Pak. J. Bot., 56(3): DOI: http://dx.doi.org/10.30848/PJB2024-3(16)

ANALYSIS OF GENETIC DIVERSITY AND RELATEDNESS AMONG CULTIVATED AND WILD MULBERRY CULTIVARS/ACCESSIONS USING RAPD MARKERS

GHULAM ALI BAJWA[1*], SAAD HUSSAIN SHAH[2], UZAIR HUSSAIN SHAH[1] AND MUHAMMAD ATIF MAJEED[1]

[1]Pakistan Forest Institute, 25130 Peshawar, Pakistan
[2]Institute of Biotechnology and Genetic Engineering, The University of Agriculture, 25130 Peshawar, Pakistan
**Corresponding author's email: gabajwa64@gmail.com*

Abstract

Mulberry with cosmopolitan distribution has a great economic value. The genetic diversity in mulberry, identified based on morphological characters, is highly ambiguous due to remarkable morphological plasticity. Molecular markers are meritoriously employed to identify genetic diversity and relatedness in living organisms. This study was conducted using six RAPD primers to decipher genetic diversity and relatedness among 18 mulberry accessions including 13 indigenous, four cultivated and nine wilds, and five overseas cultivated. Altogether 112 distinct bands were obtained, ranging from 270 to 600 base pair. Seventy-six bands (67.86%) were polymorphic ($\bar{x}$ = 12.67 primer^{-1}). Five primers yielded > 0.5 polymorphic information content. All accessions were genetically different with genetic similarity coefficient varying from 0.364 to 0.933. The accessions were divided into four principal clusters with genetic distance of 41.6% to 92.3%. Genetic diversity was greater relatively in cultivated accessions compared to wild ones. Tropical and subtropical ecotypes were richer in mulberry genetic resources compared to temperate ecotypes. The cluster analysis unraveled a mixed genetic relatedness in cultivated and wild accessions. Accessions in temperate ecotype were concentrated in one cluster, while accessions in tropical and subtropical were concentrated broadly in two clusters. CMP-16 (cultivated) and STP-18 (wild) were genetically faraway from each other. PFI-1 (cultivated) and OKP-18 (wild) were related distantly with overseas accessions MLJ-85 (Japanese origin) and HSC-85 (Chinese origin), respectively. Based on these findings, it is recommended that above mentioned four indigenous accessions may be conserved in their respective ecotypes for future breeding programmes.

Key words: Characterization, Ecotype, Genetic analysis, Growth habit, Mulberry.

Introduction

Mulberry is a multipurpose, fast growing perennial tree species of great economic value. The oligophagous Mulberry Silkworm Moth (*Bombyx mori* L.) eats solely leaves of some species of genus *Morus*. Apart from the food of silkworm, the genus provides nutrient-rich forage for livestock. Mulberry berries are widely used in food industries for making fruit juices, jams, marmalades, wines, etc. The fruit is categorized as super food, consumed either fresh or dried, due to its exquisite taste and odour, congenial colour, low-fat content, and highly nutritious (Zhang *et al*., 2018). All plant parts including root, stem, bark, leaf and fruit have therapeutic properties and used as herbal medicine for the ages. The leaf and fruit contain polyphenolic compounds, flavonoids, amino acids, vitamins and minerals. These compounds are endowed with bioactivities importantly like anti-coagulant, anti-diabetic, anti-hyperlipidemia, anti-neoplastic, anti-oxidative, non-steroidal anti-inflammatory and anti-obesity (Lee *et al*., 2020; Insang *et al*., 2022). Mulberry is also a good source of timber particularly for sports goods, furniture, musical instruments, and fuelwood. It is a good ecological tree species since it is a net mitigator of greenhouse gases. It can sequester from 93.95 to 321.7-ton CO_2 ha^{-1} year^{-1} (Li *et al*., 2020; Bajwa *et al*., 2021).

Mulberry has a cosmopolitan distribution; growing in wild and also cultivated in Africa, Asia, Europe, Latin America and Western Hemisphere. It has adapted in tropical to subarctic regions and flourishes well from lowlands up to altitude of 4,000 m above mean sea level (Orhan *et al*., 2020). Mulberry is cultivated either through seed or by vegetative propagation. It outcrosses in nature frequently and can easily be hybridized artificially as well. Moreover, polyploidy is in abundance in mulberry. It is amenable to management operations, such as training and is quick to rejuvenate (Rohela *et al*., 2020).

Mulberry belongs to genus *Morus* (Urticales: Moraceae) and has multicentric origin (Hou, 1994). More than 150 species of genus *Morus* were enlisted in Kewensis Index since first taxonomic report by Linnaeus in 1753. These species were described and named using morphological traits. High levels of plasticity in morphology however became cause of obscure taxonomy. Majority of the specific names recorded earlier in Kewensis Index happened to be either synonyms or categorized as varieties, while some of the species have been shifted to other related genera (Zhao *et al*., 2005). Apart from taxonomic confusion, morphological traits are poor parameters for analysis of genetic diversity, detecting genetic composition of hybrids and patterns of introgressive hybridization (Andersen & Lübberstedt 2003; Botton *et al*., 2005). Thus morphology is not reliable for characterization of mulberry genetic resources and their conservation to meet the future challenges (Wang *et al*., 2017; Pádua, 2018).

The molecular approaches developed for analyzing genetic diversity, and establishing genomic relationships have several merits. The molecular markers are firm and indifferent to environmental changes, and thus provide quick and dependable information for genetic analysis. A small amount of plant material at any developmental stage is enough to conduct complete genetic analysis (Vijayan, 2007). Previously, researchers have identified genetic diversity and genetic relatedness in mulberry genotypes using molecular markers including AFLP, ISSR, RAPD, RFLP and SSR or microsatellites (Kafkas *et al*., 2008; Hu *et al*., 2014; Orhan *et al*., 2020; Park *et al*., 2020; Akzad *et al*., 2021).

