Assessing genetic diversity in Iranian native silkworm (*Bombyx mori* L.) strains and Japanese commercial lines using AFLP markers

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Abstract
Three Iranian native strains and three Japanese commercial lines of the silkworm *Bombyx mori* were analyzed using amplified fragment length polymorphism (AFLP) markers. A set of ten *Pst*I/*Taq*I primer combinations amplified a total of 322 bands out of which 251 (%78) were polymorphic. Estimates of Nei's gene diversity for all loci in individual strains and commercial lines indicated a higher degree of genetic similarity within Japanese commercial lines than the Iranian native strains. The highest and the least degree of gene diversity were related to the Khorasan Orange strain (*h*=0.1812) and P107 commercial line (*h*=0.0804), respectively. The dendrogran constructed using the unweighted pair-group method with arithmitic average based on nai's genetic distance revealed two distinct groups as Khorasan native and Japanese commercial lines. The distinct clustering of these two sets of strains and lines reflects differences of the geographical origin and morphological, qualitative and quantitative traits associated with them.
Keywords: Genetic diversity; Silkworm; *Bombyx mori* L.; AFLP Markers.

INTRODUCTION

Contrary to phenotypic characterization, genetic characterization is free of environmental influences, which means greater accuracy in the data generated. This is fundamental when decisions have to be made in conservation programs or on the utilization of animal genetic resources. Thus, techniques used in the analysis of genetic variability are essential ingredients for conservation programs and in making rational breeding decisions (Egito *et al*., 1999).

The silkworm, *Bombyx mori* L., domesticated for silk production comprises a large number of geographical races and commercial lines which show substantial variation in their qualitative and quantitative traits. The traditional breeding activities involving methods like hybridization between members of elite groups are adding new varieties every year (Reddy *et al*., 1999b; Nagaraju, 2002; Mirhoseini *et al*., 2004). At present, in the silkworm, traits such as cocoon shape, cocoon colour, silk fiber length, larval duration, together with many other ethological traits, are used to differentiate varieties and selection of parental strains. But the silkworm varieties, particularly those that have been bred from crosses involving many varieties, cannot be distinguished unambiguously by the use of conventional characteristics. Therefore, it is apparent that the use of molecular markers could provide a solution to the problem, by providing unique DNA profiles. Such varietal DNA profiles would be useful in producing reliable estimates of genetic diversity, for the selection of parents to develop elite hybrids, and to protect silkworm breeder's rights (Reddy *et al.*, 1999b; Mirhoseini, 1998; 2002; Nagaraju and Goldsmith, 2002).

Molecular marker assisted selection is expected to increase the speed and precision in silkworm breeding processes to integrate the desired characters from native varieties into elite varieties. DNA fingerprinting, first described by Jeffreys *et al*. (1985), is now commonly used to study genetic variability and to analyze pedigree relationships in a wide variety of organisms including insects (Nybom, 1991; Blanchetot and Gooding, 1994; Dallas, 1988; Georges *et al*., 1988). Also several investigations have been established using various molecular techniques to analyze the silk-

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worm (Nagaraju *et al*., 2002; Nagaraju and Goldsmith, 2002; Chatterjee and Pradeep, 2003; Chen *et al*., 2003; Lu *et al*., 2003; Cheng *et al*., 2004; Lu *et al*., 2004; Miao *et al*., 2005; Goldsmith *et al*., 2005; Mirhashemi *et al*., 2007).

Amplified fragment length polymorphism (AFLP) technique has been recognized as a reliable and efficient DNA marker system, compared with RFLP, RAPD or microsatellites (Vos *et al*., 1995; Powell *et al*., 1996; Russell *et al*., 1997; Pejic *et al*., 1998).

In the present study, we report the feasibility of using the AFLP method to analyze the diverse genotypes of the silkworm in order to augment marker resources for silkworm genetic analysis.

MATERIALS AND METHODS

Silkworm strains: Three Iranian native silkworm strains including the Khorasan Lemon, Khorasan Orange and Khorasan Pink and three Japanese commercial lines including P31, P103 and P107 which differ from each other in a number of characteristics such as larval signs, egg color and cocoon shell color and shape were used in the present study. These strains and commercial lines were sampled from the Iran Silkworm Research Center located in Rasht (Center of Guilan province).

