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Full Length Research Paper

Evaluation of genetic diversity in *Pyrus* germplasm native to Azad Jammu and Kashmir (Northern Pakistan) revealed by microsatellite markers

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Pyrus **germplasm of Azad Jammu and Kashmir (56 accessions) and East Malling Research Station Kent, UK (8 reference cultivars) were characterized using 12 microsatellite markers assessing genetic diversity. In total, 9 primer pairs revealed 106 putative alleles that ranged from 7 to 19, averaging 11.8 alleles per locus. The size of amplified fragments ranged between 83 and 328 bp, while polymorphism information content varied from 0.780 to 0.918, averaging 0.844. Similarity coefficients ranging from 0.00 to 1.00 was observed in KT53 (Btung) and between BG21 and MZ26 which are both locally called 'Kotharnul'. UPGMA cluster analysis of similarity grouped all the genotypes into 3 main clusters, two identical groups and four accession/varieties which fell independently based on their pedigree and geographical origin. Similarity data illustrated an unambiguous genetic diversity and relationship among different ecotypes and varieties in a dendrogram. The most phenotypically variable accessions from different geographical regions fell into two homogeneous groups, 'A 'and 'B', with some branched accessions. Among reference varieties, 5 distantly fell into one cluster, 2 branched with the lowest affinity to each other and '*Pendula*' existed independently showing the highest diversity. The local accession KT53 (Btung), representing the highest genetic diversity to all genotypes, had a unique genetic base.**

Key words: Genetic diversity, gene pool, simple sequence repeat (SSR), pear.

INTRODUCTION

The genus *Pyrus* belongs to the subfamily Pomoideae of family *Rosaceae*, and its two main species, *Pyrus communis*. L. and *Pyrus pyrifolia* (Burm) are comercially grown throughout the temperate zones of the world. Pear stands second in ranking after apples as the most important tree fruit in the world, grown in all the continents of the world. About 72% of all commercially cultivated species of genus *Pyrus* are native to Asia and the United States containing 1,500 clones, in which 40% of these holdings are Asian pear cultivars (Nee et al., 2002). Overall, world pear production reached 19.5 million metric tonnes in 2006 (FAO, 2006).

The genetic variability in *Pyrus* germplasm has been accumulated through hybridization and naturally, through seed based propagation. Moreover, systematic characterization and evaluation in pear is still confined for the selection of desirable types. A large amount of genetic diversity exists in plant species, particularly in fruit crops in the state of Azad Jammu and Kashmir (AJK), which

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Abbreviations: PIC, Polymorphism information contents; **CTAB,** cetyl trimethyl ammonium bromide; **SSR,** simple sequence repeats; **PCR,** polymerase chain reaction; **RFLP,** restriction fragment length polymorphism; **AFLP,** amplified fragment length polymorphism; **RAPD,** random amplified polymorphic DNA.

geographically lies between the two major centres of origin of temperate fruit plant species (that is, the Caucasus Mountains and China). Like other temperate fruit species, cultivated pear, wild relatives and naturalised forms found in this area are yet to be explored. A recent report (Volk et al., 2006) shows the existence of a high diversity at the molecular level in wild pear (*P. communis*) that has still not been exploited.

Though, morphological and phenological characterization provides basic data about the ecotypes, it is still not sufficient to assess genetic diversity in pear genotypes because of low differentiating traits among species and varieties (Yu and Kuan, 1963; Yuan and Du, 1980). It is difficult to distinguish genotypes because of the influence of environment and localities on their phenotypical behaviours. Moreover, a lengthy and expensive evaluation is required during the vegetative growth period to obtain morphological data (Rajapakse et al., 1995). In the past, physiological and protein based tools have also been used to estimate the genetic diversity in *Pyrus* species on a limited scale (Jang et al., 1991, 1992; Chevreau et al., 1997). However, different DNA based genetic marker, that is, RAPD, AFLP and RFLP can be used also to identify genetic variability among pear genotypes. Additionally, DNA based genetic markers have been reported for the purpose of selection and characterization in Japanese pear (Inoue et al., 2006). Genetic variability of *Pyrus* and several other related species has been assessed using DNA markers based on PCR, such as simple sequence repeats (SSR) (Wunsch and Hormaza, 2002a; Yamamoto et al., 2002a; Brini et al., 2008). However, opportunities to compare results between collections in confirming the trueness to name are hampered as different laboratories tend to use different sets of microsatellites (Wunsch and Hormaza, 2002b; Liebhard et al., 2002; Fernandez-Fernandez et al., 2006). Microsatellites or SSR markers have been proven to be highly useful in this type of application due to their reproducibility, codominance and polymorphism (Powell et al., 1996), as well as their transferability among related species and genera (Yamamoto et al., 2001; Wunsch and Hormaza, 2002b). SSR markers, developed from *P. pyrifolia* (Yamamoto et al., 2002b, c), have been used for the genetic characterization of European and Asian pear accessions (Kimura et al., 2002; Ghosh et al., 2006). On the basis of these studies in *Pyrus* and other species, the present work was planned to test the ability of SSR markers. These markers have widely been used in mapping, fingerprinting and diversity studies. Only 10 SSR primer pairs from European pear have been reported to date as most work focused on Japanese pear (Yamamoto et al., 2002a). Although, approximately 75% of the SSRs developed in *P. pyrifolia* are polymorphic in European pear, there is still a need for more co-dominant markers of *Pyrus* (Yamamoto et al., 2002a,b,c). Unfortunately, *Pyrus* genotypes existing in the State of AJK (Northern Pakistan) have not been thoroughly studied genetically, even when the genetic diversity of several *Pyrus* species has been well evaluated. The objective of the present study was to investigate the potential and limits of the molecular technique in assessing the genetic diversity and relationship among local accessions collected from the State of AJK in comparison with 8 control/ reference samples of diverse origin. This was first attempted in the study of genetic diversity at the molecular level in local pear genotypes that are naturalized in mountainous areas of Northern Pakistan.

