultivars, and this further allocated into sub-sub clusters namely, (B-2-2-2A and B-2-2-2B). And sub-sub clusters B-2-2-2A consisted 3 banana cultivars and categorized into sub-sub clusters (A) B-2-2-2AA and B-2-2-2AB. B-2-2-2AA comprised Basrai 85% similar with other five banana cultivars and B-2-2-2AB contained two banana cultivars ADI and Jafa 86% similarity at molecular level with other three banana cultivars. Sub-sub cluster B-2-2-2B consisted three banana cultivars and further divided into Sub-sub cluster (B) B-2-2-2BA and B-2-2-2BB. B-2-2-2BA consisted of three cultivars of local Banana NIGAB-3, NIGAB-2 at 88% similar at molecular level with other one banana cultivar. The dendrogram showed NIGAB-3 and NIGAB-2 are very much closely related to each other. B-2-2-2BB contained one banana cultivars NIGAB-1 showed 86% similarity at molecular level. (Fig 4.41)

The result analysis showed the maximum genetic distance 34% was observed in group A between two local cultivars Dajiao and Fenjiao. Minimum genetic distance 12% was observed in group B between two local cultivars NIGAB-2 and NIGAB-3 on the bases of forty ISSR markers which is useful for the genetic diversity of fourteen banana cultivars from Pakistan.

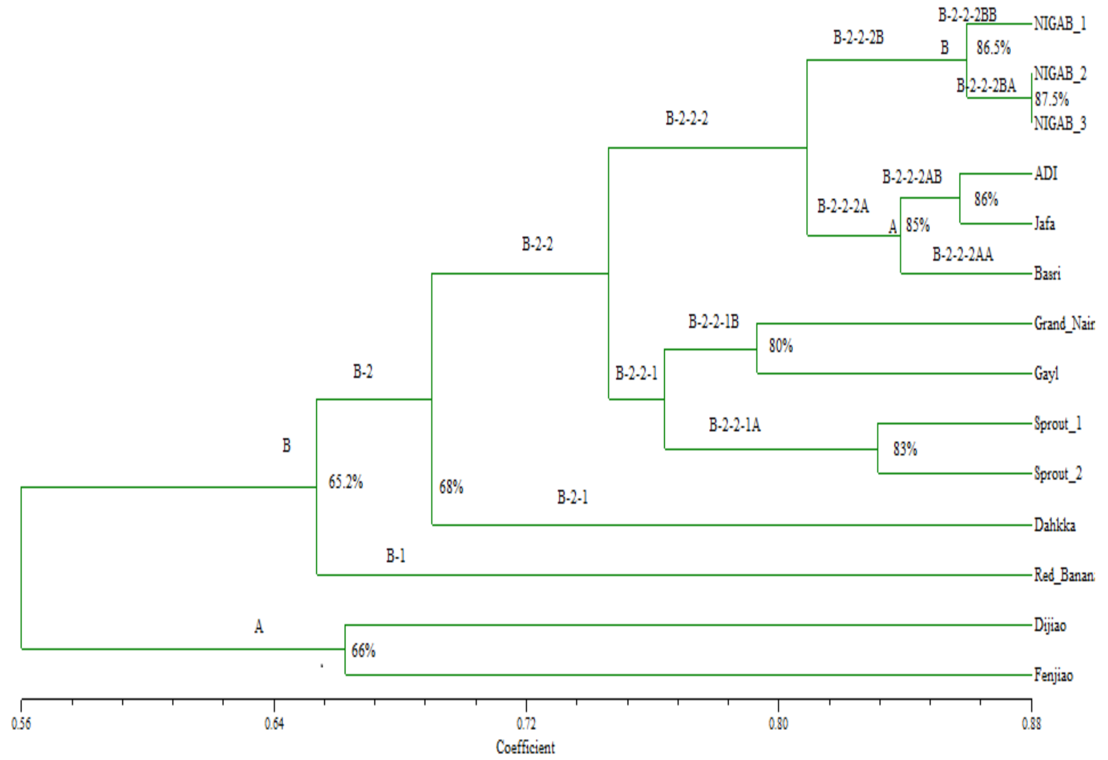


Figure 4.41: Dendrogram generated relationship between fourteen banana cultivars from Pakistan