Genomic DNA extraction: Genomic DNA was isolated by the method of Suzuki *et al*. (1972) and as modified by Nagaraja and Nagaraju (1995). DNA was quantified using a known standard (λDNA) on agarose gels.

AFLP analysis: The original AFLP procedure as described by Zabeau and Vos (1993) and Vos *et al*. (1995) was followed with minor modification. The restriction enzymes used were *Pst*I and *Taq*I, which produce polymorphic DNA fragments in the silkworm (Tan *et al*., 2001). Table 1 shows *Pst*I and *Taq*I adapters and primers with some modifications as implemented by Tan *et al*. (2001).

The primer-*Pst*I-01 and primer-*Taq*I-01 were used for the preamplification reaction. The 25 µl PCR preamplification reaction system contained 2 µl of digested and ligated DNA diluted 1:5 V/V H_2O, 50 ng of both primer-*Pst*I-01 and primer-*Taq*I-01, 1 unit *Taq* polymerase, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$ 1X PCR buffer. The preamplification reaction conditions and steps employed were as described by Vos *et al*.

Table1. Adapters and primers used in AFLP analysis.

Name		Sequence
Adapters *Pst*I	*Pst* top strand	5′-GACGTGACGGCCGTCATGCA
	Pst bottom strand	5′-TGACGGCCGTCACG
Adapters *Taq*I	*Taq* top strand	5′-GACGATGAGTCCTGAG
	Taq bottom strand	5′-CGCTCAGGACTCAT
Primers *Pst*I	P01	5′-GACGGCCGTCATGCAG
	P21	5′-GACGGCCGTCATGCAGTA
	P22	5′-GACGGCCGTCATGCAGAT
	P23	5′-GACGGCCGTCATGCAGTC
	P31	5′-GACGGCCGTCATGCAGAAC
	P32	5′-GACGGCCGTCATGCAGAGA
	P33	5′-GACGGCCGTCATGCAGATG
	P34	5′-GACGGCCGTCATGCAGAAG
Primers *Taq*I	T01	5′-GATGAGTCCTGAGCGA
	T21	5′-GATGAGTCCTGAGCGATA
	T22	5′-GATGAGTCCTGAGCGAAT
	T23	5′-GATGAGTCCTGAGCGATC
	T24	5′-GATGAGTCCTGAGCGATG
	T35	5′-GATGAGTCCTGAGCGATAC

Selective nucleotides are shown as bold letters.

(1995). After the preamplification reaction, the reaction mixture was diluted to 150 µl with double distilled H_2O (dd H_2O). The 10 µl PCR selective amplification system contained 1 µl of product from the diluted preamplification reaction, 25 ng of both selective primers, 0.3 units of *Taq* DNA polymerase, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$ 1X PCR buffer. All amplification reactions were performed in a Touch gene model thermocycler (Techne).

Initially, three individuals from three studied strains were chosen to test the variation of 66 primer combinations (data not shown). With these individuals the polymorphism rates and the total number of bands with the 66 primer combinations were evaluated. The most useful primer combinations were considered those having the highest polymorphism rate that also generate a reasonable number of clearly detectable total fragments. Using results from the evaluation of 66 primer combinations based on 3 individuals, the ten most-polymorphic primer combinations, producing clearly readable bands, were selected for the subsequent analyses with at least 30 samples.

Gel analysis: An equal volume of loading buffer (Formamide 10 ml, Xylene cyanol FF 10 mg, Bromophenol blue 10 mg, 0.5 M EDTA pH 8.0 200 µl) was added to each sample, and denatured at 95°C for 3 min then placed on ice for 2 min before loading. 4 µl of samples loaded onto 6% denaturing polyacrylamide gel matrix (7 M Urea; 19:1 Acrylamide: bis; 1X TBE buffer). The electrophoresis parameters were set to 75 Watt, 50°C and a run time of 1.5 hours was selected. Bands detected by the silver staining procedure (Promega, Technical manual No.023) and gel images were scanned and saved as jpeg files for scoring and further analysis. Two typical gels are shown in Figure 1a,b.