MATERIALS AND METHODS

Plant material

Leaves of 56 local ecotypes/accessions were collected from different localities of AJK (Northern Pakistan) and transported to UK, at the University of Sussex, School of Life Sciences, Department of Biological Sciences, for evaluation. The samples were stored at - 80°C temperature until DNA was extracted. For a comparative study, fresh leaves from the eight reference varieties, 'Abbe Fetel', 'Doyenne du Comice', 'Conference', 'Passe Crassane' and 'Williams' (all *P. communis*), 'Hosui' (*P. pyrifolia*), 'Pendula' (*Pyrus salicifolia)* and 'Chantecler' (*Pyrus calleryana),* were collected from East Malling Research Station in the National Fruit Collection at Brogdale, UK and frozen in liquid nitrogen.

Genomic DNA extraction and microsatellite analysis

Genomic DNA of local accessions was extracted using a DNeasy Plant Mini and DNeasy Plant Maxi Kit (Qiagen Inc., Mississauga, Ont.), while DNA of reference varieties was extracted following a CTAB protocol as described by Fernández-Fernández et al. (2006).

Choice of primers and optimization of PCR

Twelve SSR primer pairs as shown in Table 1 were selected from each linkage group (where possible) as a subset of markers based on robustness, preferably single locus, and which have been proven to be polymorphic in previous studies (Liebhard et al., 2002; Fernandez-Fernandez et al., 2006). PCR conditions were optimised to obtain strong and reliable amplification. The DNA samples were amplified with the chosen primers in multiplex reactions using a thermocycler machine and ensuring the presence of the selected control samples within each plate.

PCR reactions were carried out in 12.5 □l volumes containing 2.5 ng of DNA, 20 mM Tris-HCl (pH 8.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM of each dNTP, 0.25 units of *Taq* polymerase (Invitrogen) and 2 µM of each forward and reverse primer, 0.25 unit *Taq* DNA polymerase and 3.125 ng template DNA. Forward primers were labelled with the fluorescent dyes 6-FAM, NED, VIC or PET (ABI) and the tetraplex system was used. The thermal cycler was programmed and the PCR conditions comprised an initial denaturation at 94°C for 05 min, touchdown phase 94°C for 30 s, annealing temperature at 55°C for 45 s (0.5°C decreased per cycle ×10 cycles), 72°C for 60 s, 94°C for 30 s, 50°C for 45 s × 15 cycles, 72°C for 60 s followed by the final extension at 72°C for 10 min and held for 4°C.

Data analysis

After amplification, PCR products were loaded onto an ABI automated sequencer so that the alleles could be sized. The quality and

Table 1. Passport data of 56 *Pyrus* accessions/ wild genotypes collected from five districts of the State of Azad Jammu and Kashmir.

Accession number	Local name of the accession	Area explored/ location	Altitude (m)	Rain fall (mm)
RT1	Khurolli	Rawalakot	1200 - 2700	1600
RT2	Bagugosha	Paniolla		
RT3	Glass	Parat		
RT4	Pathar nakh	Pothi		
RT5	Kotharnul	Khurick		
RT6	Khurolli	Duraik		
RT7	Desi nash	Hussain kot		
RT8	Btung	Bunjosa		
RT9	Btangi	Tolipeer		
RT10	Kashmiri nakh	Sigholla		
RT11	Glass	Charh		
RT12	Bagugosha	Hajira		
RT13	Frashishi	Hajira		
BG14	Desi nashpati	Bagh	1200 - 2700	1600
BG15	Btung	Arga		
BG16	Khurolli	Dhirkot		
BG17	Btangi	Sudhongulli		
BG18	Kashmiri nakh	Malot		
BG19	Kotharnul	Chattar		
BG20	Desi nakh	Dhull Road		
BG21	Kotharnul	Peer Seydhan		
BG22	Khurolli	Shairutarah		
BG23	Kashmiri nakh	Khotta Farward		
BG24	Bagugosha	Kurshidabad		
BG25	Frashishi	Peer Seydhan		
MZ26	Kotharnul	Muzaffarabad	600 - 4500	900
MZ27	Desi nashpati	Ghariduptta		
MZ28	Glass	Hutian		
MZ29	Kashmiri nakh	Chakar		
MZ30	Raj btung	Chukothi		
MZ31	Raj btung	Chanari		
MZ32	Frashishi	Leepa		
MZ33	Btangi	Monjhohi		
MZ34	Pathar nakh	Challa Bondhi		
MZ35	Desi nakh	Kirn		
MZ36	Pathar nakh	Kale		
MZ37	Raj btung	Authmuqam		
SD38	Btangi	Sudhnoti	600 – 1800	800
SD39	Desi nakh	Goha		
SD40	Frashishi	Trarkhal		
SD41	Desi nakh	Bloch		
SD42	Khurolli	Jundalli		
SD43	Pathar nakh	Jundalli		
SD44	Bagugosha	Mung		
SD45	Kashmiri nakh	Thorar		
SD46	Glass	Tahain		
SD47	Desi nakh	Thorar		
SD48	Nashpati	Mung		
SD49	Frashishi	Azadputtan		