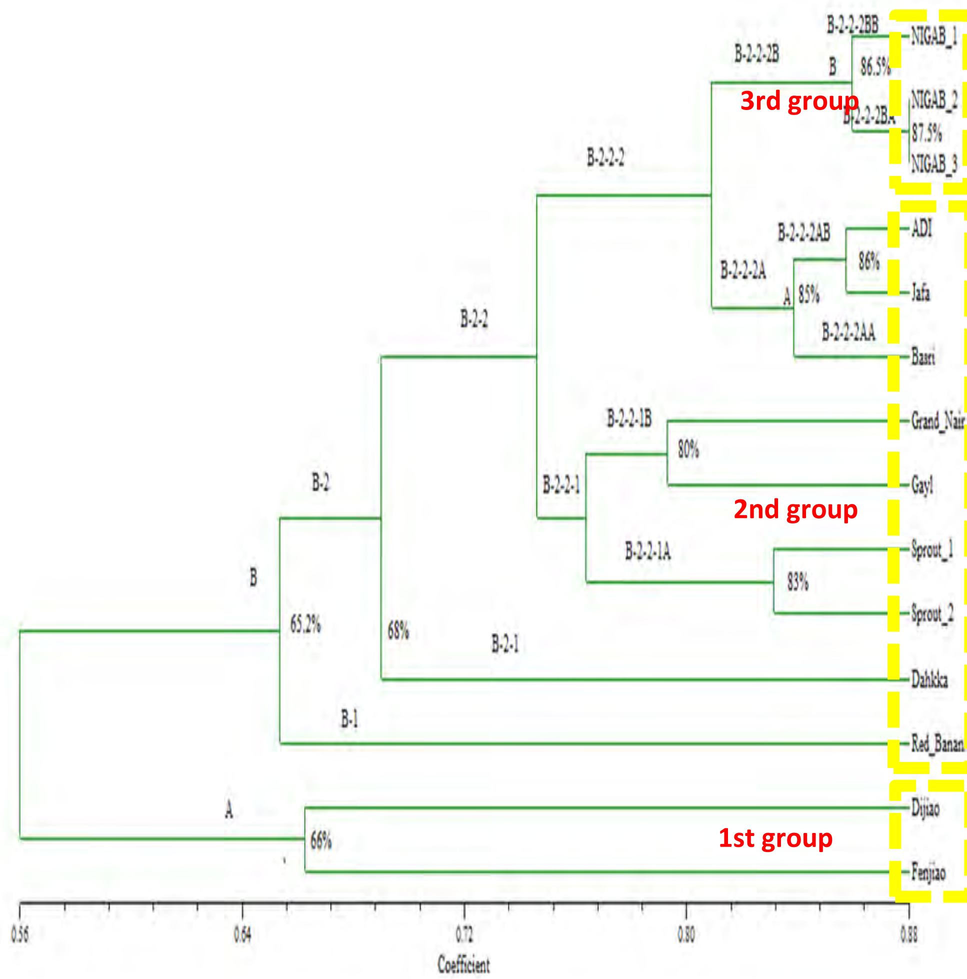


Figure 4.42: Dendrogram generated genetic relationship between fourteen banana cultivars from Pakistan into three main groups

