Department of Biotechnology, Bioinformatics and Pharmacy

Jaypee University of Information Technology,

Waknaghat

Study of Genetic Diversity in Mulberry from Ladakh

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Submitted in partial fulfillment of the Degree of

Bachelor of Technology

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CERTIFICATE

This is to certify that the work titled "Study of Genetic Diversity in Mulberry plant from Ladakh" submitted by **Ms. Nivedita Jamwal and Mr. Manish Gupta** to the Jaypee University of Information Technology, Waknaghat in partial fulfillment of the requirement for the award of the degree of Bachelor of Technology in Biotechnology is a record of bona fide research work carried out by them under my guidance and supervision and no part of this work has been submitted to any other university or institute for this or any ot her degree or diploma.

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Needless to say, errors and omissions are ours.

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27/5/2012

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LIST OF ABBREVIATIONS

Abbreviations	Expanded Form
SSR	Simple sequence repeat
SPAR	Single primer amplification
DAMD	Directed amplification of minisatellite DNA
NJ	Neighbour-joining
Ml	Micro litre
PCA	Principal coordinates analysis
UPGMA	Unweighted pair group method with arithmetic means

SUMMARY

Mulberry is the sole food source for mulberry silkworm and a number of indigenous and exotic varieties are used in sericulture. Studies on assessment of genetic diversity have been done amongst a few mulberry varieties using one or at the most two methods. However, no comprehensive study on a large number of varieties has been carried out. In present study, simple sequence repeats (SSR) maker were used to determine of diversity in 27 mulberry genotypes collected from seven locations of two different valleys of Leh Ladhakh regions. A cumulative analysis of the data generated by this method resulted in a neighbour-joining (NJ) tree that gave a reflection of the relatedness and affinities of the genotypes to each other, with respect to location as well as valleys. With the help of popgene software we calculated effective number of alleles and polymorphism. We have used Gen Alex software which gives us Analysis of Molecular Variance (AMOVA) which is a method of estimating population differentiation directly from molecular data and testing hypotheses about such differentiation.

Chapter 1

Introduction

Mulberry belongs to genus *Morus* and family Moraceae. *Mulberry* is a small to medium-sized monoecious or dioecious shrub or tree. *Mulberry* is distributed in tropical, sub-tropical and temperate zones in Asia, Europe, North America, Africa and South America. The trees historically have been used for sericulture especially in east, central, and south Asia. There are at least 24 species with more than 100 known cultivars It has been widely believed that *Mulberry* species originated on the low slopes of the Himalayas bordering China and India, the study of Hou suggested a multicentered origin .In India, there are mainly four species of *Morus* found viz.*Morus alba, M.indica*, M.serrata and *M. laevigata.* Among them, *M. alba* is most important species from sericulture point of view. *M.alba* is native to china but with time dispersed to different parts of world. Different white *Mulberry* (*M.alba*) genotypes have white, purple, or red colour fruits. The *Mulberry* fruits are sweet but they are very perishable.

Trans-Himalayan (Ladakh) region is popularly known as high altitude cold desert. It lies between 32° to 36° north longitude and 76° to 79° east latitude sprawls over 96,701 sq. km with low population. This part of Himalaya is rocky, barren and devoid of soil. We have surveyed the Ladakh for *Morus* and found that *Morus alba* is the main species available in Ladakh, so this study concentrates on *M. alba*. *M. alba* is found wild in three valleys of Ladakh namely Indus, Nubra and Suru. *Morus* is valued for its fruit rather than sericulture in Ladakh, but now conditions are changing as lot of other fruit options available, people are not bothering to conserve *Morus* and numbers of trees are decreasing day by day. But *Morus* is very important tree from sericulture point of view; also in future we need to have diverse germplasm available to fight changing environmental conditions. So conservation of *Morus* biodiversity is emergent tasks ahead, studies on conservation genetics have greatly increased in the last decades, mainly need to evaluate measures to reduce the impact of habitat destruction through anthropogenic action. Reductions in population size usually result in loss of genetic diversity, inbreeding and increased extinction risk.

Molecular markers have been commonly used to characterize *Mulberry* cultivars and wild accessions. For example, RAPD and inter simple sequence repeat (ISSR) markers were used to study the genetic relationships of Japanese and Indian cultivars to assess molecular variability .ISSRs were also combined with simple sequence repeat (SSR) to reveal genetic variation among wild and cultivated *Mulberry* species Indeed, ISSRs have been the most common marker system used in mulberries. Directed Amplification of Minisatellite DNA (DAMD) and Single Primer Amplification Reactions (SPAR) were also used to characterize *Mulberry* cultivars. But no study is carried till date for assessment of genetic diversity of *Morus* in Ladakh. In this study we have used SSR marker to analyze the *M. Alba* samples collected from two valleys Indus and Suru and asses the genetic diversity and population differentiation in *M. alba*

CHAPTER 2

REVIEW OF LITERATURE

Mulberry (genus *Morus*) is an economically important plant used for sericulture, as it is the sole food plant for the domesticated silkworm, *Bombyx mori*. The genus *Morus*, which is widely distributed in Asia, Europe, North and South America, and Africa, is cultivated extensively in East, Central and South Asia for silk production. A few species of mulberry are also valued for their edible fruit (*M. alba, M.indica* and *M. laevigata*), timber (*M laevigata* and *M. serrata*). Whereas it has been widely believed that mulberry species originated on the low slopes of the Himalayas bordering China and India, the study of Hou suggests a multi centered origin .Since the classification of the genus *Morus* is mainly based on morphological characteristics, considerable differences exist among systematists as to the number of species that exist in this genus. So far, more than 150 species of mulberry have been cited in the Index Kewensis, but a majority of them have been treated either as synonyms or as varieties rather than species, and some have been transferred to allied genera.

A study carried out by Koidzumi in 1917 recognised 24 species and one variety under the genus *Morus* based on the style length in female flowers and the nature of the stigma in male flowers. In contrast, more than 60 years later by analyzing the electrophoretic patterns of seven enzymes and sap proteins in 131 varieties of three mulberry species, *M. bombycis, M. alba*, and *M. latifolia* Hirano categorised them into seven varietal groups, and affinities among them. Because of environmental influence, phenotypic traits in many cases fail to serve as unambiguous markers for systematic and diversity analysis. Moreover, most of the putative mulberry species are dioecious and can cross-pollinate among themselves to produce fertile hybrids, suggesting that they have relatively close genetic relationships. Such a high degree of cross-species reproductive success is not encountered often in nature, and has thus created considerable doubt with regard to the species status of mulberry. Molecular markers successfully developed during the last two decades have largely overcome the problems that are associated with phenotype-based classification. Initially,isozymes and Restriction Fragment Length Polymorphisms (RFLPs) served as reliable markers for genetic analyses in plants. But PCR based techniques developed in recent years such as Random Amplified Polymorphic DNA (RAPDs), Inter Simple Sequence Repeats (ISSR) , Amplified Fragment Length Polymorphisms (AFLPs) and Simple Sequence Repeats (SSRs) , also called microsatellites, provide DNA markers that are dispersed throughout plant genomes and are easier to reproduce and analyse. High levels of polymorphism and their co-dominant nature have made SSRs ideal markers for studying genetic diversity in plants .However, the time and cost of identifying SSR motifs and designing primers for regions flanking SSRs have restricted the widespread use of microsatellites in plants ISSR markers, which show dominant inheritance, use SSR repeat-anchored primers and are being used as an alternate tool in diversity studies. ISSR markers are useful in detecting genetic polymorphisms among accessions by generating a large number of markers that target multiple microsatellite loci distributed across the genome.

CHAPTER 3

MATERIALS & METHODS

1. Plant material certification and collection

Twenty seven locally grown genotypes collected from 7 different villages spread across two valleys of Ladakh Trans-Himalayan region. *Mulberry* in Ladakh is having immense morphological variability like fruit shape, fruit colour etc. While selecting the plants on morphological basis, only those genotypes (from each village) were taken for the investigations, which were morphologically very distinct for above mentioned parameters so as to avoid any chance of duplication.