Table 1. Contd.

KT50	Kotharnul	Khuhi Rutta		
KT51	Kashmiri nakh	Nikyal		
KT52	Desi nakh	Nikyal		
KT53	Btung	Tattapani	600 - 1800	800
KT54	Btangi	Tattapani		
KT55	Raj btung	Mundhol		
KT56	Bagugosha	Tatrinot		

reliability of the fingerprints was assured by using standardised methodologies with optimised primers and regular control samples.

The data were compiled using GENESCAN and GENOTYPER software. Hard copies of all traces from the software were printed allowing clear comparisons between peak intensity and fragment size of the amplified products. Any variation in peak size between plates was normalised by comparing the control samples. All data were checked twice and repeats were performed on any sample, whose traces were unclear.

Amplified products from microsatellite analyses were scored qualitatively for the presence and absence of each marker allele-genotype combination. Each SSR band amplified by a given primer was treated as a unit character. Data were entered into a binary matrix as discrete variables, that is, 1 for presence and 0 for absence of the character. The PIC value of a marker was calculated according to the following formula:

$$\underset{i}{\mathbf{PIC}} = 1 - \sum_{j=1}^{n} P^2_{ij}$$

Where P_{ij} is the frequency of *j*th allele for the *i*th marker, and summed over *n* alleles.

Average number of alleles, average PIC value and average genetic similarity were computed on the basis of different pear accessions/ cultivars and microsatellite classes. Pair-wise comparisons of the ecotypes based on the proportion of unique and shared amplification alleles were used to measure the genetic similarity by Dice coefficients using Simqual sub-program in a similarity routine of NTSYS-pc software package version 2.2 (Rohlf, 2004). Estimates of genetic similarity (F) were calculated between all pairs of the genotypes according to Nei and Li (1979) based on the following formula:

$$\text{Similarity (F)} = 2N_{ab} / (N_a + N_b)$$

Where N_a = the total number of fragments detected in individual 'a', N_b = the total number of fragments shown by individual 'b' and N_{ab} = the number of fragments shared by individuals 'a' and 'b'.

The resultant similarity matrix data were employed to construct a dendrogram using Sequential Agglomerative Hierarchical Nesting (SAHN) based on unweighted pair-group method with an arithmetic average (UPGMA) to infer genetic relationships and phylogeny among the accessions. All computations were carried out using the NTSYS-pc software package version 2.2 (Rohlf, 2004).

RESULTS

Genetic polymorphism

A total of 12 microsatellite (SSR) markers were used to characterize and assess the genetic diversity of 56 pear accessions and eight reference varieties (Table 1), in which 9 primers were successfully transferred to pear and they generated right and reproducible products in 33 accessions and 8 varieties of pear, whereas 3 markers (CH01f0a, CH03d12 and EMPc117) were monomorphic or could not amplify some of the pear accessions (Table 2). A considerable level of variability was observed among different accessions as well as cultivars. In most of the cases, reference cultivars exhibited distinct banding patterns. The microsatellites exhibited several bands that were shared among the local accessions, whereas a few bands were shared among local and reference accessions of *Pyrus communis* and *P. pyrifolia.* Results from the analysis of the reference set of accessions with the standard set of SSRs are shown in Table 3. The inclusion of *P. pyrifolia* 'Hosui', *P. salicifolia* 'Pendula' and *P. calleryana* 'Chantecler' in the reference set of *P. communis* cultivars resulted in a wide range of allele sizes, as expected. Interestingly, many primers revealed characteristic fragments in reference cultivars which were not produced in any of the local accessions used. Similarly, one of the local accessions, Btung (KT53), shared very limited alleles with other accessions and was most diverse.