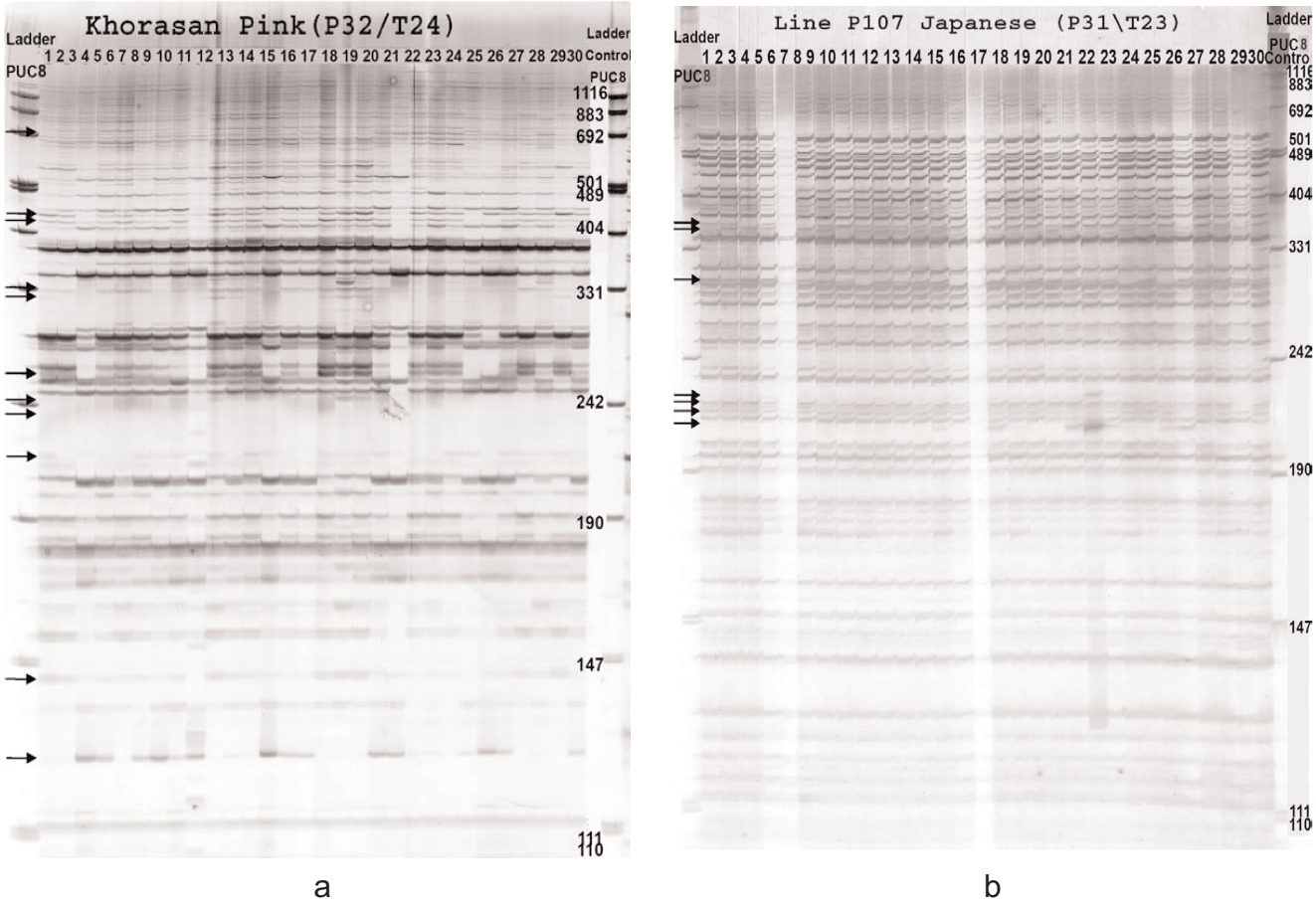

Figure 1. a) A portion of an AFLP gel generated using P32/T24 primer pair from Khorasan Pink strain. Each lane represents a single silkworm, with a total 30 silkworms shown. Highly polymorphic bands are indicated by arrows on the left margin. All bands were polymorphic when all the 183 silkworms were included (not shown). b) A portion of an AFLP gel generated using P31/T23 primer pair from P107 Japanese line. Each lane represents a single silkworm, with a total of 30 silkworms shown. Highly polymorphic bands are indicated by arrows on the left margin. All bands were polymorphic when all 183 silkworms were included (not shown). Two last lines are control and ladder PCU8.