Pakistan has diverse climatic conditions, wherein four species of mulberry (*M. alba*, *M. laevigata*, *M. nigra* & *M. rubra*) grow indigenously. Apart from indigenous species, *M. latifolia* (Japanese origin) and a few varieties of *M. alba* were introduced from Japan, Peoples Republic of China and Republic of Korea for rearing *B. mori* in early 1980s. Earlier, genetic diversity and relatedness among ten overseas mulberry accessions was studied using agronomic traits (Bukhari *et al*., 2010). However molecular markers, to the best of our knowledge, have not been used for genetic diversity analysis of mulberry genotypes in the country. Thus, we used six RAPD primers with the objective to: (i) analyze genetic diversity in 18 cultivated and wild accessions of mulberry, (ii) identify genetic diversity in different ecotypes, and (iii) establish genetic relatedness among the accessions. Specifically we estimated polymorphism, Polymorphic information content, genetic similarity and drawn a UPGMA dendrogram for cluster analysis.

Material and Methods

Plant material: The study was conducted at Pakistan Forest Institute (PFI), and Institute of Biotechnology & Genetic Engineering, The University of Agriculture, Peshawar during 2021-22. Leaves of 18 mulberry accessions were collected from different ecotypes of Pakistan during February 2021 including five overseas accessions introduced from China, Japan and South Korea and cultivated at Sericulture Research Garden (SRG), PFI, Peshawar. Out of 13 indigenous accessions four were cultivated and nine were wild. Accession-wise collected leaves were kept at − 20°C in airtight bags for isolation of DNA. Mulberry accessions, their habit of growth and description of ecotypes are summarized in (Table 1).

Extraction of DNA: Genomic deoxyribonucleic acid (gDNA) was isolated by Cetyltrimethyl ammonium bromide (CTAB) surfactant, employing slightly customized protocol developed by Doyle & Doyle (1987). Two hundred milligram leaves per accession, stored at − 20°C, were macerated in liquid nitrogen (− 192°C). A paste of macerated leaves was prepared by adding 0.5 mL of CTAB buffer (100 mM Tris Base, 4.5 mM NaCl, 0.5 mM ethylene diamine tetra acetic acid (EDTA), 2.0 g powder of CTAB and 2.0% Mercaptoethanol). The paste was transferred into 2.0 mL Eppendorf tubes and incubated at 65°C for 30 minutes in hot bath with constant mixing by inversion. In incubated paste, 500 µL of PCI solution (Phenol 25.0 µL, Chloroform 24.0 µL, Isoamyl alcohol 1.0 µL) was added, mixed using vortex mixer, and was centrifuged for 10 minutes at a speed of 13k rpm. The top aqueous layer, holding DNA, was poured into fresh Eppendorf tubes and added 600 µL ice cold Isopropanol and 28.0 µL Ammonium acetate. The suspension was mixed gently by several inversions, incubated at − 20°C overnight, and centrifuged for 12 minutes at a speed of 13k rpm for precipitation. The top layer was removed without disturbing DNA pellets at the bottom. DNA was purified by washing with 70% cold ethanol (150-250 µL) and centrifuged at 12k rpm for 5 minutes. Ethanol was removed and the pellets containing DNA were dissolved in 50.0 µL of 1X TE Buffer (Thermo Scientific). The quality of extracted DNA was checked by spectrophotometry (Thermo Scientific NanoDrop™ 2000c), diluted to a concentration of 100 ng μL^{-1}, and stored at − 20°C for RAPD based analysis.

RAPD Amplification: Six RAPD primers were used for amplifying polymerase chain reaction products. The thermal cycler method, slightly modified by Ahmad *et al*., (2012), was applied for amplification. PCR was performed using 10.0 µL reaction consisting of 5.0 µL Master Mix, 2.0 µL primer, 2.0 µL PCR water and 1.0 µL DNA. PCR thermo-profiling for amplification started with initial denaturation and denaturation for 5 minutes and 1 minute, respectively, both at 94°C. The denatured samples were run through 35 cycles of annealing each of 1 minute at 37°C - 54°C, primer annealing range (Table 2), and followed by extension and final extension for 1 minute and 10 minutes, respectively, both at 72°C. The amplicons were profiled in 1.5% agarose gel by electrophoresis. The agarose gel consisted of 90.0 mL distilled water and 1.5 g agarose in 10.0 mL 10x TE buffer (100 mM Tris-borate, 2.0 mM EDTA, and pH 8.0). The gel of 1.5% concentration was melted in a microwave oven for 30 seconds, stained with 3.0 µL Ethidium bromide and cooled down. One hundred base pair Ladder (Thermo Scientific) was used for measuring amplicon size and images of amplicons were realized using Gel Documentation System (Unitec).

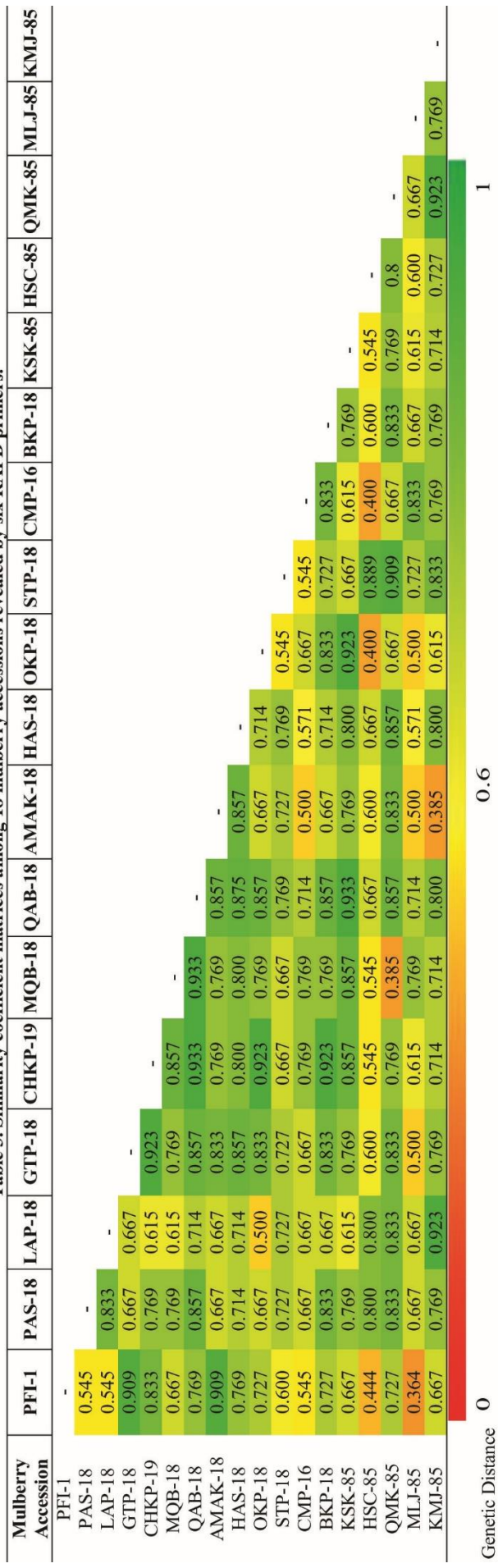
Table 1. Summary of mulberry accessions, habit of growth, vegetation type and ecotype characteristics