The level of polymorphism among the local accessions and control cultivars was evaluated by calculating the allele number and PIC values for each of the 9 SSR loci evaluated. Each of the loci differed significantly in their ability to determine variability among the genotypes. A total number of 106 different reproducible and scorable alleles were detected across 33 accessions and 8 cultivars with 9 SSR markers. The number of alleles per locus, generated by each marker, varied from 7 (CH04e03) to 19 (CH01d08) with an average of 11.8 alleles per locus (Table 3). The overall size of the alleles varied from 83 (CH05c06) to 321 bp (CH01d08) for 9 loci. All markers revealed polymorphism among the pear genotypes. However, a local collection RT6 (Khurolli) gave the highest numbers of alleles (26) (Table 2). It was closely followed by RT9 (Btangi) producing 23 alleles, while the accession SD39 (Desi nakh) and two cultivars (Chantecler and Hosui) gave the least number of alleles (that is, 15). The level of polymorphism among the 33 accessions and 8 cultivars was evaluated by calculating PIC values for each of the 9 SSR loci. The PIC values varied among loci and ranged from 0.780 (EMPc11) to 0.918 (CH01d08)

Table 2. Number of alleles per accession, group /varieties, on the basis of 0 and 1 SSR analysis.

Names of accessions/ varieties	Group/ individual/control	Number of alleles
RT1	A-Group	18
RT2	A-Group	18
RT4	A-Group	18
RT7	A-Group	18
RT12	A-Group	18
RT13	A-Group	18
BG14	A-Group	18
BG19	A-Group	18
BG24	A-Group	18
BG25	A-Group	18
MZ34	A-Group	18
SD42	A-Group	18
SD47	A-Group	18
RT8	B-Group	18
RT10	B-Group	18
RT11	B-Group	18
BG16	B-Group	18
BG17	B-Group	18
SD40	B-Group	18
SD41	B-Group	18
SD46	B-Group	18
KT50	B-Group	18
KT52	B-Group	18
MZ26	Individual	17
RT3	Individual	16
RT6	Individual	26
RT9	Individual	23
BG15	Individual	19
BG18	Individual	18
BG21	Individual	17
MZ32	Individual	21
SD39	Individual	15
KT53	Individual	16
Abbe Fetel	Control	16
Chantecler	Control	15
Comice	Control	17
Conference	Control	16
Hosui	Control	15
Passe Crassane	Control	16
Pendula	Control	17
Williams	Control	16

with an average of 0.844 per locus (Table 3). A random selection of the amplified fragments was tested by sequencing in confirming the contained SSRs. Three of these loci did not show any bands in the accessions and presented a pattern of stutters to the left of the main peak, with possible 'null' alleles. Therefore, parameter values of these loci were not included in the calculation of average values.

Genetic relationships among genotypes

A similarity matrix based on the proportion of shared SSR alleles was used to establish the level of relatedness

Table 3. Details of SSR markers used, indicating their linkage group on pear chromosomes, number of alleles detected, allele size range and polymorphism information content (PIC).

Marker	Linkage group	Forward primer	Reverse Primer	Dye EMR	Polymorphic Alleles	Size range (bp)	Difference (bp)	PIC value
CH01d08	15	CTCCGCCGCTATAACACTTC	TACTCTGGAGGGTATGTCAAAG	Npig	19	239 - 321	82	0.918
CH03g07	3	AATAAGCATTCAAAGCAATCCG	TTTTTCCAAATCGAGTTTCGTT	F	15	198 - 264	66	0.877
CH04e03	5	TTGAAGATGTTTGGCTGTGC	TGCATGTCTGTCTCCTCCAT	P	7	177 - 227	50	0.813
CH02b10	2	CAAGGAAATCATCAAAGATTCAAG	CAAGTGGCTTCGGATAGTTG	P	12	112 - 160	48	0.858
CH01d09	12	GCCATCTGAACAGAATGTGC	CCCTTCATTCACATTTCCAG	V	12	122 - 172	50	0.890
GD96	17	CGGCGGAAAGCAATCACCT	GCCAGCCCTCTATGGTTCCAGA	N	10	139 - 195	56	0.794
CH05c06	16	ATTGGAACTCTCCGTATTGTGC	ATCAACAGTAGTGGTAGCCGGT	F	12	83 - 119	36	0.858
EMPc11	11	GCGATTAAAGATCAATAAACCCATA	AAGCAGCTGGTTGGTGAAAT	N	10	123 - 171	48	0.780
GD147	13	TCCCGCCATTTCTCTGC	GTTTAAACCGCTGCTGCTGAAC	P	9	112 - 164	52	0.809
CH01f07a	10	CCCTACACAGTTTCTCAACCC	CGTTTTTGGAGCGTAGGAAC	V	-	175 - 214	39	-
CH03d12	6	GCCCAGAAGCAATAAGTAAACC	ATTGCTCCATGCATAAAGGG	F	-	84 - 162	78	-
EMPc117	7	GTTCTATCTACCAAGCCACGCT	CGTTTGTGTTTTACGTGTTG	V	-	82 - 142	60	-
Total	-			-	106	-	-	-
Average	-			-	11.8	-	-	0.844

between the local accessions and reference cultivars, while the UPGMA dendrogram was constructed from the genetic similarity coefficients. Both analyses revealed a varying degree of genetic relationship for cultivars belonging to different species and the accessions used. Pair-wise genetic similarity coefficients of accessions and cultivars estimated by Dice's coefficient (Nei and Li 1979) varied from 0.00 to 1.00 (Table 4). Thirteen accessions in group-A and ten in group-B were the closest genotypes and they exhibited 100% similarity among themselves. Likewise, BG21 (Kotharnul) and MZ26 (Kotharnul) were also analogous and displayed 100% similarity. The lowest level of genetic similarity (0.00%) was obtained between 'Conference' and group-B accessions, 'Conference' and RT3 (Glass), 'Hosui' and 'Abbe Fetel' and SD39 and KT53 (locally called Btung).