Table 1 Locations of collection of 27 Genotypes of mulberry

Sample	No.of Samples	Locations
1-4	4	Skurbuchan
5-8	4	Domkhar
9-12	4	Achinathang
13-16	4	Beama
17-20	4	Sanjole
21-24	4	Battalic
25-27	3	achkamal

Table 2 Geographically distinct valleys from where 27 Mulberry genotypes were collected.

Valley	NO. Of sample	Sample
Indus	**20**	**1-20**
Suru	**7**	**21-27**

2.1 Genomic DNA Extraction

DNA extraction from plant tissue can vary depending on the material used. Essentially any mechanical means of breaking down the cell wall and membranes to allow access to nuclear material, without its degradation is required. For this, usually an initial grinding stage with liquid nitrogen is employed to break down cell wall material and allow access to DNA while harmful cellular enzymes and chemicals remain inactivated. Once the tissue has been sufficiently ground, it is then be re-suspended CTAB buffer. In order to purify DNA, insoluble particulates are removed through centrifugation while soluble proteins and other material are separated through mixing with chloroform and centrifugation. DNA is then precipitated from the aqueous phase and washed thoroughly to remove contaminating salts. The purified DNA is then re-suspended and stored in TE buffer or sterile distilled water. To check the quality of the extracted DNA, a sample is run on an agarose gel, stained with ethidium bromide, and visualised under UV light.

2.1 Solutions for DNA extraction

10% (w/v) CTAB (N-cetyl N, N, N-trimethyle-ammonium bromide) - 10 gm of CTAB was dissolved in 70 ml of water at 65°C then final colume was made to 100 ml.

0.5 MEDTA (pH 8.0) - 93.05 gm of ethylene diamine tetra acetate was added to 400 ml of water. The pH of solution was adjusted to 8.0 by addition of NaOH. The final volume was made to 500 ml and sterilized by autoclaving.

5 M NaCl - 146.1 gm sodium chloride was dissolved in 400 ml of water and the final colume was made to 500 ml and sterilized by autoclaving.

Phenol : chloroform : isoamyl alcohal (25:24:1) – 750µl buffered phenol (pH opproximately 7.8), 720 µl chloroform and 30 µl isoamyl alchol were mixed by vortexing. The resulted solution was kept at 4°C in coloured container.

1M Tris - 60.58 gm Tris base was dissolved in 300 µl of water and the pH was adjusted to 8.0. The final volume was than made to 500 µl and sterilized by autoclaving.

DNA extraction buffer - The DNA extraction buffer contained 100 mM Tris-Hcl, 20 mM EDTA (pH-8.0), 1.4 M NaCl, 2.0 (w/v) CTAB and 0.2% (v/v), 2-Mercapto ethanol, 1% PVP

2.2 Procedure

1. Grind 1g of leaf sample with the help of liquid nitrogen and suspend the powder in 500 µl of CTAB buffer. Transfer CTAB/plant extract mixture to a microfuge tube.
2. Incubate the CTAB/plant extract mixture for about 1 hour at 60o C in a re-circulating water bath.
3. Add equal volume (500 µl) of Chloroform : Iso Amyl Alcohol (CI) to the CTAB/plant extract mixture and centrifuge at 1200 rpm for 10 minutes, Transfer the supernatant to clean microfuge tubes.
4. Transfer the upper aqueous phase only (contains the DNA) to a clean microfuge tube.and add $1/6^{th}$ volume of ice cold isopropanol to each tube and incubate at -20 oC for 1 hour
5. Centrifuge at 12,000 rpm for 10 minutes. Discard the supernatant and wash the pallet with 70% ethanol once.
6. After the wash, spin the DNA into a pellet by centrifuging at 13000 rpm for 1 min. Remove all the supernatant and allow the DNA pellet to dry (approximately 15 min). Do not allow the DNA to over dry or it will be hard to re-dissolve.
7. Re-suspend the DNA in sterile DNase free water

2.3 Electrophoresis of DNA in agarose gel

The genomic DNA was was electrophorised in 0.8 per cent agarose gel under submerged conditions to check r quality and assessment of quantity. 1X TAE buffer was used as gel and tray buffer. Before loading the samples in the wells, 4 µl of loading dye was added to each sample. The solutions for the agarose gel electrophoresis were prepared as under:

Ethidium Bromide - 100 mg of ethidium bromide (10 mg/ml) was added to 10 ml of sterile water, and stirred on a magnetic stirrer until the dye was completely dissolved. The container was wrapped in aluminium foil or the solution was transferred to a dark bottle and stored at room temperature.

6X loading Dye – Dissolved 0.25% bromphenol blue, 0.25% xylene cynol FF and 30% glycerol in water. kept at 4°C till use .

Agarose Gel (0.8%) – 1.6 gm agarose was poured in 200 ml of 1X TAE buffer and boiled for 3-5 minutes. 3 µl ethidium bromide solution was added when temperature reaches 50-60°C approximately. This solution was now poured into the casting tray containing a comb for gelling.

2.4 Examining the quality of DNA

The quality of genomic DNA was checked by observing the electrophorased DNA in Biorad GelDoc system. The DNA samples seen as clear compact bands were considered of good quality whereas the sample sowing diffused smear were considered as fragmented and sheared DNA. The DAN was again isolated from sample deviating considerably.

2.5 DNA quantification

The DNA in the sample was quantified by comparing visually the thickness of DNA band with that of λ DAN bands of known concentration which was electrophoreased along with genomic DNA. The working solution of DNA was prepared from the original sample suitable to carry out PCR reaction.

2.5.1 Procedure:

1. After the gel has cooled completely and solidified the combs was removed and the tray inserted properly into the gel chamber.
2. Poured enough 1X TAE buffer into the chamber to cover the gel and fill the wells.
3. Load 5µL DNA with 1.5µL loading dye.
4. Loaded 1 µL lambda DNA with final concentrations of (200ng, 100, 50 per µL)
5. The gel is then run at 100V for around 30 minutes.
6. The gel is then studied under UV light.

PCR-Polymerase Chain Reaction

Material required

Components required per PCR vial:

COMPONENTS	VOLUME
PCR buffer	2.5 µL
dNTPs	0.5 µL
DNA	1 µL
Taq polymerase	0.25 µL
Forward primer	1.5 µL
Reverse primer	1.5 µL
dH2O	17.75 µL
Total	**25 µL**

in the PCR based techniques diluted DNA is mixed with a master mix comprising the PCR buffer, DNTPS, primer, water and the Taq polymerase enzyme in a PCR eppendorf tube .The mixture is loaded into the PCR. The PCR is pre-programmed for appropriate number of cycles and temperature variations depending on the technique. After required cycles, the samples are subjected to electrophoresis, either AGE or PAGE, depending on the technique. The staining is done for revealing the banding pattern.Gene amplification of 27 Morus alba samples were carried out under following PCR conditions:

Initial Denaturing: 94°C 5 minutes

Denaturing: 94°C 1 minute

Annealing: TA °C 1 minute 35 cycles

Extension: 72°C 2 minutes

Final Extension: 72°C 7 minutes

CHAPTER 4

RESULTS AND DISCUSSIONS

1. Quantification of DNA samples using different concentrations λ DNA

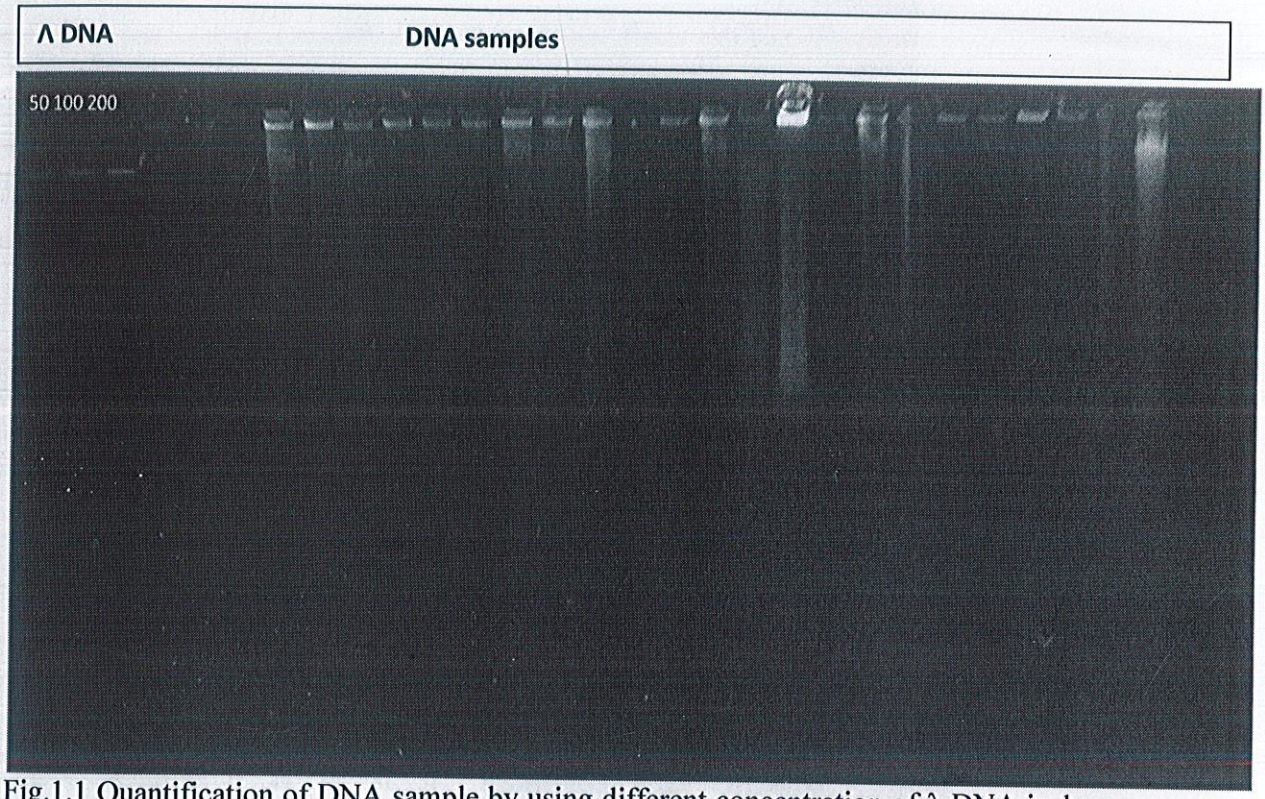


Fig.1.1 Quantification of DNA sample by using different concentration of λ DNA is done for checking the presence of the DNA in the sample and also for the quantification of the DNA in the given sample.AS we can see here DNA is present in all sample.

2 Primer standardization

Primer standardization is a process in which we determine the temperature for every primer at which they can amplify best. We have done this standardization for all 10 Primer.

Fig. 2.1 standardization for primer 1, temperature range from 43° to 53°c.

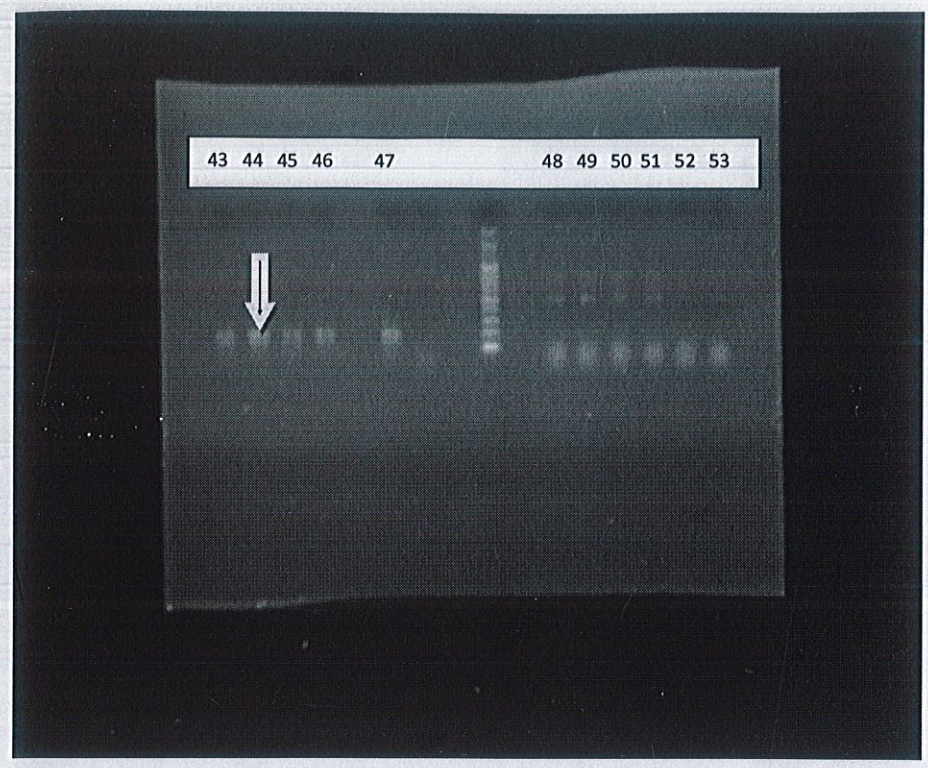


Fig. 2.2 standardization of primer, temperature range from 48° to 52°c.

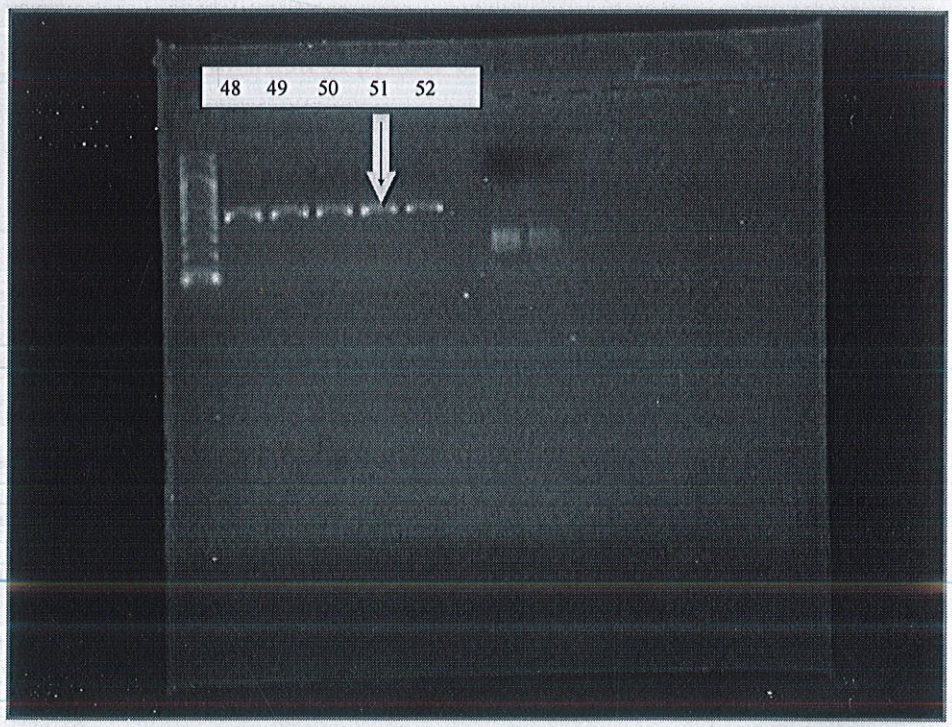


Fig. 2.3 standardization of primer 4, temperature range from 41° to 46°c.