CHAPTER 5

DISCUSSION

5. DISCUSSION

For any germplasm characterization, first step is to analyze the material morphologically as well as molecular level. (Patrício *et al*., 2016). In the past, cultivars have been broadly categorized by morphological traits including; plant leaf, fruit, and seed characteristics. Diversity studies in *Musa* have been carried out by using different molecular techniques, like morphologically characters by (Simmonds, 1962; Simmonds and Weatherup, 1990), cytogenetics (Cheesman, 1948 and Osuji *et al*,. 1997), isozymes (Bhat *et a*l., 1992), molecular cytogenetics (Osuji *et al*,. 1998), cryptic developmental stages (Trontelj and Fišer, 2009) and intergenic spacers evaluated (Lanaud *et al*., 1992). These methods in *Musa* species especially at large scales required speaslized expertise, is too time consuming, costly for routine monitoring, which, the results are obtain based on morphological parameters are not broad (Carugati *et al*., 2015). By the use of morphological technique, sometimes differentiating among banana cultivars becomes very difficult. The morphological based approaches s not always easy because influence of environmental factors for the identification and diversity analysis (Lopez-Vizcón and Ortega, 2012).

In conclusion, the ISSR technique has been demonstrated in the present study to be a useful tool in determining the molecular diversity in banana cultivars grown in Pakistan. The present study showed that each ISSR primer could generate polymorphisms among the fourteen banana cultivars. We differentiated cultivars according to banding patterns with the help of ISSR markers. In some cases these results correlate with the morphological classification of selected banana cultivars grown in Pakistan, whilst in other cases showed that there were significantly different genotypic and morphological classifications. This study also showed that the collected banana cultivars grown in Pakistan were differentiated on the basis of ISSR analysis divided into groups and clusters. These ISSR markers target the region of banana genome on their non-coding regions and detected the percentage of total polymorphism about 44.57%. ISSR primers have produced 260 scorable bands and 109 were polymorphic. The size of DNA amplified bands ranged between (100-1500-bp) and detected a higher level of polymorphism percentage about 44.57%. The result analysis showed polymorphism which was identified with the help of markers, based on the Jaccard's coefficient similarity index. Two main groups A and B were classified and identified

in the UPGMA analysis (fig 4.41) while on the basis visual appearance groups (ISSR data analysis), three prominent and distinct group (fig 4.42) were visible i.e. the group having Diajiao and Fenjiao, the group having NIGAB cultivars while the rest of all were categorized in same group. Furthermore, Diajiao and Fenjiao seems to appear the first group, then rest of cultivars and finally the group with NIGAB cultivars, respectively. Discussing first group A, 34% variation was observed between two local cultivars Dajiao and Fenjiao and 66% similar at molecular level with other banana cultivars. The middle group was separated from both by 67% from first group while 86 % from third group. Minimum genetic distance at molecular level 12% was observed in group B between two local cultivars NIGAB-2 and NIGAB-3 and 88% similar at molecular level with other banana cultivars. Although, NIGAB-2 and NIGAB-3 on the bases of ISSR fell in a same cluster, NIGAB-2 and NIGAB-3 showed close relationship with each other at molecular level, that's why both fell in a same cluster (Fig 4.41), although both cultivars NIGAB-2 and NIGAB-3 were significantly different to each other based on morphological classification when these results compared with previous studies similar findings were also observed between some of the banana cultivars namely Grand Nain, and Raja Udang (Dwarf Cavendish) which were designated by (Simmond, 1966) as having the AAA genomic constitution based on morphological characterization, fell in separate clusters but on molecular analysis both cultivars fell in same cluster (Loh *et al*., 2000). According to (Loh *et al*., 2000), it is possible that the differences in phenotypic characters may not be due to euploidy differences but rather due to allelic differences in single or multiple genes.

This finding is congruent with previous studies that have successfully used ISSR markers to acquire genetic diversity information of banana, such as (Chandra *et al*., 2018), who were able to find 109 bands that were polymorphic and also showed 53.83% polymorphism. Our results on ISSR analysis of banana cultivars grown in Pakistan confirmed and conflict starkly in others when compared to the previous studies on characterization of banana cultivars based on molecular markers. Furthermore, this study provided evidence of a genetic diversity showed 44.57% among the selected cultivars proving that this germplasm can be used for further analysis. The results of present study for genetic diversity in germplasm included two groups A and B which showed the genetic variation occurred that cultivated germplasm could be different from each other. The data analysis showed Fenjiao and Dajiao cultivars were totally in

different grouped from other banana cultivars, Dajiao showed no close relationship to Fenjiao. Our results when compared to the previous studies (Long *et al*., 2009 and Yi *et al*., 2002), similar type of results were observed.