Cluster analysis clearly separated and effectively differentiated 41 genotypes, including 8 reference cultivars, into 3 clusters that comprised two identical groups and four independently falling cultivars/accession, based on similarity coefficient levels according to their pedigree and geographic origins (Figure 1).

Cluster I: The largest cluster consisted of 28 local accessions including two identical groups separated from other clusters at a similarity value of 0.40. The first group included a total of 13 accessions, denoted as group-A (Figure 1) along with two other closely associated acces-sions, MZ32 (Frashishi) and RT6 (Khurolli), separated from the homogenous group-A with a similarity value of 0.92 and 0.64, respectively. Ten accessions fell in the second identical group-B along with three more acces-sions, RT3 (Glass), BG21 (Kotharnul) and MZ26 (Kotharnul), that are branched closely and related with this group (Figure 1). Furthermore, BG21 (Kotharnul) and MZ26 (Kotharnul) had 100% similarity with each other.

Cluster II: Three accessions namely RT9, BG15 and SD39 (locally called Btangi, Btung and Desi nakh, respectively) fell in cluster II and were separated from each other at the similarity coefficient levels of 0.90 (RT9 and BG15) and 0.79 (SD39), thereby showing a high level of genetic relationship. However, a considerable level of genetic variability was found between the accessions of cluster I and those in cluster II (Figure 1).

Cluster III: This cluster consisted of all European cultivars (*P. communis*) namely 'Abbe Fetel', 'Comice', 'Conference', 'Passe Crassane' and 'Williams' and one accession BG18 (Kashmiri nakh). All these varieties had close affinity with each other and distantly separated from the local

Table 4. Coefficients of similarity for 33 pear accessions with 8 representative varieties based on SSR analysis.

No.	Accession	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	A-Group	1.000																			
2	B-Group	0.500	1.000																		
3	RT3	0.486	0.973	1.000																	
4	RT6	0.636	0.636	0.667	1.000																
5	RT9	0.341	0.488	0.476	0.531	1.000															
6	BG15	0.162	0.324	0.316	0.400	0.905	1.000														
7	BG18	0.216	0.108	0.158	0.267	0.333	0.368	1.000													
8	BG21	0.541	0.973	0.947	0.667	0.476	0.316	0.158	1.000												
9	MZ26	0.541	0.973	0.947	0.667	0.476	0.316	0.158	1.000	1.000											
10	MZ32	0.923	0.615	0.600	0.723	0.364	0.200	0.250	0.650	0.650	1.000										
11	SD39	0.182	0.303	0.294	0.439	0.789	0.882	0.294	0.294	0.294	0.222	1.000									
12	KT53	0.059	0.059	0.057	0.143	0.154	0.171	0.114	0.057	0.057	0.054	0.129	1.000								
13	Abbe Fetel	0.353	0.059	0.114	0.286	0.103	0.057	0.286	0.114	0.114	0.324	0.065	0.063	1.000							
14	Chantecler	0.061	0.121	0.118	0.098	0.211	0.235	0.176	0.118	0.118	0.111	0.200	0.000	0.000	1.000						
15	Comice	0.400	0.114	0.111	0.326	0.150	0.056	0.222	0.167	0.167	0.368	0.063	0.121	0.485	0.000	1.000					
16	Conference	0.235	0.000	0.000	0.286	0.154	0.171	0.171	0.057	0.057	0.216	0.194	0.125	0.313	0.065	0.424	1.000				
17	Hosui	0.061	0.061	0.059	0.049	0.158	0.118	0.176	0.059	0.059	0.056	0.000	0.000	0.000	0.133	0.063	0.065	1.000			
18	Passe rassane	0.353	0.118	0.114	0.381	0.154	0.114	0.286	0.171	0.171	0.378	0.129	0.000	0.375	0.065	0.485	0.500	0.065	1.000		
19	Pendula	0.171	0.057	0.056	0.140	0.050	0.056	0.167	0.056	0.056	0.158	0.063	0.000	0.121	0.063	0.235	0.182	0.000	0.182	1.000	
20	Williams	0.294	0.059	0.114	0.524	0.308	0.343	0.229	0.114	0.114	0.324	0.387	0.063	0.438	0.000	0.364	0.563	0.000	0.625	0.182	1.000

accession (BG18) at different similarity co-efficient values of 0.29, 0.22, 0.17 and 0.23, respectively (Table 4). However, local accession (BG18) exhibited very low genetic relationship with other local collections and also from other control varieties. Moreover, this accession branched with the European group of genotypes, but did not show a strong genetic relation within them (Figure 1). Consequently, this indicated diversity in its origin.