S. #	Accession code	Growth habit	Ecotype description
1.	PFI-1	Cultivated	Sub-tropical broad leaved; MTR = 3 - 39°C; MAP = 470 mm; Elev. = 357 m masl
2.	PAS-18	Wild	Tropical thorn; MTR = 9 - 44°C; MAP = 160 mm; Elev. = 65 m masl
3.	LAP-18	Wild	Sub-tropical broad leaved; MTR = 6 - 40°C; MAP = > 800 mm; Elev. = 225 m masl
4.	GTP-18	Wild	Sub-tropical broad leaved; MTR = 5 - 41°C; MAP = 400 mm; Elev. = 235 m masl
5.	CHKP-19	Wild	Dry temperate (Steppe); MTR = − 5 - 22°C; MAP = > 1200 mm; Elev. = 1,494 m masl
6.	MQB-18	Cultivated	Dry temperate (desert); MTR = − 2 - 34°C; MAP = 13 mm; Elev. = 1,700 m masl
7.	QAB-18	Wild	Dry Temperate; MTR = −3 - 36°C; MAP = 260 mm; Elev. = 1,680 m masl
8.	AMAK-18	Cultivated	Moist Temperate; MTR = − 2 - 30°C; MAP = >1500 mm; Elev. = 1,445 m masl
9.	HAS-18	Wild	Mediterranean tropical; MTR = 11 - 40°C; MAP = 180 mm; Elev. = 13 m masl
10.	OKP-18	Wild	Sub-tropical broad leaved; MTR = 7 - 41°C; MAP = 500 mm; Elev. = 110 m masl
11.	STP-18	Wild	Sub-tropical broad leaved; MTR = 5 - 40°C; MAP = > 1,000 mm; Elev. = 250 m masl
12.	CMP-16	Cultivated	Sub-tropical broad leaved; MTR = 5 - 41°C; MAP = > 700 mm; Elev. = 225 m masl
13.	BKP-18	Wild	Tropical thorn; MTR = 8 - 42; MAP = 200 mm; Elev. = 160 m masl
14.	KSK-85	Cultivated	Introduced from South Korea and cultivated in SRG, PFI, Peshawar; MTR = 3 - 39°C; MAP = 470 mm; Elev. = 357 m masl
15.	QMK-85	Cultivated	-do-
16.	HSC-85	Cultivated	Introduced from China and cultivated in SRG, PFI
17.	MLJ-85	Cultivated	Introduced from Japan and cultivated in SRG, PFI
18.	KMJ-85	Cultivated	-do-

MAP = Mean annual precipitation; MTR = Mean temperature regime; masl = mean above sea level

Table 3. Similarity coefficient matrices among 18 mulberry accessions revealed by six RAPD primers.

Mulberry Accession	PFI-1	PAS-18	LAP-18	GTP-18	CHKP-19	MQB-18	QAB-18	AMAK-18	HAS-18	OKP-18	STP-18	CMP-16	BKP-18	KSK-85	HSC-85	QMK-85	MLJ-85	KMJ-85
PFI-1	-																	
PAS-18	0.545	-																
LAP-18	0.545	0.833	-															
GTP-18	0.909	0.667	0.667	-														
CHKP-19	0.833	0.769	0.615	0.923	-													
MQB-18	0.667	0.769	0.615	0.769	0.857	-												
QAB-18	0.769	0.857	0.714	0.857	0.933	0.933	-											
AMAK-18	0.909	0.667	0.667	0.833	0.769	0.769	0.857	-										
HAS-18	0.769	0.714	0.714	0.857	0.800	0.800	0.875	0.857	-									
OKP-18	0.727	0.667	0.500	0.833	0.923	0.769	0.857	0.667	0.714	-								
STP-18	0.600	0.727	0.727	0.727	0.667	0.667	0.769	0.727	0.769	0.545	-							
CMP-16	0.545	0.667	0.667	0.667	0.769	0.769	0.714	0.500	0.571	0.667	0.545	-						
BKP-18	0.727	0.833	0.667	0.833	0.923	0.769	0.857	0.667	0.714	0.833	0.727	0.833	-					
KSK-85	0.667	0.769	0.615	0.769	0.857	0.857	0.933	0.769	0.800	0.923	0.667	0.615	0.769	-				
HSC-85	0.444	0.800	0.800	0.600	0.545	0.545	0.667	0.600	0.667	0.400	0.889	0.400	0.600	0.545	-			
QMK-85	0.727	0.833	0.833	0.833	0.769	0.385	0.857	0.833	0.857	0.667	0.909	0.667	0.833	0.769	0.8	-		
MLJ-85	0.364	0.667	0.667	0.500	0.615	0.769	0.714	0.500	0.571	0.500	0.727	0.833	0.667	0.615	0.600	0.667	-	
KMJ-85	0.667	0.769	0.923	0.769	0.714	0.714	0.800	0.385	0.800	0.615	0.833	0.769	0.769	0.714	0.727	0.923	0.769	-



Marker coding and data analysis: All distinctive and reproducible bands were counted in binary coding of 0 and 1; where 0 and 1 meant absent and present, respectively. Polymorphism was estimated based on total and polymorphic bands scored by each primer. Genetic similarity coefficients among accessions were calculated using complete linkage of the number of common bands employing equation developed by Nei & Li (1979). Genetic diversity expressed as polymorphic information content (PIC) was computed following formula derived by Anderson *et al*., (1993) and genetic relatedness among mulberry accessions was established by cluster analysis. Minitab Statistical Software ver.17 was used for finding the squared Euclidean distance matrix data sets and drawing a dendrogram based on an unweighted pair-group method of arithmetic mean (UPGMA).