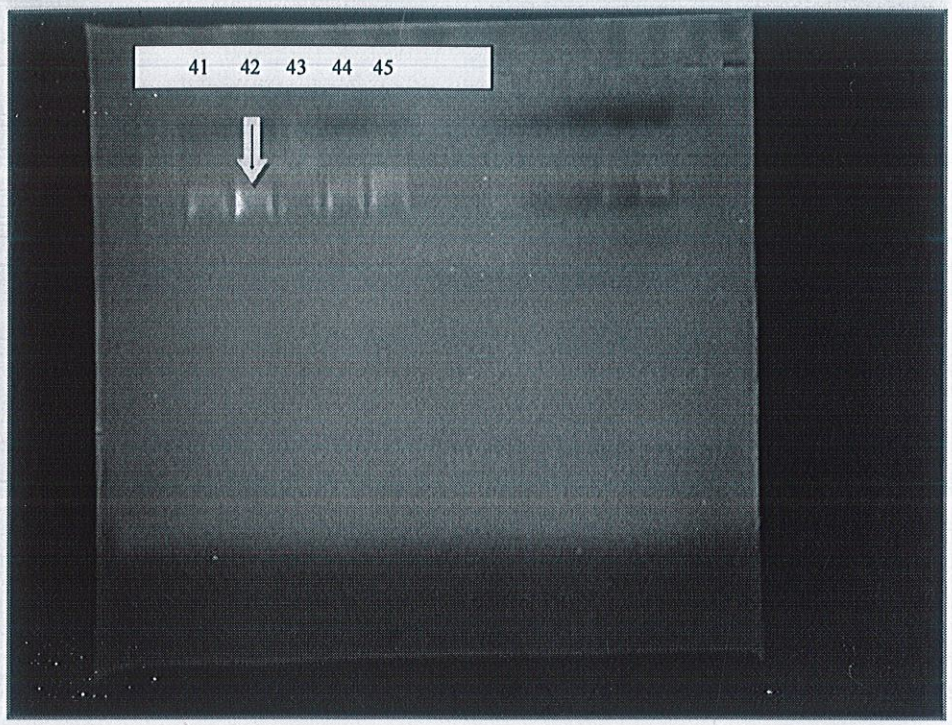


Fig. 2.4 standardization of primer 5 and 6,temperature range from 52° to 63°c.

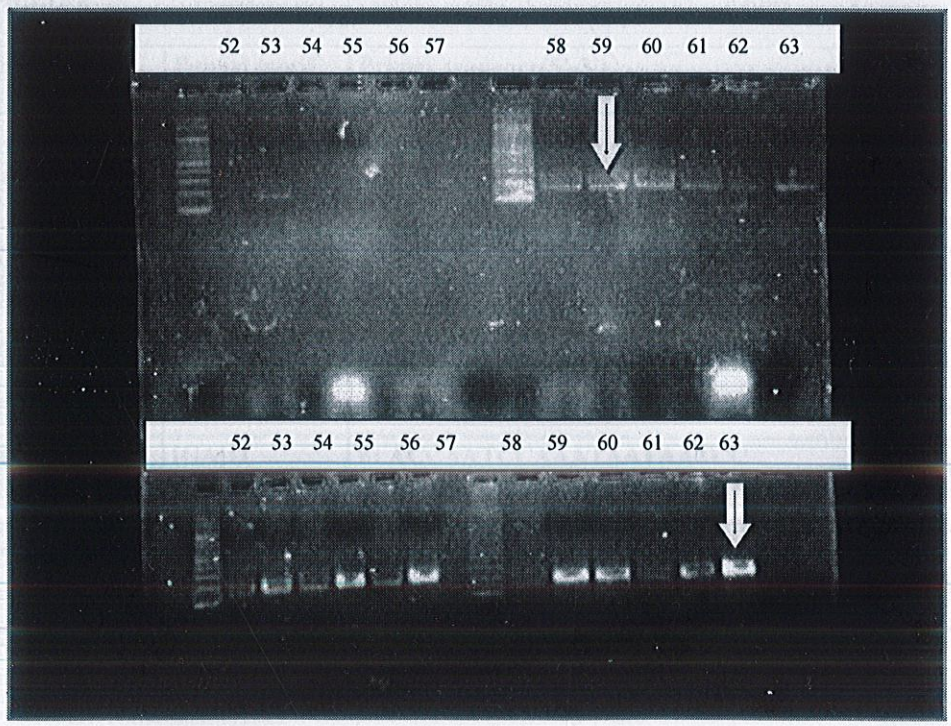
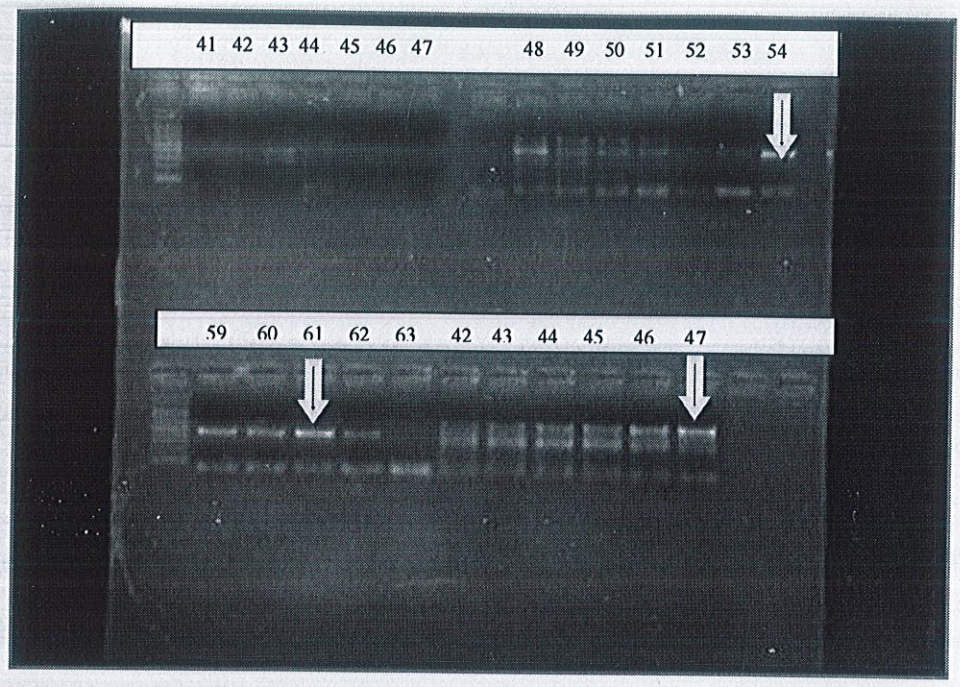


Fig. 2.5 standardization of primer 7,8 and 9.



As we can see from the above figures we have done standardization process for 10 primers out of which 8 primers were successfully amplified at different temperature. standardization temperature for primer 1 to 9 (except primer 3) is mention in following table.

Table 3 standardization temperature and primer sequence of SSR markers-

Marker	Repeat motif	Primer sequence(5'-3')	T m (°C)	T a	amplification
MFC-1	$(CT)_{13}$	F: ACTAGACTGAAAAAACATTGC R: TGAGATTGAAAGGAAACGAG	61.9 56.9	44	Amplified
MFC-2	$(AC)_{18}$ $(AT)_{7}$	F: GCTTCCGATGCTGCTCTTA R: TCGGAGACTTTTGTTCAAT	62.6 58.5	51	Amplified
MFC-4	$(AT)_{4}$ $(AC)_{11}$	F: CCAAACTTTTAGATACAACTT R: TTTCTCAACATATTAACAGG	58.4 54.5	45	amplified
MFC-5	[GA]13	F: ACCAATCCAAATAATAATCC R: ACACGCTTACTAGAATTACC	58.3 58.7	59	Amplified
MFC-6	[TAA]3,[GT]8	F: AGGCTACTTCAGTGCTACA R: GCCATAAGTAATAAAAACC	61.0 61.9	63	amplified
MFC-7	$[AG]_{11}$	F: CACAATCAAAATAGTTACCG R: AGCGAAGACAGTTACAAAGC	58.2 54.1	54	amplified