The three remaining reference cultivars (‘Pendula’, ‘Chantecler’ and ‘Housi’) and one local accession (KT53) made solitary groups, with each having an individual accession or cultivar. A single cultivar named as 'Pendula' (*P .salicifolia*) showed the highest genetic variability to the representative samples, as well as to all local collections with similarity coefficient values that ranged from 0.05 to 0.23 with all other genotypes. In the same pattern, genotype KT53 (Btung) had the lowest similarity, which ranged from 0 to 0.39 for all the genotypes that were analysed and showed the most diverse and variable genetic background. Further, a solitary cultivar ‘Chantecler’ (*P. calleryana*) branched distantly from an individual cultivar ‘Hosui’ at 0.133 coefficient of similarity value (Table 4). However, both cultivars were different from local accessions as well as other reference cultivars, and as a result, they were well isolated from them and from one another.

DISCUSSION

In DNA based techniques, microsatellite/SSR markers are the most preferable markers used to detect a high degree of polymorphism and identification for genus *Pyrus.* Genetic diversity and relationships among the different pear cultivars by using SSRs has been previously reported (Kim et al., 2000; Kimura et al., 2003). In the present study, 9 primers generated right and reproducible products using 33 accessions and 8 cultivars of pear genotypes, whereas 3 markers (CH01f0a, CH03d12 and EMPc117) were monomorphic or could not amplify some of the pear accessions. A considerable level of genetic variation among pear

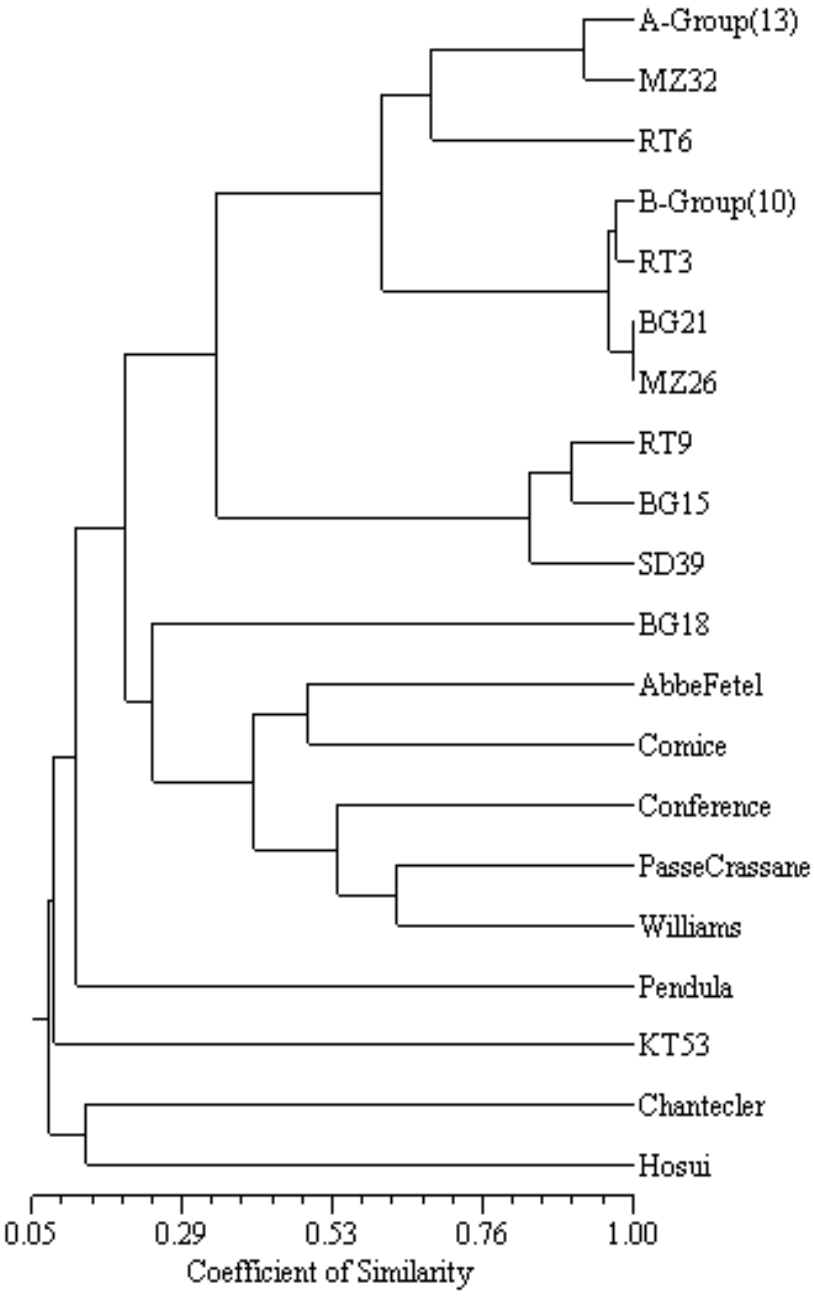

Figure 1. Dendrogram showing the genetic diversity/similarity among 33 local collections and 8 reference varieties of pear, based on Dice's coefficient of similarity analysis using nine microsatellite primer pairs. A-Group comprised 13, while B-Group comprised 10 accessions.

genotypes indicated that the nine SSR markers were enough to identify these accessions and cultivars. Moreover, SSRs have already been used for cultivar identification and construction of genetic linkage maps of species belonging to family *Rosaceae* such as *Malus* (*Malus domestica*) (Guilford et al., 1997; Gianfranceschi et al., 1998) and *Prunus* species (Cipriani et al., 1999).