Results

Quality of isolated DNA was good with an optical density (OD) ranging from 1.62 to 2.15 and concentration of 275.7 ng μL^{-1} to 1411.4 ng μL^{-1}. Optical density was > 2.0 in more than 60% accessions. The best OD was found in LAP-18, while the poorest OD was found in PFI-1. DNA concentration was > 500 ng μL^{-1} in more than 72% accessions. The highest and the lowest DNA concentration was found in KSK-85 and AMAK-18, respectively.

All the tested primers amplified DNA fragments with reproducible and distinct bands (Fig. 1). The amplicon size was 270 to 600 base pair. Primer OP-AP12 amplified larger sized fragments, while primer OP-G11 amplified smaller sized fragments. In accession QAB-18 and HAS-18 eight loci were amplified, while in HSC-85 four loci were amplified.

Summary of amplified bands, polymorphic bands and PIC is presented in Table 2. Overall, 112 monomorphic bands were amplified, ($\bar{x}$ = 18.67 bands primer^{-1}). Primer OP-G19 amplified the highest number of monomorphic bands, while three primers (OP-B8, OP-G3 & OP-G11) amplified equal number of monomorphic bands. Out of the total bands, 76 bands were polymorphic (67.86%), ($\bar{x}$ = 12.67 primer^{-1}). Three primers amplified bands all of polymorphic nature. Primer OP-G19 produced the lowest polymorphism, followed by primer OP-B1. In seven accessions 100% bands were of polymorphic nature, while in four accessions > 60% bands were of polymorphic nature. PIC was ranged from 0.480 to 0.753, ($\bar{x}$ = 0.612 primer^{-1}). Primer OP-G3 gave the best PIC, while primer OP-B1 gave the least PIC.

The pair-wise genetic similarity matrices are summarized in (Table 3). A large variability was observed in genetic similarity (GS), ranging from 0.364 to 0.933 among the accessions. Overall mean similarity coefficient was 0.725. GS among nine wild accessions, collected from tropical, subtropical, moist temperate and dry temperate ecotypes, varied between 0.400 and 0.933. The GS within tropical and subtropical ecotypes varied between 0.500 and 0.833, while the GS within temperate ecotypes varied between 0.769 and 0.933. Genetic variability (GS = 0.364

to 0.933) in cultivated indigenous accessions was relatively greater compared to the wild accessions. The GS further revealed that cultivated indigenous accessions have moderate genetic similarity with that of overseas accessions. Two wild dry temperate accessions (QAB-18 CHKP-19) related closely, while cultivated dry temperate accession MQB-18 was related the most with overseas accession KSK-85 (Korean origin). Contrarily, two sub-tropical accessions (CMP-16, cultivated and STP-18, wild) were genetically faraway from each other. Similarly, three indigenous accessions PFI-1, MQB-18 (cultivated) and OKP-18 (wild) showed exquisite genetic distance with overseas accessions MLJ-85 (Japanese origin), QMK-85 (Korean origin) and HSC-85 (Chinese origin), respectively. Indigenous accession AMAK-18 was at a distance with overseas accession KMJ-85 (Japanese origin).

Summary of cluster analysis of 18 accessions is presented in (Fig. 2). Cluster analysis showed a genetic similarity range of 41.55% to 92.26%. Primarily, 18 accessions were grouped into four clusters consisting of 2 to 6 accessions. Cluster I (C-I) & Cluster III (C-III) were separated at 58.45% genetic distance from Cluster II (C-II) & Cluster IV (C-IV). While C-I and C-III were separated from each other at 33.33% genetic distance. C-II and C-IV were separated from each other at 50.0% genetic distance. C-I included four indigenous accessions: PFI-1, AMAK-18, GTP-18 and HAS-18. Within C-I, PFI-1 and AMAK-18 were further separated from GTP-18 and HAS-18 at 79.99% similarity. GTP-18 showed 85.36% genetic similarity with HAS-18, while PFI-1 showed 92.26% genetic similarity with AMAK-18. Seventy five percent accessions in C-I were cultivated. C-II was the most diverse with a genetic similarity between 75.36% and 92.26%. This cluster included three indigenous wild accessions (PAS-18, LAP-18 & STP-18) and three overseas cultivated accessions (HSC-85, QMK-85 & KMJ-85). C-II was further divided into three sub-clusters at 75.36% GS. Within C-II, PAS-18 was remotely related to other five accessions, while overseas accessions QMK-85 (Korean origin) and KMJ-85 (Japanese origin) were closest with each other. Indigenous wild accession STP-18 showed 91.84% GS with overseas accession HSC-85 (Chinese origin). C-III included five indigenous (CHKP-19, MQB-18, QAB-18, OKP-18 & BKP-18) and one overseas accession KSK-85 (Korean origin). C-III was further sub-divided in three sub-clusters, each comprising two accessions. About sixty-six percent accessions in C-III were wild. MQB-18 showed 91.84% GS with QAB-18, while CHKP-19 showed 92.26% GS with BKP-18. C-IV included two accessions; one indigenous cultivated accession CMP-16 and one overseas accession MLJ-85 (Japanese origin) with 83.33% GS between themselves. Accessions belonging to dry temperate ecotype were confined to C-III, while nearly 89% wild accessions belonging to tropical and sub-tropical ecotypes were confined to C-II and C-III.