MFC-8	[CA]9,TA[CA]14, [TA]6	F: GTGGCGTCGTCTCTAATAAT R: TATTCTATGCTGTCTTATGTCA	49.0 50.0	61	amplified
MFC-9	[AC]15TC[AC]8 [AT]7	F: GATATTTTCATGTTTAGTTTG R: GAGGATAGACCAACAACAAC	55.0 49.0	47	amplified

3. SSR fingerprinting for 27 DNA samples-

Now we have amplified our 27 DNA samples for 1 to 9 primer (except primer 3) at standard temperature of each SSr marker and then run those DNA samples at metaphore gel and visualize them with the help of Gel doc system. Results showed in the following figures for 8 primers-

Fig. 3.1 result for primer 1 at 44°c

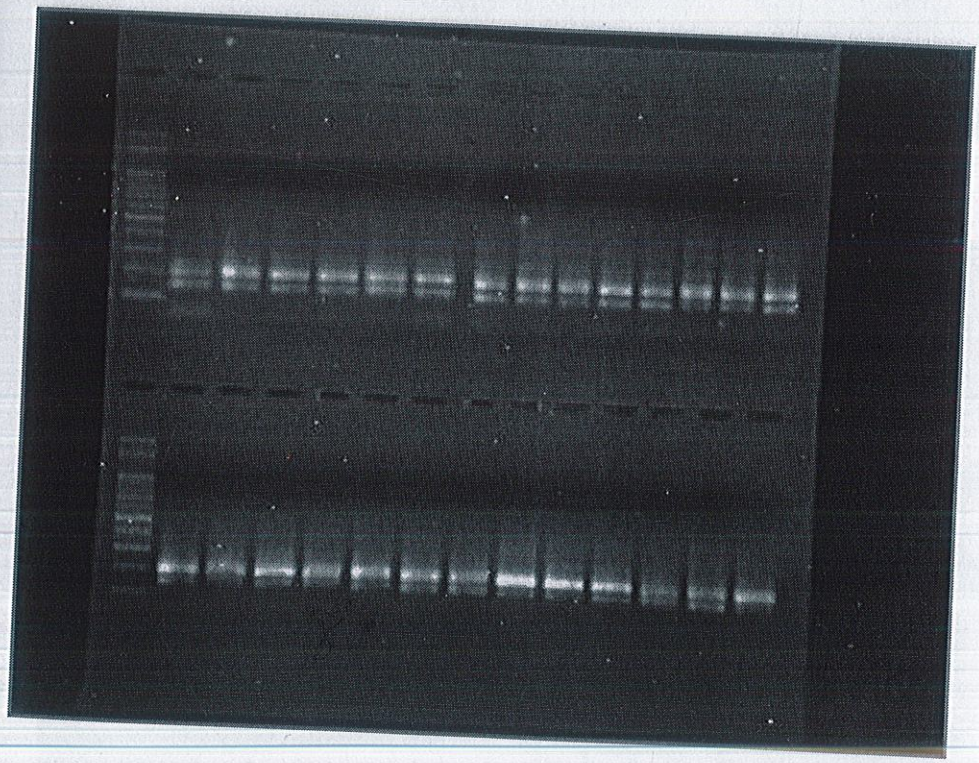


Fig. 3.2 result for primer 2 at 51°c for 27 DNA samples

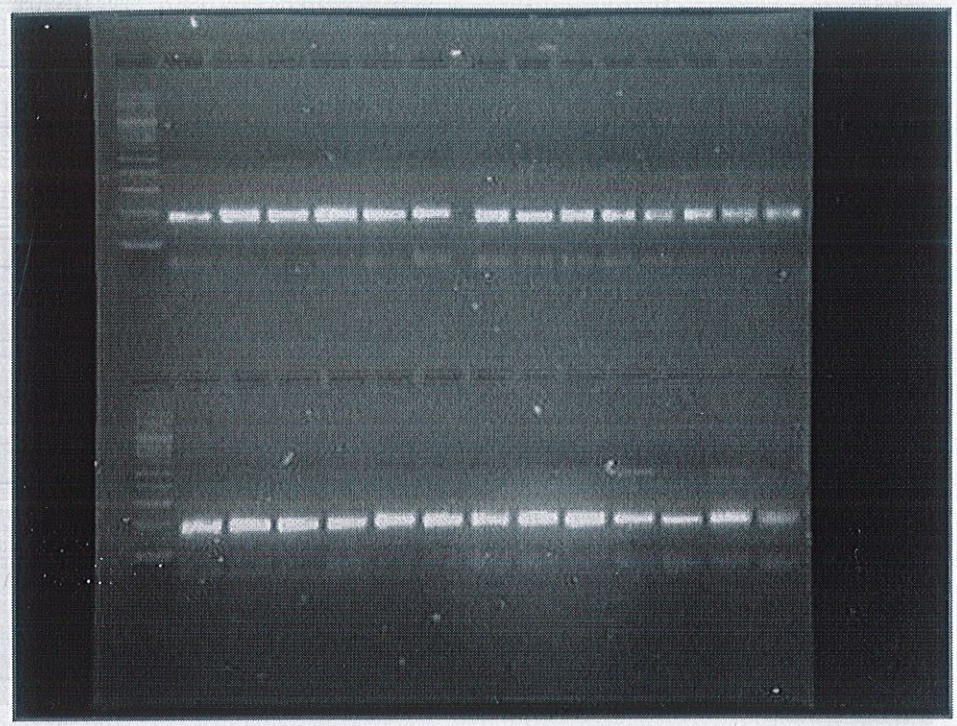


Fig. 3.3 result for primer 4 at 45°c for 27 DNA samples.

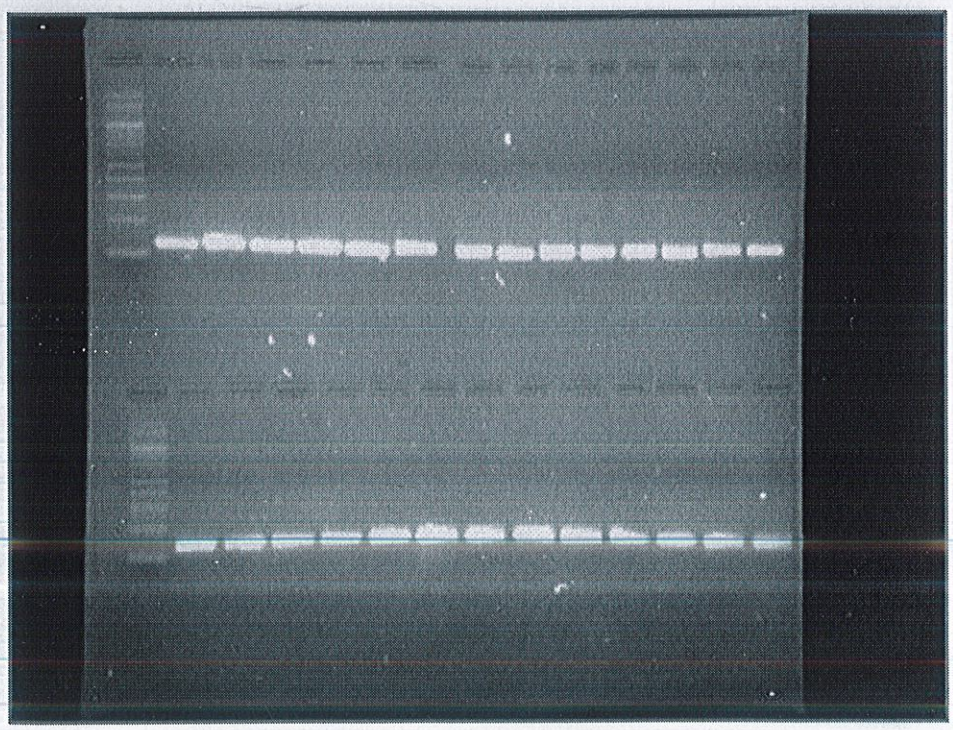


Fig. 3.4 result for primer 5 at 59°c for 27 DNA samples.