Results indicated that a few polymorphic microsatillite markers (9) could differentiate a number (41) of pear genotypes with an average of 11.8 alleles per locus, which is close to 14.8 alleles per locus that was previously reported by Kimura et al. (2002), who analysed 60 pear accessions with 9 markers. The variation in the number of allele produced by SSR markers demonstrates heterozygosity in different alleles at a given locus, in which the heterozygosity could reflect greatly the state of

genetic variability (Gregorius et al., 1986; Miller and Tanskley, 1990). Moreover, lower number of alleles per locus in the present investigation has been previously reported by Guilford et al. (1997) and Gianfranceschi et al. (1998) in apple (7.1) and in 34 cultivars of *Pyrus* species (4.3) by Yamamoto et al. (2001) by using microsatellite markers. Wunsch and Hormaza (2007) also studied 7 SSRs marker developed in apple to identify the 63 European pear cultivars, and a total number of 46 alleles were amplified with an average of (6.6) allele per locus. In addition to these, seven SSRs amplified a total of 36 fragments in 25 local genotypes with 6 common varieties of pear (Brini et al., 2008). All these amplified fragments produced different fingerprinting pattern that allowed all the varieties analysed to be distinguished. It is concluded that 9 primer pairs gave reproducible and highly polymorphic DNA amplification band patterns that were selected to construct a fingerprinting in distinguishing *Pyrus* genotypes.

Number of genotypes with null alleles at 12 microsatellite markers was 23 accessions out of 56. Such accessions showed high deficit of heterozygosity, but did not produce alleles, which was perhaps due to alleles drop out or as a result of limited sample sizes. Some alleles presented a pattern of stutters to the left of the main peak, with possible 'null' alleles (Liebhard et al., 2002). Hokanson et al. (2001) also reported that large number of accessions showed null genotypes among 142 *Malus* accessions, evaluated by microsatellites primer pairs. Comparatively, lower number of alleles was common among the local accessions and reference/ control cultivars of pear. However, larger number of accessions with diverse geographical origin resulted in high level of diversity and polymorphism in *Pyrus* germplasm (Yamamoto et al., 2001). The accessions, BG18 (Kashmiri nakh) and KT53 (Btung), shared very limited number of fragments with all other local accessions, as well as *P. communis and P. pyrifolia* cultivars, thereby presenting their distant association with the local and European groups. The microsatellite assay generated accession and cultivar-specific alleles in some of the genotypes screened. These may be used as DNA fingerprints for accession/cultivar identification.

A high value of polymorphism information contents (PIC) was found in most microsatellite loci indicating that this could be a valid tool for discrimination of pear germplasm. All micosatellite loci scored in this study were highly polymorphic, displaying high value of PIC (0.780 to 0.918). The high value confirmed the finding of Belaj et al. (2007) who observed the high value of PIC (from 0.598 to 0.925), while studying genetic diversity and population structure of wild olives.

The microsatellite data demonstrated a clear division and sufficient amount of molecular variation among the local accessions and reference varieties. Furthermore, lack of genetic difference in the accessions of both group-A and group-B, could be due to either extremely low difference between the accessions at the DNA level and / or that the ancestors of given accessions were same. These accessions were also very similar in morphological and agronomic traits that strengthen the supposition at DNA level.

The accessions and cultivars were clustered and grouped mainly on the basis of their relatedness and differences according to their pedigrees and geographical features located at different regions. In dendrogram, all accessions (33) and 8 reference cultivars of pears were grouped. These accessions/ecotypes were collected on the basis of phenotypical and morphological variation and their parental and pedigree relations were unknown. Out of 8 varieties, five were clustered into one group namely 'Abbe Fetel', 'Comice', 'Conference', 'Passe Crassane' and 'Williams' of European origin, and the rest three were from Chinese and Japanese origin.

The cluster analysis revealed that genotypes could be classified into 3 main clusters, in which two homogenous groups and some genotypes fell independently. All these were separated at various affinities levels in dendrogram. Other workers (Kawata et al., 1995; Iketani et al., 1998; Oliveira et al., 1999; Monte-Corvo et al., 2000) also grouped and divided *Pyrus* into various groups using RFLP or RAPD markers. These results support the traditional view that genus *Pyrus* consists of two geographic species groups namely, occidental pears and oriental pears (Rubtsov, 1944; Layne and Quamme, 1975).