Racharak and Eiadthong. (2007) used six ISSR primers to estimate the genetic diversity of both wild and cultivated banana. The results were also in line with present study in term of genetic diversity. He was able to differentiate the accessions on the bases of similarity index which ranged among 0.47 to 0.93 and the average genetic distance similarity was 0.67. Compared to this study similarity index ranged was between 0.68 - 0.88 and the average 0.72. A bit of variations of results in both the studies may be due to difference in marker selection and germplasm used. Moreover in both the cases the dendrogram clearly revealed indistinct classification when examined. Lamare and Rao. (2015) also studied genetic diversity using total of 58 ISSR primers and UBC-835 detected a higher level of polymorphism. Analysis showed maximum genetic variation within the cultivars identified which is supported by the AMOVA analysis (88 %). Similar finding were also observed in this study and UBC-835 detected a higher level of polymorphism in fourteen banana cultivars and analysis revealed 44.57% polymorphism and 34% diversification among them in term of genetic diversity.

So far ISSR markers are used in many crops and have found many positive results in terms of genetic diversity and trait identification for varietal improvement. ISSR is simple and easy molecular technique used for genetic diversity in different banana cultivars. ISSR technique is also very reliable, cost-effective, simple, fast, and highly discriminative (Khadke *et al*., 2015). ISSR has been widely utilized for DNA/ gene analysis in various aspects of biological sciences. Through applying this technique, many accessions of banana can be characterized in a shorter period of time (Mosa *et al.,* 2018). The results obtained from the investigation were helpful for the new cultivars identification and selection of parents suitable for creating of mapping population. Furthermore. It will also help for the identification of duplications among the cultivars in tissue culture germplasm banks and in the fields. Especially in Pakistan where no previous work or information is available as we were able to separate Dajiao, Fenjiao and NIGAB cultivars, also able to differentiate between broad base germplasm. The present investigation was primarily focused on the study of genetic diversity among

the Pakistani banana cultivars grown in Pakistan. Using ISSR molecular markers it was revealed that significant amount of genetic diversity existed in the cultivars studied. Therefore, understanding genetic variation between and within the genomic groups is very important for the evaluation of plants with superior traits. The findings derived out of this study will provide useful information for future identification of duplications among the accession in tissue culture germplasm banks and conservation strategies of banana genetic resources in Pakistan.

CHAPTER 6
CONCLUSION AND RECOMMENDATION

6. CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Banana cultivars from Pakistan could not be differentiated morphologically due to influence of environmental factors and visual parameters were very close. By using ISSR markers closely related banana cultivars were differentiated and total number of cultivars grown in Pakistan were found to be fourteen. These cultivars were divided into two groups A and B. This study indicated that at molecular level, the cultivars in group A 66% were similar with other cultivars and 34% genetically differed from other cultivars proving the broad base of germplasm. The cultivars in group B 88% similar to other cultivars and 12% variation existed among the genotype, which differentiate from other cultivars, based on ISSR markers used.

6.2 Recommendation

In this study we were able to differentiate between the selected germplasm. Moreover ISSR was fruitful for cultivar identification as well. Furthermore due to high success of ISSR in genetic diversity and cultivar identification it is recommended that all the indigenous germplasm must be evaluated taking this study as bases. Moreover, the mentioned ISSR showed also be further used for Pakistani germplasm as this work is unique of its kind as no literature or research is previously reported in Pakistan. Apart from this these cultivar must be sequenced and advance analysis in term of SNPs, proteomics and transcriptomes should be used for further evaluation. Along with it trait specific study must be carried out in order to work on specific yield and other traits in breeding programs. As we were able to develop strategies to evaluate the germplasm using ISSR and are also working on different aspect of banana, this lab must be used as a reference lab for collaboration.

CHAPTER 7
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7. REFERENCES

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