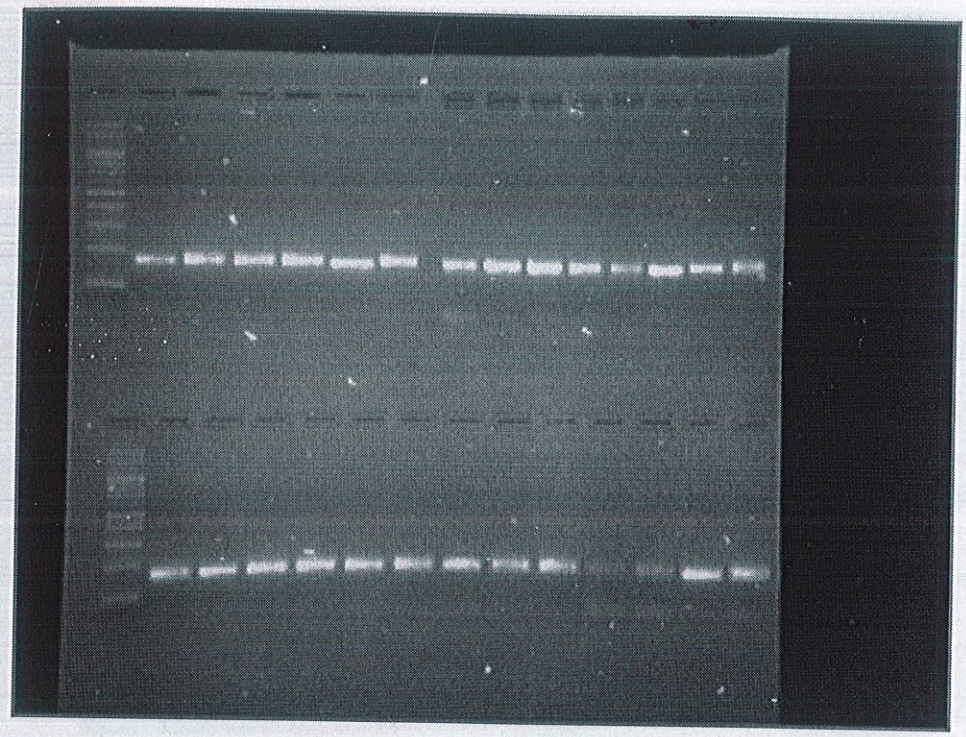


Fig. 3.5 result for primer 6 at 63°c for 27 DNA samples

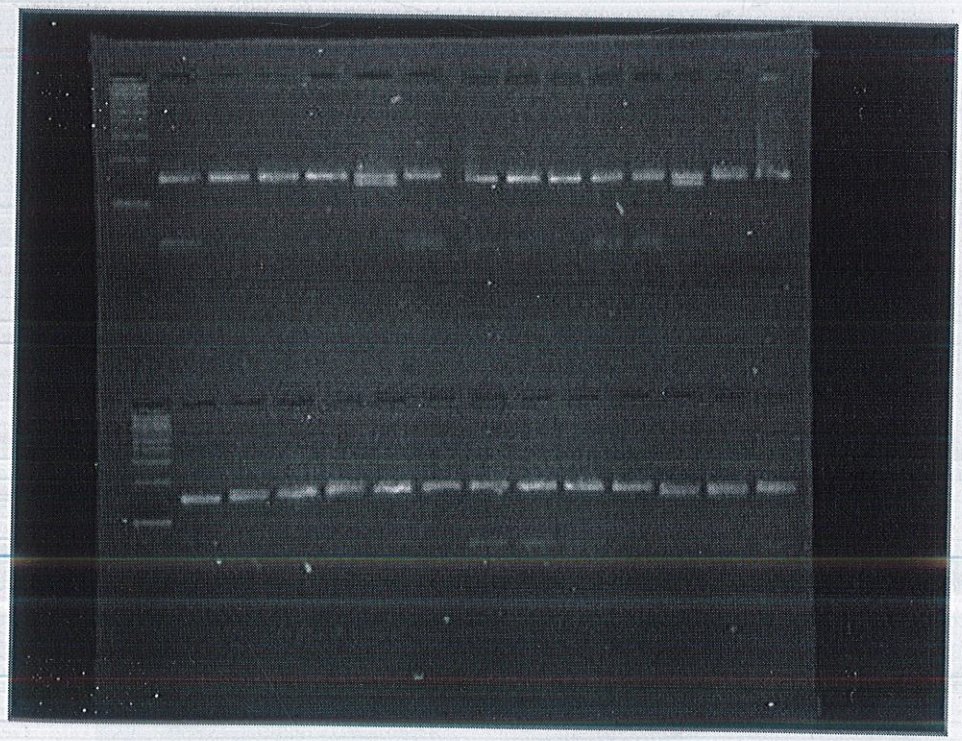


Fig. 3.6 result for primer 7 at 54°c for 27 DNA samples

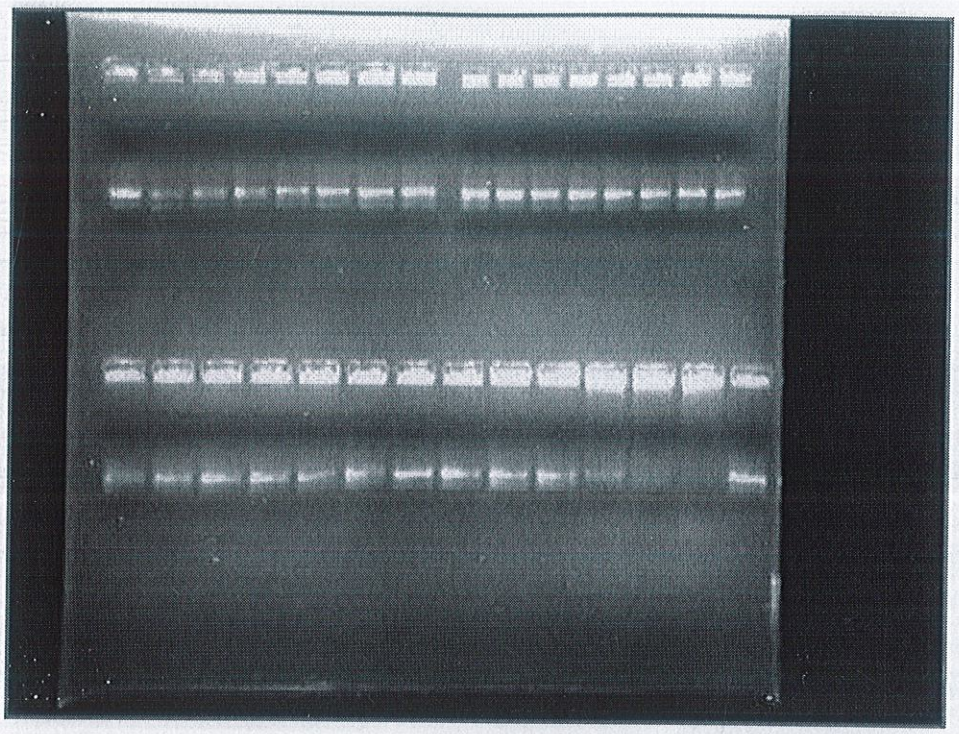


Fig. 3.7 results for primer 8 at 61°c for 27 DNA samples

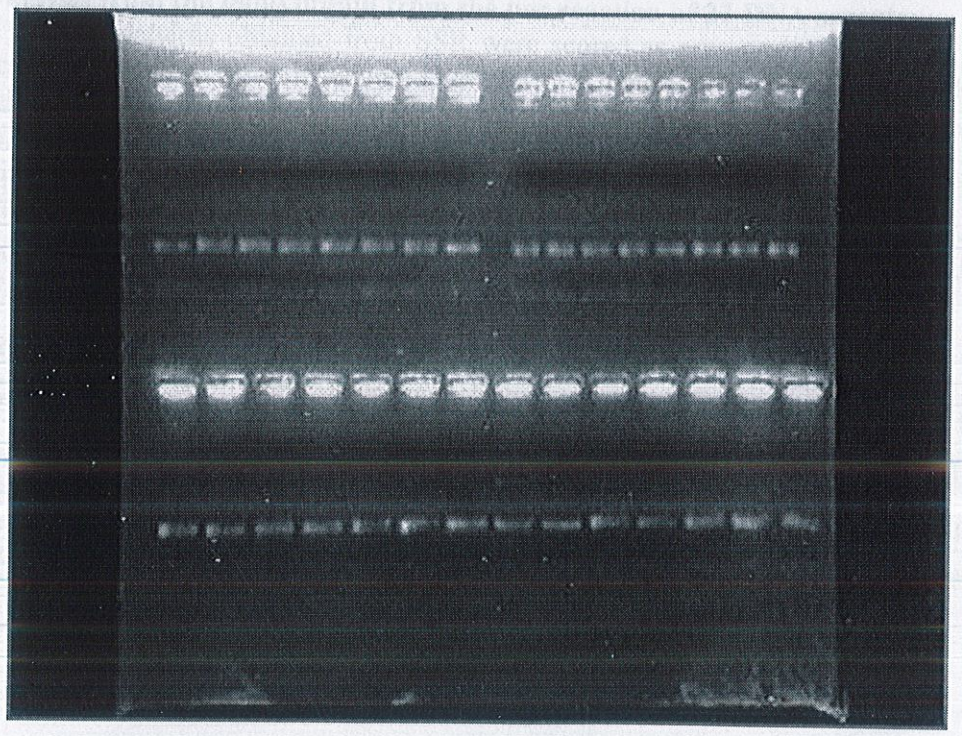
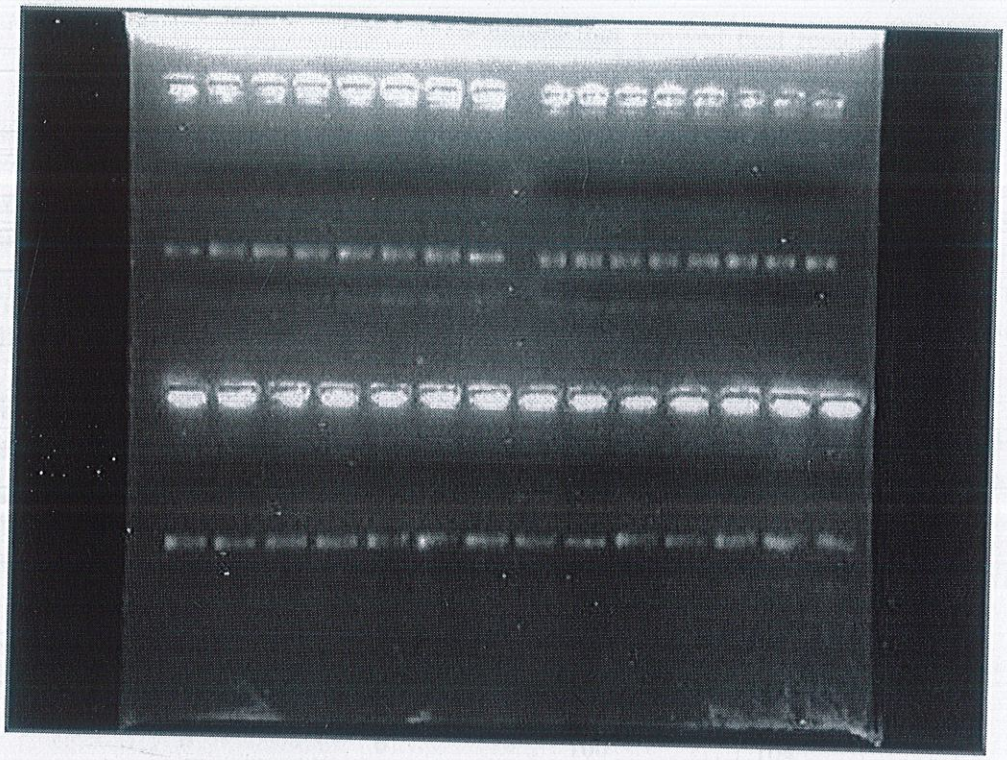


Fig. 3.8 results for primer 9 at 47°c for 27 DNA samples.



4. Analysis from the band obtain from the pcr reaction of 27 DNA samples

The banding patterns obtained from SSR were scored as present (1) or absent (0), each of which was treated as an independent character.Pop gene was used to calculate the different genetic diversity parameters: observed number of alleles (Na), effective number of alleles (Ne), Nei's genetic diversity (H), Shannon's information index (I) (Lewontin, 7), number of polymorphic loci (NPL) and percentage polymorphic loci (PPL) across all the fifteen populations were also analyzed. Within species diversity (Hs) and total genetic diversity (Ht) (Nei, 9) were calculated within the species and among population using POPGENE software.

Table 4 Showing Genetic diversity in 27 samples of *M. alba*

Sr no	Sample size	Na* (mean ±Sd)	Ne (mean ± SD)	h (mean ±SD)	I (mean ±SD)
1	27	1.7941±0.4104	1.4833±0.3739	0.2792±0.1931	0.4154±0.2668

5. Genetic Diversity:

Out of 10 primers used 8 primers were chosen that showed best resolution in amplification profiles, a total of 31 loci were obtained out of which 27 were polymorphic i.e. percentage of polymorphic loci is 87.09%. Each primer yielded 1 to 5 loci with average of 3.9% loci per primer. Resolving power of primer ranged from 2.1 to 7.7 . At the population level, the percentage of polymorphic loci per population ranged from 17.65% to 50.00% with an average of 35.02%. two valleys not showing much difference in genetic diversity parameters. The reason may be because both Indus and suru valley is not separated by any natural barrier so there is no restriction of gene flow. also migration of *mulberry* depends on the human migration due to absence of the natural barrier human migration is more compared to other valleys.

Table 5 Showing total number of loci, polymorphic loci and resolving power for 8 SSR primers.

marker	Total number Of loci	Number of Polymorphic loci	Percentage of Polymorphic loci	Total number of fragment amplified	Resolving power
1	3	2	66.7	78	5.8
2	6	6	100	104	7.8
4	4	2	50	83	6.2
5	4	4	100	28	2.1
6	5	4	80	62	4.6
7	3	3	100	45	3.4
8	3	3	100	47	3.5
9	3	3	100	37	2.8

6. Genetic relationship:

The dendrogram was constructed by using UPGMA cluster analysis on the basis of Jaccard's coefficient. Principal coordinate analysis performed using GenAlex software also strengthens finding of dendrogram.genotypes from Indus and Suru valley are inter mixed, there is no clear cut separation between them. Number of cluster less than the number of populations sampled.