The accessions 13 and 10 in the number found in identical groups A and B, respectively, fell in cluster I and were separated from each other at a similarity value of 0.40. Most of these were phenotypically and morphologically variable to each other, but genetically, they showed identical patterns in cluster analysis and were identical and homogeneous. These accessions in each group might belong to the same clones or differ in only single gene mutations which could not be detected by SSR analysis (Testolin et al., 2000). Further, MZ32 (Frashishi) had a very close affinity to the homogeneous group-A, and RT6 (Khurolli) was also included in cluster I and shared a higher level of similarity coefficient (0.63). The above mentioned accessions found in various ecological and geographical zones of the State of AJK were variable to each other in phenotypical as well as morphological traits, which were possibly due to environmental conditions prevailing in those areas. Chamberlain and Hubert (1998) reported that the phenotypical differences might have been determined by relatively few genes which could not be reflected in the molecular results. However, the accessions of MZ32 and RT6 (Frashishi and Khurolli) although had some resemblance in their fruit shapes with varieties of cluster III, fell outside and showed a high level of diversity with varieties of 'Williams', 'Comice', 'Passe Crassane', 'Abbe Fetel' and 'Conference' with lower affinities which ranged from 0.32 to 0.52. Moreover, these two have relatively similar genetic background with accessions of group-A, which

might have originated from the same gene pool and slight variation due to transmission of a few alleles from parents to its progeny. Three other accessions of this cluster, that is, RT3 (Glass) and two identical accessions with 100% similarity between them (BG21 and MZ26) and locally called the same name as Kotharnul, were also similar in phenotypical and morphological traits. Moreover, these had the highest affinities (0.97) and were tightly associated with accessions of group-B in cluster analysis, irrespective of their phenotypical, morphological and ecological dissimilarity. These results also indicate inter population genetic variation within local collection. These results are in accordance with Chaparoo et al. (1994) and Dirlewanger et al. (1998) who reported very high similarity coefficient in *Prunus persica* cultivars (0.994).

Three genotypes, RT9 (Btangi), BG15 (Btung) and SD39 (Desi nakh), showing a parent-offspring relationship were grouped into cluster II at a very close position (about 0.79 to 0.90). These obtained results were quite reasonable and the morphological and genetic relationship suggest a common lineage that might be a transmission of the single allele from parents to their progeny as a result of natural cross pollination among these genotypes. However, these were genetically far distant to other local accessions and reference varieties, which thus fell in a separate cluster. Commercially, these genotypes were not so important with respect to fruit quality, but were found in wild forms as hardy plants and resistant against diseases, used as rootstocks for commercial varieties of pears in this region. Furthermore, it is possible that the wild group of accessions could be related to the wild *P. ussuriensis.* Phenotypically, these accessions could also be very close to *Pyrus aromatica* grown in a wild form in Iwate, Aomori and Akita Japan (Kikuchi, 1948). It is interesting to note that, due to the flourishing trade between Pakistan and China and the geographical and climatic similarity for a long period of time, it could be hypothesized that the progenitors of *Pyrus* germplasm collected from Northern Pakistan, particularly RT9, BG15, SD39 and KT53 (Btangi, Btung, Desi nakh and Btung, respectively), might be from ancient China. It is considered that all the species in *Pyrus* are inter-crossable and there are no major incompatibility barriers to inter-specific hybridization in *Pyrus* (Westwood and Bjornstad, 1971; Bell and Hough, 1986). It would be appropriate to conclude that majority of accessions found in the State of AJK, which are adjacent to China, have similarity with those originated from East Asia and China. This hypothesis was also supported by Kikuchi (1948) and Yu (1979).

Cluster analysis of the estimated genetic similarity, further illustrated the third main cluster composed of 5 cultivars, namely ‘Comice’, ‘Abbe Fetel’, ‘Conference’, ‘Passe Crassane’, ‘Williams’ and one local accession BG18 (Kashmiri nakh) that is distantly branched with these reference varieties. This cluster analysis revealed that higher genetic similarities among references in European varieties might be due to the fact that they belong to the same gene pool and share common progenitors. These results are also supported by Wunsch and Hormaza (2007) who reported a higher genetic similarity in newly bred varieties, 'Williams', 'Conference' and 'Comice', which share a common origin and the same progenitors. An independently existing variety called 'Pendula' and two more reference varieties namely 'Chantecler' and 'Hosui' that are both distantly associated to each other, were analyzed as control samples. All these were genetically diverse from the local collection as well as European group of varieties.

A very distinct ecotype that existed independently (KT53) and which was locally called Btung, showed a very unique genetic base and the highest dissimilarity with all accessions as well as varieties. Although some accessions with the same local names had a close relationship in their phenotypical and morphological traits, their genetic background and origin have not been understood. It is possible that it might be a mutant clone or some other possibility due to allopolyploid origin of the Maloideae subfamily of *Rosaceae* (Layne and Quamme, 1975). Moreover, DNA sequence analysis confirmed the polyploidy origin of the Maloideae subfamily of *Rosaceae*, via aneuploidy from an ancestor with chromosome number 9 (Evans and Campbell, 2002). This study indicated the existence of a high level of genetic diversity among the *Pyrus* ecotypes. The grouping and unique genetic background of these ecotypes, based on SSRs, need to be exploited with a larger number of molecular markers to evaluate accurate genetic diversity among pear genotypes.

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