Statistical analysis: The gel images were scored using a binary scoring system that recorded the presence and absence of bands as 1 and 0, respectively.

The following indices calculated using the POPGENE program (Population Genetic Analysis) version 1.31 (Yeh *et al*., 1999) were used to estimate the information content of AFLP markers: (a) number of polymorphisms identified per primer pair (*Np*); (b) effective number of alleles (ne) (c) an assay efficiency index (Shannon's index) of the information carried by AFLP markers per assay (corresponding to a primer combination). Assay efficiency index calculates the number of effective alleles (Morgante *et al*., 1994) identified in a single experiment, corresponding to a primer pair in the case of the AFLP technique.

Also the genetic distances between strains and commercial lines were calculated using the POPGENE program. This program establishes standardized genetic distance matrices (Nei, 1972) and matrices of genetic distances corrected for small samples (Nei, 1978). The method proposed by Nei (1972) is one of the most used to obtain genetic distances between populations (Lynch and Milligan, 1994). Genetic variation between individuals for each strain and commercial line was estimated using gene diversity (*h*) indices based on Nei (1973). According to Weir (1996) this is the most adequate method to study unique populations.

Cluster analysis was performed based on the Nei's standard genetic distance using the unweighted pair group method using the arithmetic means (UPGMA) algorithm (Sneath and Sokal, 1973) provided the TFPGA program (Tools for Population Genetics Analyses*,* version 1.3) (Miller, 1997). Also bootstraping on UPGMA trees was conducted using TFPGA.

The analysis of molecular variance (AMOVA) (Excoffier *et al*., 1992) was used to separate the variance between and within strains and lines using the ARLEQUIN program (Schneider *et al*., 2000). Although this test was not originally designed for analysis of dominant data, it has been used in other similar studies (Tansley and Brown, 2000).

RESULTS

AFLP analysis of 183 individuals including at least 30 individuals from each six studied strains and commercial lines using ten pairs of primers, provided a total of 322 markers of which 251 (77.95%) were polymorphic (Table 2). The primers differed in their ability to detect polymorphism within strains and commercial lines. The average overall strains and lines with polymorphic bands amplified per primer pair combination varied from 18 (P21/T21) to 35 (P31/T24) and for individual strains and lines from 0 (P21/T21 in P103 and P33/T22 in P31) to 18 (P31/T24 in Khorasan Orange) (Table 3).

If we consider each strain and line as a population, then when all populations were used, the greater part of total genetic variability (73.38%) was due to differences among populations while the remaining 26.62% was due to differences within populations. When only the native strains were used, the "among population variability" decreased to 58.20% while the "within population variability" increased to 41.80% (Table 2).

The number of polymorphisms observed within strains and lines ranged from 50 (P107), with an average number per primer pair ranging from 5.0 ± 4.6 to 117 (Khorasan Orange), giving an overall average of 11.7 ± 3.5 (Table 3).

The gene diversity index was calculated for all strains and lines, considering them as unique populations. This analysis was based on the mean allelic frequency of the 251 AFLP markers, which varied from 100 bp to 1,000 bp. P107 presented the lowest gene diversity (*h*= 0.0804) (Table 3).

Within strains and lines, no significant differences in the average gene diversity were observed. Gene diversity values ranged between 0.0804, in P107, and 0.1812 in the Khorasan Orange (Table 4).

Table 2. Analysis of molecular variance (AMOVA) of the six strains and lines studied, using 251 AFLP markers(1).

Source of variation	DF	SS	MS	VC	%Total
Between all strains and lines	5	5813.839	1162.7678	37.68541	73.38*
Within strains and lines	177	2419.861	13.6715	13.67153	26.62*
Between native strains(2)	2	1584.028	792.014	24.99563	58.20*
Within native strains	90	1615.994	17.9554	17.95549	41.80*

(1)DF: degrees of freedom; SS: sum of squares; MS: mean square; VC: variance components. (2)Only native breeds were considered. * Significant at 5% level of probability.