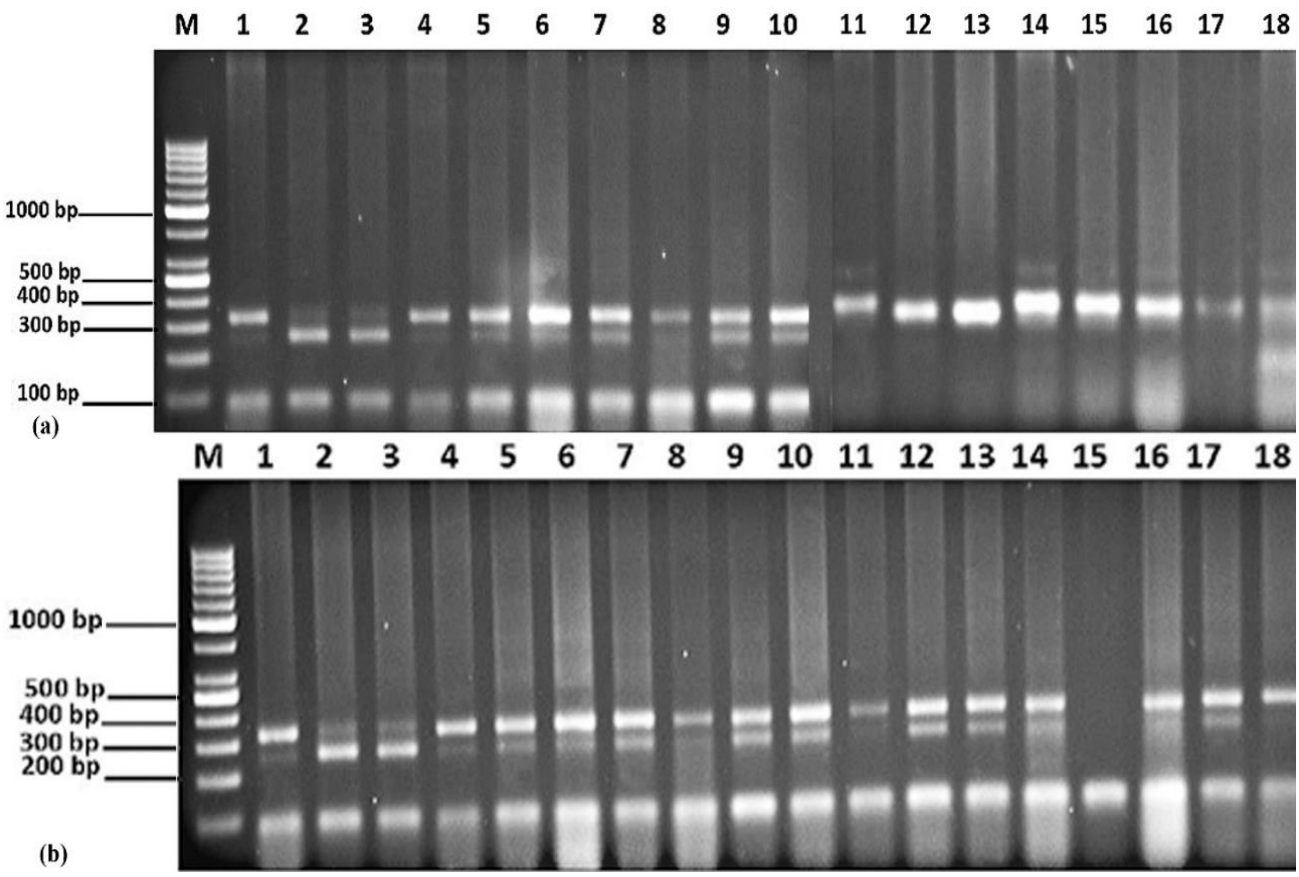


Fig. 1. Gel photographs of PCR products amplified by RAPD primers (a) = OP-B1, and (b) = OP-G19. (Lanes are labelled with number as per accession number listed in Table 1).

Table 2. Summary of sequence, annealing temperature, band size, polymorphism and polymorphic information content of six RAPD primers.

Primer code	Sequence 5′→3′	T_m (°C)	TB (No.)	Band size Min-Max (bp)	PB (No.)	Polymorphism (%)	PIC
OP-B1	GTTTCGCTCC	43	25	290 – 355	11	44.0	0.480
OP-B8	GTCCACACGG	48	15	300 – 320	15	100.0	0.653
OP-G3	AGACCCTCCA	44	15	295 – 380	11	73.33	0.753
OP-G11	TGCCCGTCGT	54	15	270 – 290	15	100.0	0.651
OP-AP12	GTCTTACCCC	40	16	500 – 600	16	100.0	0.605
OP-G19	GTCAGGGCAA	37	26	290 – 500	8	30.77	0.528
Total			112	-	76	67.86	3.670
Mean			18.67	-	12.67	67.86	0.612

PB = Polymorphic bands; TB = Total bands; Tm = Annealing temp, PIC = Polymorphic information content