Dendrogram consolidates the trend obtained in genetic diversity parameter

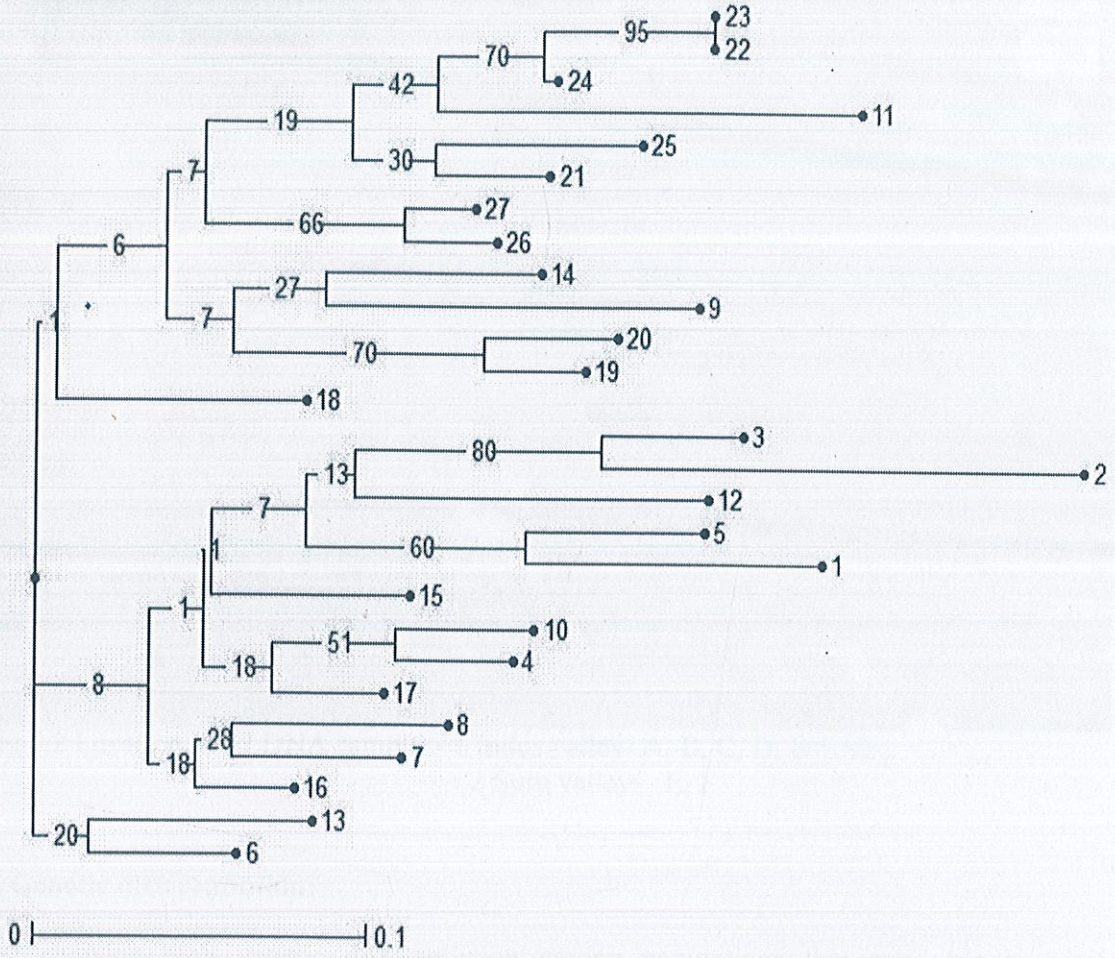


Fig 6.1 Dendrogram showing relationship between 27 *M. alba* samples.

This tree shows us the relationship between the 27 DNA samples which were taken from two different valleys. From tree we can see that sample 1 to 20 except 9,14 and 11 have common ancestors. While on other side sample 21 to 27 and sample 9, 11 and 14 have common ancestors. The reason behind this might be gene flow or human migration. Suru valley is located on highway and location of sample 9, 11 and 14 is connected with this highway. That's why human migration is easy between these two points and this is the main reason of this relation between two different valley's samples

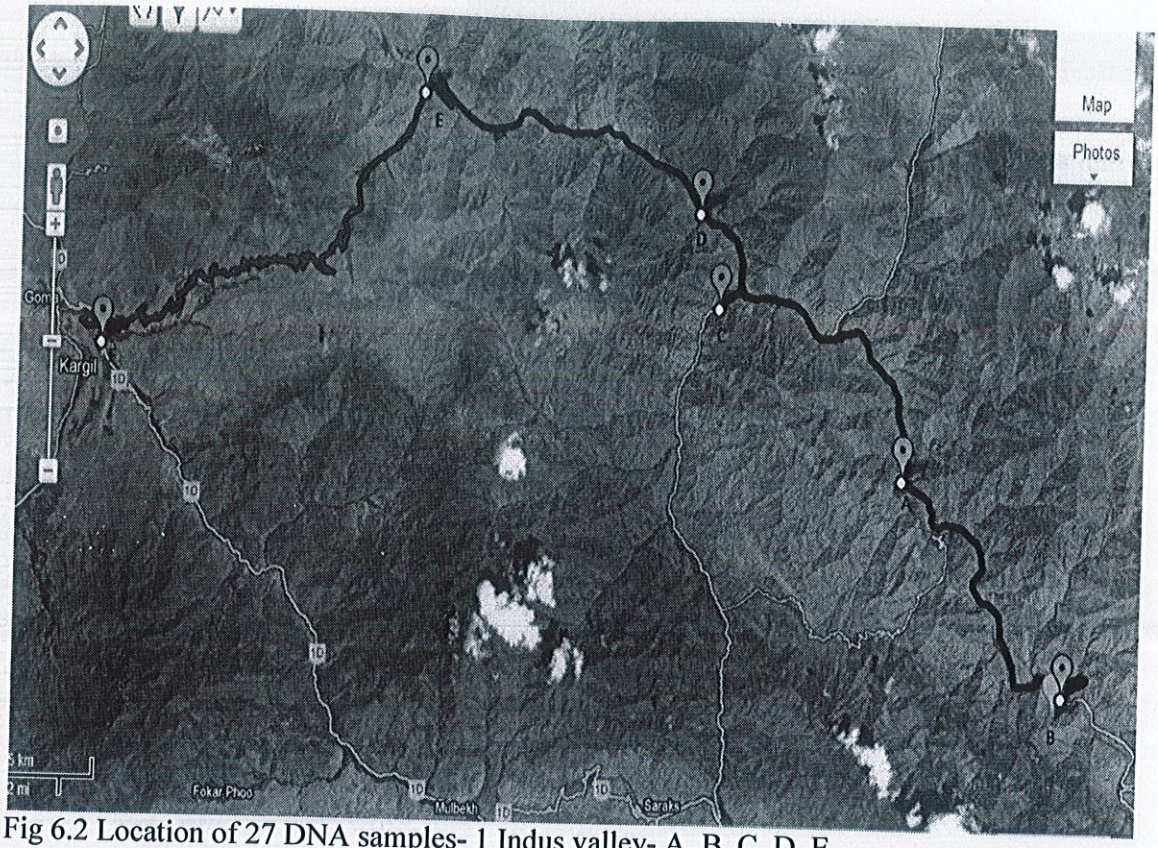


Fig 6.2 Location of 27 DNA samples- 1 Indus valley- A, B, C, D, E
2 Suru valley- E, F

7. Genetic differentiation:

Gst showing high genetic differentiation among populations this may due to patchy distribution of *Mulberry* in Ladakh, most of the populations are separated by unfertile and barren land which restricts gene flow, which is evident by lower value of Nm (gene flow) among populations. Nm is consistent with Gst value, normally region with higher Gst have lower Nm value and region with lower Gst have higher Nm (Suru valley and Indus valley).

Sample size	Ht (mean±SD)	Hs (mean±SD)	Gst	Gcs	Nm (Gst)	Nm (Gcs)	Hc
27	0.2792±0.0373	0.1596±0.0141	0.4284	1	0.6671	0	0

8. Analysis of Molecular Variance (AMOVA)

According to Analysis of molecular variance (AMOVA) variability within populations is highest (72%) while variability among populations (28%) and lower.

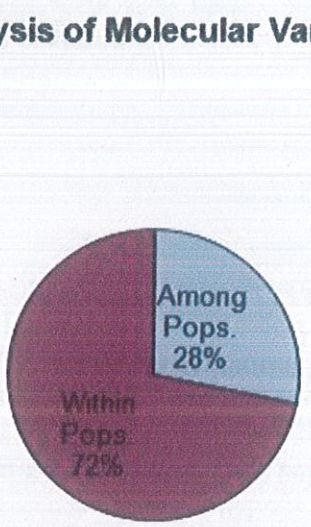


Fig.4.1 Showing analysis of molecular varience (AMOVA).

conclusion

Overall result suggests that genetic diversity and genetic differentiation is low in studied area but gene flow is moderate. The potential vulnerability of this species to increase in inbreeding, future conservation efforts should be directed towards ensuring pollen and seed dispersal among different populations

CHAPTER 5

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APPENDIX-I

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