Table 3. Average diversity values detected with ten primer pairs for six strains and lines.

Strains and Lines	N	Primer pair/Total number of loci																					
		P21/T21 (40)		P31/T24 (28)		P22/T24 (22)		P23/T23 (29)		P32/T24 (23)		P33/T22 (24)		P31/T22 (29)		P31/T23 (39)		P34/T22 (31)		P23/T35 (33)		Average over loci	
		Np	*h*	*Np*	*h*	*Np*	*h*	*Np*	*h*	*Np*	*h*	*Np*	*h*	*Np*	*h*	*Np*	*h*	*Np*	*h*	*Np*	*h*	*Np*	*h*
Kh. Le.	33	12	0.2186	11	0.1202	8	0.1656	7	0.1106	9	0.1671	8	0.1647	5	0.0581	10	0.1055	12	0.1900	11	0.1518	93	0.1396
Kh. Or.	30	9	0.1929	18	0.1942	9	0.1850	12	0.2250	13	0.2295	10	0.1689	6	0.0773	16	0.2112	13	0.2061	11	0.1510	117	0.1812
Kh. Pi.	30	10	0.2219	15	0.1419	15	0.2676	10	0.1160	11	0.1729	8	0.1481	9	0.1322	16	0.1930	10	0.1681	9	0.0877	113	0.1602
P31	30	1	0.0277	10	0.1001	4	0.0852	1	0.0221	5	0.0927	0	0.0000	7	0.0936	11	0.1406	5	0.0961	7	0.1050	51	0.0824
P103	30	0	0.0000	13	0.1282	5	0.1095	5	0.0843	5	0.0975	5	0.1097	5	0.0583	14	0.1466	15	0.1957	7	0.0928	74	0.1081
P107	30	1	0.0071	3	0.0257	2	0.0321	3	0.0563	4	0.0785	3	0.0511	4	0.0477	8	0.0999	5	0.0861	17	0.2741	50	0.0804
A.O.S.L.	183	18	0.3558	35	0.3797	20	0.4222	21	0.4062	21	0.3773	21	0.4018	29	0.3524	31	0.3427	25	0.4322	30	0.3868	251	0.3837

(N=sample size; *Np*=number of polymorphic loci; *h*=gene diversity (Nei, 1973); A.O. S. L. =Average over strains and lines)
(Kh.Le.=Khorasan Lemon; Kh.Or.=Khorasan Orange; Kh.Pi.=Khorasan Pink)

Table 4. Mean and standard deviation (SD) of gene diversity (*h*), average number of effective alleles per locus (ne) and Shannon's Information Index calculated within and across six strains and lines studied.

Strains and lines	*h*	ne	Shannon's Information Index
Khorasan Lemon	0.1396±0.2001	1.2472±0.3693	0.2044±0.2866
Khorasan Orange	0.1812±0.2136	1.3249±0.4019	0.2639±0.3039
Khorasan Pink	0.1602±0.2069	1.2863±0.3927	0.2357±0.2936
P31	0.0824±0.1701	1.1473±0.3135	0.1197±0.2441
P103	0.1081±0.1811	1.1875±0.3308	0.1604±0.2615
P107	0.0804±0.1697	1.1454±0.3173	0.1166±0.2425
Across strains and lines	0.3837±0.1316	1.6852±0.2997	0.5606±0.1635

No marker diagnostic for strain and line identification was detected and the observed differences among strains and lines consisted of AFLP allele frequency differences. Across strains and lines, the Shannon's Information Index was 0.5606, indicating that 56.06 effective alleles (mean: 1.6852 alleles per locus) were on average identified per primer pair. Within strains and lines, Shannon's Information Index values were slightly lower, but still ranged from 0.1166 to 0.2639, with an average number of effective alleles ranging from 1.1454 to 1.3249, excluding monomorphic bands from computation (Table 4).