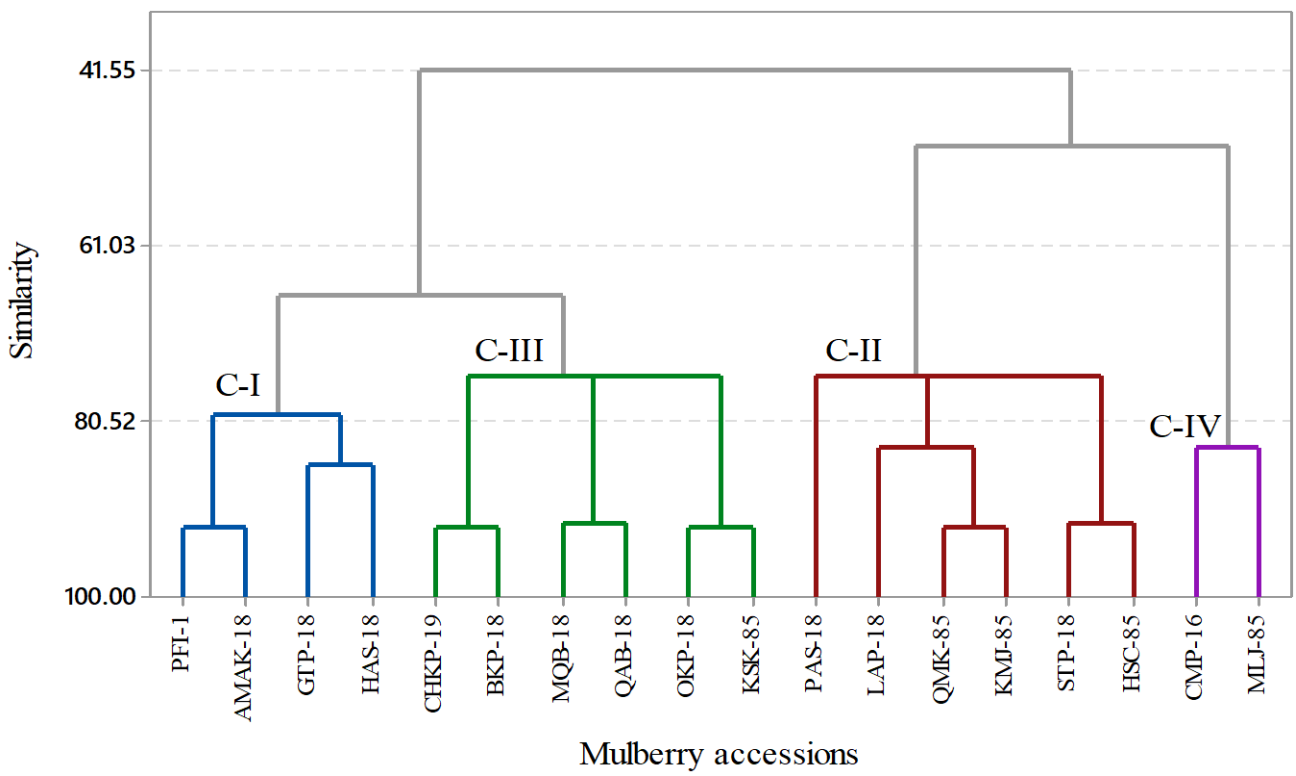


Fig. 2. UPGMA dendrogram exhibiting genetic relatedness of 18 mulberry accessions (C-I = Cluster I; C-II = Cluster II; C-III = Cluster III; C-IV = Cluster IV).

Discussion

An advance knowledge of genetic divergence in mulberry accessions is important for conservation and future breeding programmes. Precise information of genetic diversity within ecotype has paramount importance to ascertain what to conserve, and where to conserve. Genetic diversity is determined traditionally by phenotypically. However, due to high degree of morphological plasticity in mulberry this method is highly unreliable. The use of molecular techniques for genomic studies has definite advantages over all other methods. Among molecular markers, the PCR-based RAPD analysis is a simple and a powerful tool and does not require priori knowledge of the target genome. It is used often to detect genetic diversity and genetic relationship (Akzad *et al*., 2021).

Our findings reveal that the tested primers are useful for deciphering genetic diversity and establishing genetic relatedness as well in mulberry genotypes. The six RAPD primers amplify altogether 112 bands of 270 to 600 bp size, out of which 67.86% are polymorphic in nature. Apart from polymorphism, PIC is another reliable criterion applied for suggesting usefulness of a molecular marker (Orhan *et al*., 2020). The primers used in this study provide valuable data regarding PIC. Based upon categorization of molecular primers as described by Botstein *et al*., (1980), > 83% of our primers turned out to be highly informative (PIC > 0.5), while < 17% primers are fairly informative (PIC = 0.48).

Polymorphism and PIC expose a substantial level of genetic diversity amongst 18 mulberry accessions, either cultivated or wild, belonging to different ecotypes. The polymorphism disclosed by our study is comparable with Kalpana *et al*., (2012). Kalpana and his research team found 66.67% polymorphism via 40 RAPD primers in 16 Korean mulberry cultivars belonging to *M. alba*. Our findings of polymorphism of 12.67 bands primer^{-1} are greater compared to Awasthi *et al*., (2004) and Sheet *et al*., (2018). Awasthi and his research team obtained a sum total of 128 discrete bands using 19 RAPD markers in 15 mulberry species with a polymorphism of 6.26 bands primer^{-1}. Sheet and his research group achieved 59.62% polymorphism using ten RAPD primers in nine cultivated Korean mulberry cultivars. Our primers disclose PIC ranging from 0.480 to 0.753, ($\bar{x}$ = 0.612 primer^{-1}). The present mean PIC is greater compared to Sheet *et al*., (2018), who obtained PIC ranging from 0.173 to 0.792, ($\bar{x}$ = 0.457). Contrarily, Ozrenk *et al*., (2010) reported greater polymorphism (86.0%) than ours. Ozrenk and his research group obtained this polymorphism using six RAPD primers while analyzing genetic diversity amongst 47 mulberry genotypes collected from Eastern Anatolia-Turkey. The variation in polymorphism and PIC obtained by above mentioned researchers using different primers may be assigned, besides other factors, to incomplete annealing primer to DNA template. A compatible quantitative ratio between DNA template and a primer is vital for consistent RAPD-PCR products (Ali *et al*., 2006). A molar excess of primers against DNA template is necessary for complete annealing and to avoid self-annealing as well.

All under study accessions are genetically different from each other. The primers generate similarity coefficient between 0.364 and 0.933 ($\bar{x}$ = 0.725), indicating a substantial genetic diversity amongst the accessions. These findings validate a high-degree of genetic diversity in the accessions either cultivated or wild, collected from different ecotypes. We find higher genetic diversity compared to previously reported by Sheet *et al*., (2018). They obtained genetic similarity coefficients ranging from 0.361 to 0.790 ($\bar{x}$ = 0.575) in nine mulberry cultivars. Present findings further highlight higher genetic diversity in cultivated accessions compared to wild accessions. Moreover, tropical and subtropical ecotypes harbour greater genetic diversity compared to temperate ecotypes. Broadly these findings are in agreement with that of Awasthi *et al*., (2004), who reported greater genetic divergence in cultivated species of mulberry. Zhao *et al*., (2007b) formerly compared genetic diversity among 19 cultivated and eight wild mulberry accessions in China using ISSR and SSR markers. They found cultivated accessions different genetically from wild accessions.

The UPGMA dendrogram confirms genetic diversity patterns and relatedness amongst the accessions. As a whole, 18 accessions are divided into four main divergent clusters. The accessions collected from dry temperate ecotype are clustered in C-III. Accessions MQB-18 and QAB-18, collected from almost same altitude, are the closest genetically. Accession AMAK-18 (cultivated) collected from moist temperate is at a good distance from the accessions of dry temperate ecotype. Eighty-nine percent wild accessions belonging to tropical and sub-tropical ecotypes are grouped into C-II and C-III. Overseas accessions fall in one main cluster except MLJ-85 that lies with indigenous accession CMP-16 (cultivated). Among overseas accessions QMK-85 relates closely with KMJ-85. All the accessions fall in one principal cluster that indicates that they have a common ancestor. While down to the cluster and sub-cluster levels, there are further grouping that means these accessions have evolved in different environments which diverged them. The close relationship between CMP-16 and MLJ-85 suggests either there is convergent evolution in these loci or they possess a common ancestor, which could be natural phenomenon or breeding.

Separation of accessions across ecotypes, especially tropical and subtropical in two clusters, shows existence of a considerable genetic diversity in the ecotypes. Our findings of high genetic diversity within ecotypes are in conformity with Vijayan *et al*., (2004). They grouped genotypes of genus *Morus* collected from different geographical locations in different clusters due to different levels of genetic diversity within a geographical location. Zhao *et al*., (2007a) analyzed eight populations, comprising 66 varieties, of mulberry in different ecotypes in China using 12 ISSR primers. They found relationship among mulberry varieties within ecotype with some anomalies, such as, genotypes from the same district were placed in distant clusters. Similarly, Kafkas *et al*., (2008) found weak genetic relatedness within geographical regions amongst 43 mulberry accessions belonging to three species. They found strong clustering of accessions around their respective species instead of their geographical regions. Likewise, Park *et al*., (2020) found assorted genetic relatedness among 48 mulberry (*Morus* spp.) genotypes collected from different regions. They recorded a weak mutual genetic relatedness among overseas genotypes.

Our study is the first attempt to ascertain mulberry genetic resources in different ecotypes in the country using RAPD markers. The current band profiles amplified using six RAPD markers are useful to compare the patterns of unknown varieties and also to predict their ancestry. This study discloses all together a significant genetic diversity in cultivated and wild accessions. The cultivated accessions PFI-1 and MLJ-85 (Japanese origin) are distantly related, while the wild indigenous accession OKP-18 of sub-tropical ecotype is related distantly with HSC-85 (Chinese origin). The indigenous cultivated accession CMP-16 and wild sub-tropical accession STP-18 are genetically the farthest from each other. These remotely related accessions are highly likely to produce more productive and hardy varieties through transgressive breeding. These accessions, therefore, should be conserved in their respective ecotypes for the benefit of breeders working globally.

Conclusions

The results showed that the six RAPD primers were polymorphic and highly informative with PIC ranging from 0.480 to 0.753. The similarity coefficient of 0.364 to 0.933 showed the existence of a considerable genetic diversity among 18 accessions. Cultivated accessions were genetically more diverse compared to wild ones. Tropical and subtropical ecotypes were richer in mulberry genetic resources compared to temperate, both moist and dry ecotypes. Altogether, accessions are divided in four distinct genetic groups. Indigenous accessions CMP-16 and STP-18 are genetically faraway from each other. Similarly, accessions PFI-1 and OKP-18 are distantly related with overseas accessions MLJ-85 (Japanese origin) and HSC-85 (Chinese origin), respectively. These findings recommend *in-situ* conservation of four remotely related indigenous mulberry accessions for future breeding programmes at national and international level as well.

Acknowledgements

This research was financed by Government of Khyber Pakhtunkhwa-Pakistan under Development Project titled "Mapping, Digitization, Value Addition and Marketing of NTFP in Collaboration with NTFP Directorate Forest Department" (Scheme code: 386/180436).

References

Ahmad, Z., A.S. Mumtaz, M. Nisar and N. Khan. 2012. Diversity analysis of chickpea (*Cicer arietinum* L.) germplasm and its implications for conservation and crop breeding. *Agri. Sci*., 5(3): 723-731. https://doi.org/10.4236/as.2012.35087

Akzad, M.B., S. Nuraeni and S.H. Larekeng. 2021. Detecting DNA polymorphism on mulberry (*Morus* sp.) using RAPD and ISSR markers. *Plant Cell Biotechnol. Mol. Biol*., 22 (35&36): 106-111.

Ali, B.A., T.H. Huang, H.H. Salem and Q.D. Xie. 2006. Influence of thermal cycler day-to-day reproducibility of random amplified polymorphic DNA fingerprints. *Biotechnol*., 5(3): 324-329. https://doi.10.3923/biotech.2006.324.329

Andersen, J.R. and T. Lübberstedt. 2003. Functional markers in plants. *Trends Plant Sci*., 8(11): 554-560. https://doi.org/ 10.1016/j.tplants.2003.09.010

Anderson, J.A., G.A. Churchill, J.E. Autrique, S.D. Tanksley and M.E. Sorrells. 1993. Optimizing parental selection for genetic linkage maps. *Genome*, 36(1): 181-186.

Awasthi, A.K., G.M. Nagaraja, G.V. Naik, S. Kanginakudru, K. Thangavelu and J. Nagaraju. 2004. Genetic diversity and relationships in mulberry (genus *Morus*) as revealed by RAPD and ISSR marker assays. *BMC Genet*., 5(1): 1. https://doi.org/10.1186/1471-2156-5-1

Bajwa, G.A., M. Umair, Y. Nawab and Z. Rizwan. 2021. Morphometry of leaf and shoot variables to assess aboveground biomass structure and carbon sequestration by different varieties of white mulberry (*Morus alba* L.). *J. For. Res.,* 32(4): 2291-2300. https://doi.org/10.1007/s11676-020-01268-7.

Botstein, D., R.L. White, M. Skolnick and R.W. Davis. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Amer. J. Hum. Genet.,* 32(3): 314-331.

Botton, A., G. Barcaccia, S. Cappellozza, R.D. Tos, C. Bonghi and A. Ramina. 2005. DNA fingerprinting sheds light on the origin of introduced mulberry (*Morus* spp.) accessions in Italy. *Genet. Resour. Crop Evol.,* 52(2): 181-192. https://doi.org/10.1007/s10722-003-4429-x

Bukhari, S.S.B., G.A. Bajwa and M. Ali. 2010. Genetic diversity and relatedness in exotic mulberry (*Morus*) germplasm accessions. *Pak. J. Forest*., 60(1): 1-12.