The estimates of genetic distances between the strains and lines were calculated to help in the study of genetic relationships and genetic divergence between pairs of strains and lines for standard genetic distances (Dp) (Nei, 1972) and those corrected for small populations (Dc) (Nei, 1978). Estimates of standard genetic distance (Dp) using AFLP data ranged from 0.2279 for the most closely related strains (Khorasan Lemon and Khorasan Orange) to 0.6395 in the most divergent strains and lines (Khorasan Lemon and P103) (Table 5).

The dendrogram of the standard genetic distance (Dp) matrix generated by the UPGMA method (Nei, 1972) as well as the bootstrapping proportions shown in Figure 2, resolved the six silkworm strains and lines into two major clusters, one comprising Khorasan strains and the other with commercial Japanese lines. Identical topology was also observed with Nei's 1978 corrected genetic distance (Dc; results not shown).

When all 183 individuals were analyzed together, any overlap of individuals belonging to different strains and lines was evident and those belonging to the same strain and line clustered together in the same clade in the dendrogram (results not shown).

DISCUSSION

AFLP is a technology increasingly adopted for the investigation of biodiversity in a wide variety of microbial, plant and animal species. For this investigation, we selected ten highly informative primer pairs which permitted the identification of 251 polymorphic

Table 5. Genetic distances between six strains and lines of *Bombyx mori* estimated using Nei (1972, 1978) methods, from AFLP data, using the POPGENE program. The standard genetic distances (Nei, 1972) are below the diagonal, and corrected distances (Nei, 1978), above the diagonal.

Strains and lines	Khorasan Lemon	Khorasan Orange	Khorasan Pink	P31	P103	P107
Khorasan Lemon	-	0.2245	0.2390	0.5569	0.6372	0.5291
Khorasan Orange	0.2279	-	0.2560	0.5269	0.5202	0.5241
Khorasan Pink	0.2420	0.2597	-	0.5244	0.6007	0.4977
P31	0.5590	0.5297	0.5268	-	0.2906	0.4538
P103	0.6395	0.5232	0.6033	0.2924	-	0.3970
P107	0.5312	0.5268	0.5001	0.4553	0.3988	-

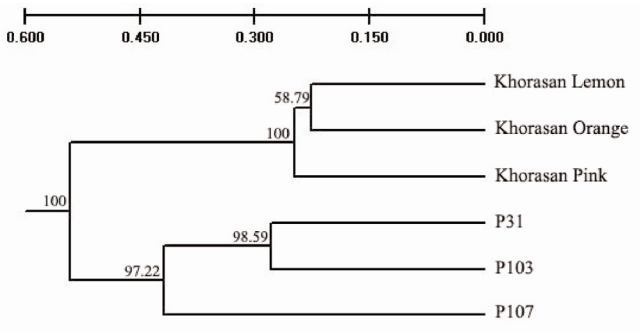

Figure 2. Dendrogram generated by the UPGMA method for Nei's (1972) standard genetic distance, using the TFPGA program. The confidence value of each node was estimated from 10,000 permutations.

markers, with an average of 25.1 markers and 56.06 effective alleles per assay.

According to decrease in the sampling error, at least 30 individuals from each strain and line and 50 markers as loci have been studied to estimate genetic diversity. One of the main attractions of the AFLP method is its high multiplex ratio (Powell *et al.*, 1996; Rafalski *et al*., 1996), which means that a large number of amplification products are generated in a single reaction.

The average number of effective alleles per locus over all the primers was 1.68, which confirms good influence of the alleles on high polymorphism and estimation of genetic diversity as considered being close to the real number of alleles i.e. 2. It is necessary to point that the effective allele number at individual loci is directly related to polymorphism.

As shown in table 2, the genetic variability within populations was much lower than between populations and the greater part of genetic variability was due to differences between populations. This may be explained by the conservation method that has been carried out by the Iran Silkworm Research Center as the gene bank association to conserve these strains and lines. With respect to low effective population size and the fact that each female mates only with one male, and in conclusion all their offsprings are full-sibs, then the inbreeding rate is very high. On the other hand it could cause more differentiation among these strains and lines (Falconer, 1989). These results are consistent with those obtained from morphological, protein and RAPD (Mirhoseini, 1998) and ISSR marker studies (Balvasi, 2003) of silkworm and differ from other studies that have used RAPD markers on rare and endangered *Leucadendron elimense* (Proteaceae) (Tansley and Brown, 2000) and Brazilian native bovine breeds (Serrano *et al*., 2004), where the greater part of genetic variance is due to differences between individuals within breeds.

As estimates of genetic distances shown in table 5, among Khorasan strains, the highest divergence was observed between the Khorasan Orange and Khorasan Pink (Dp= 0.2597) that is in agreement with results collected on genetic variation using ISSR markers in the study of five Iranian native silkworm strains (Balvasi, 2003). Among Japanese commercial lines, the highest divergence was observed between the P31 and P107 (Dp= 0.4553). It was also observed that the average genetic distances between Japanese lines presented higher values compared to the Khorasan strains, making it evident that "within genetic variability" of Japanese lines is lower than that of Khorasan strains.

The dendrogram of the genetic distance matrix generated by the UPGMA method (Nei, 1972) is shown in Figure 2. It is apparent that the AFLP markers used in this study have sufficient ability to discriminate the Khorasan native strains from the Japanese commercial lines. The distinct clustering of Khorasan strains and Japanese lines reflects the geographical origin and, morphological, qualitative and quantitative traits associated with these two sets of strains and lines. Khorasan Lemon and Khorasan Orange strains are grouped in one cluster separated from the Khorasan Pink strain, which is consistent with grouping based on morphological characteristics (Mirhoseini, 1998).

Silkworm strains have been reared in different regions of the world and different strains have evolved because of changes in their phenotype and genotype over a period of time. Based on one hypothesis, all the strains during a long period have differentiated from a monovoltine Chinese variety (Chatterjee and Data, 1992).

The power of DNA fingerprinting in estimating the genetic relationship of populations in various species has been well demonstrated (Castagnone-Sereno *et al*., 1993; Meng *et al*., 1996; Nagaraja and Nagaraju, 1995). Nagaraju *et al*. (2001) compared RFLPs and three PCR based techniques (RAPD, SSRs and ISSR-PCR) for genetic analysis of the silkworm, of which the RAPD, ISSR-PCR and RFLP assays clearly separated the diapausing and non-diapausing silkworm varieties.

The dendrogram obtained from individual hierarchical cluster analysis of the Khorasan native silkworm strains and Japanese commercial lines using the UPGMA method based on data from AFLP markers, clustered all individuals belonging to the same strain and line in the same clade in the dendrogram (data not

shown), whereas the earlier analysis using RAPD markers on individuals from the same strains and lines (Mirhoseini, 1998) did not lead to such consistent separation of the strains and lines. The AFLP markers resulted in a more consistent pattern confirming its high reliability. This may be due to the selection that has been carried out, as well as the work carried out by the Breeder's Association to maintain these strains and lines.

In silkworm rearing, environmental conditions are very determinative, characteristics like compatibility with environmental conditions especially resistance to regional diseases are very important. Therefore the eco-races of each region must be identified in order to use their valuable genes for commercial lines and varieties (Mirhoseini *et al*., 2004).

Molecular AFLP data give no information on unique and valuable traits existing within strains and those maintained so far by natural or human selection. Therefore, they alone give insufficient indication of the strains to be conserved for the preservation of biodiversity. However, they may concur to the correct choices, revealing unexpected patterns of genetic variation when historical information on breed origin is scarce or uncertain and in evaluating the genetic diversity of strains at the genome level. In this view, they are well suited for integration of demographic, phenotypic and available molecular information at loci having adaptive value (e.g. QTL) (Ajmone-Marsan *et al*., 2001).

The fact that the AFLP method resolves native and commercial lines-specific amplification products makes it useful in augmenting marker resources for the silkworm genome mapping programme. In the mapping strategy, the genetically diverse strains of silkworm, which possess highly contrasting qualitative and quantitative traits, are chosen as parental genotypes to raise the required mapping population. The strain-specific profiles and pattern similarity within the strains make the method invaluable in addressing problems involved in breeders' rights, genetic homozygosity of the strains, marker-assisted breeding and cross-breeding strategies (Reddy *et al.*, 1999b).

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