Doyle, J.J. and J.L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.,* 19(1): 11-15.

Hou, Y.J. 1994. *Mulberry Breeding*. Sericulture Department, Zhejiang Agri. Uni., Hangzhou, China.

Hu, D., P. Zhang, Y.L. Sun, S. Zhang, Z. Wang and C. Chen. 2014. Genetic relationship in mulberry (*Morus* L.) inferred through PCR–RFLP and *trn*D-*trn*T sequence data of chloroplast DNA. *Biotechnol. Equip.,* 28(3): 425-430. https://doi.org/ 10.1080/13102818. 2014.928980

Insang, S., I. Kijpatanasilp, S. Jafari and K. Assatarakul. 2022. Ultrasound-assisted extraction of functional compound from mulberry (*Morus alba* L.) leaf using response surface methodology and effect of microencapsulation by spray drying on quality of optimized extract. *Ultrason. Sonochem.,* 82: https://doi.org/10.1016/j.ultsonch.2021.105806

Kafkas, S., M. Özgen, Y. Doğan, B. Özcan, S. Ercişli and S. Serçe. 2008. Molecular characterization of mulberry accessions in Turkey by AFLP markers. *J. Amer. Soc. Hort. Sci.,* 133(4): 593-597.

Kalpana, D., S.H. Choi, T.K. Choi, K. Senthil and Y.S. Lee. 2012. Assessment of genetic diversity among varieties of mulberry using RAPD and ISSR fingerprinting. *Sci. Hort.,* 134(2): 79-87.

Lee, S., M.S. Lee, E. Chang, Y. Lee, J. Lee, J. Kim, C.T Kim, I.H. Kim and Y. Kim. 2020. Mulberry fruit extract promotes serum HDL-cholesterol levels and suppresses hepatic microRNA-33 expression in rats fed high cholesterol/ cholic acid diet. *Nutrients,* 12(5): 1499. https://doi.org/ 10.3390/ nu12051499

Li, Y., Y. Wang, Q. He and Y.L. Yang. 2020. Calculation and evaluation of carbon footprint in mulberry production: a case of Haining in China. *Int. J. Environ. Res. Public Health*, 17(4): 1339. https://doi.org/10.3390/ijerph1704133917

Nei, M. and W. Li. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. (Genetics) USA,* 76(10): 5269-5273.

Orhan, E., M. Akin, S.P. Eyduran and S. Ercisl. 2020. Molecular characterization of mulberry genotypes and species in Turkey. *Not. Bot. Hort. Agrobot.,* 48(2): 549-557. https://doi.org/ 10.15835/nbha48211928

Ozrenk, K., G.R. Sensoy, C. Erdinc, M. Guleryuz and A. Aykanat. 2010. Molecular characterization of mulberry germplasm from Eastern Anatolia. *Afr. J. Biotechnol*., 9(1): 1-6. https://doi.org/10.5897/AJB09.997

Pádua, J.G. 2018. Conservation of crop genetic resources in Brazil in the context of the target 9 of the global strategy for plant conservation. *Rodriguésia*, 69(4): 1557-1565. https://doi.org/ 10.1590/2175-7860201869406

Park, E.J., M.U. Kang, M.S. Choi, G.B. Sung and S.K. Nho. 2020. Analysis of the genetic relationship among mulberry (*Morus* spp.) cultivars using inter-simple sequence repeat (ISSR) markers. *Int. J. Indust. Entomol.*, 41(2): 56-62.

Rohela, G.K., S.P. Muttanna, R. Kumar and S.R. Chowdhury. 2020. Mulberry (*Morus* spp.): an ideal plant for sustainable development. *Trees, Forests & People*, 2 https://doi.org/10.1016/j.tfp.2020.100011

Sheet, S., K. Ghosh, S. Acharya, K.P. Kim and Y.S. Lee. 2018. Estimating genetic conformism of Korean mulberry cultivars using random amplified polymorphic DNA and inter-simple sequence repeat profiling. *Plants*, 7(1): 21. https://doi.org/10.3390/plants7010021

Vijayan, K. 2007. Molecular markers and their application in mulberry breeding. *Int. J. Indust. Entomol.,* 15(2): 145-155.

Vijayan, K., P.P. Srivastava and A.K. Awasthi. 2004. Analysis of phylogenetic relationship among five mulberry (*Morus*) species using molecular markers. *Genome*, 47(3): 439-448.

Wang, Z., Y. Zhang, F. Dai, G. Luo, G. Xiao and C. Tang. 2017. Genetic diversity among mulberry genotypes from seven countries. *Physiol. Mol. Biol. Plants*, 23(2): 421-427. https://doi.org/10.1007/s12298-017-0427-x

Zhang, H., Z.F. Ma, X. Luo and X. Li. 2018. Effects of mulberry fruit (*Morus alba* L.) consumption on health outcomes: a mini-review. *Antioxidants*, 7(5): 69. https://doi.org/10.3390/antiox7050069

Zhao, W., Y. Pan, Z. Zhang, S. Jia, X. Miao and Y. Huang. 2005. Phylogeny of the genus *Morus* (Urticales: Moraceae) inferred from ITS and *trn*L-F sequences. *Afr. J. Biotechnol*., 4(6): 563-569.

Zhao, W., Y. Wang, T. Chen, G. Jia, X. J. Qi, Y. Pang, S. Wang, Z. Li, Y. Huang, Y. Pan and Y.H. Yang. 2007a. Genetic structure of mulberry from different ecotypes revealed by ISSRs in China: an implication for conservation of local mulberry varieties. *Sci. Hortic*., 115(1): 47-55. https://doi.org/10.1016/j.scienta.2007.07.017

Zhao, W., Z.H. Zhou, X. Miao, Y. Zhang, S.B. Wang, J.H. Huang, H. Xiang, Y. Pan and Y. Huang. 2007b. A comparison of genetic variation among wild and cultivated *Morus* species (Moraceae: *Morus*) as revealed by ISSR and SSR markers. *Biodiv. Conserv*., 16(2): 275-290. https://doi.org/10.1007/s10531-005-6973-5.

(Received for publication 13 May 2022)