STUDY OF GENETIC DIVERSITY IN PRUNUS SP. (SWEET CHERRY) FROM HIMACHAL PRADESH.

By

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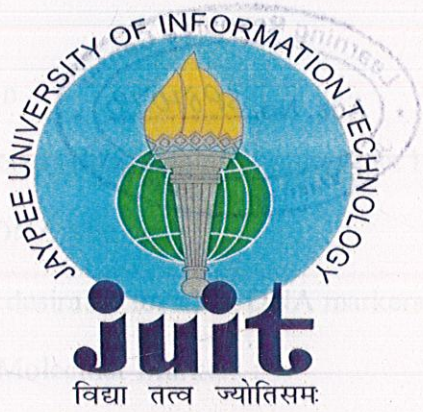

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CERTIFICATE

List of Novel Primers Designed from the Flanking Regions of SSR Repeats present in *Prunus sp.*This is to certify that the work entitled **"Study of Genetic Diversity in *Prunus sp.* (sweet cherry) from Himachal Pradesh"** submitted by **Paramdeep Singh** and **Tanya Jain** in partial fulfillment for the award of degree of Bachelor of Technology in Biotechnology in 2011 of **Jaypee University of Information Technology , Waknaghat** is a bonafide record of his / her original work carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Dr. Harvinder Singh

Senior Lecturer

Date : 24th May, 2011

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LIST OF ABBREVIATIONS

ROS	Reactive Oxygen Species
RFLP	Restriction Fragment Length Polymorphism
RAPD	Random Amplified Polymorphic DNA
SSR	Simple Sequence Repeat
AFLP	Amplified Fragment Length Polymorphism
DNA	Deoxyribonucleic Acid
PCR	Polymerase Chain Reaction
TAE	Tris Acetate EDTA
EDTA	Ethylene Diamine Tetra Acetate
NCBI	National Centre for Biotechnology Information
MISA	Microsatellite Identification Tool
dNTP	Deoxyribonucleotide Triphosphate
ul.	Micro litre
%	Percentage
ml.	Milliliter
M	Molarity

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Abstract

Prunus sp. is rapidly developing as economical tree species of returning farming land to forestry and the ecological restoration and reconstruction has gained high economic benefits. But also, some problems have appeared, such as pollenizer is not or unreasonably disposed, resulting in the trees of 8-9 years older has flowered but not fruited; meanwhile varieties are too confused and some cultivars have unclear genetic relationship, which has created serious impact on intensive management and breeding work. Genetic characterization of 9 *Prunus* sp collected from NBPGR, Himachal Pradesh region were analyzed using 17 PCR markers (10 RAPDs and 7 SSRs). RAPD analysis yielded 58 polymorphic fragments (100%), with an average of 5.8 polymorphic fragments per primer. SSR analysis produced 14 polymorphic (93.3%), with an average of 2.1 polymorphic fragments per primer. The genetic diversity was high between the species (Nei's genetic diversity = 0.34 and Shannon's information index = 0.395) as measured by combination of both RAPD and SSR markers. The mean coefficient of gene differentiation (Gst) was 0.446, indicating 65.4% of the genetic diversity resided within the genotypes. RAPD markers were found more efficient with regard to polymorphism detection, as they detected 100% in comparison to 93.3% for SSR markers. Clustering of genotypes within groups was not similar when RAPD and SSR derived dendrogram were compared, whereas the pattern of clustering of the genotypes remained more or less the same in SSR and combined data of RAPD+SSR. Insilico analysis of SSRs derived from ESTs suggested *Prunus* species being rich in monomers followed by dimers and tetramers. A/T; AG/CT; AAG/CTT simple sequence repeats were observed to be present in high frequency.

Chapter 1

INTRODUCTION

1.1 Description:

Wild cherry (*Prunus avium*) belongs to the family Rosaceae and it is a diploid with a chromosome number of 2n=2x=16. The domesticated form cultivated for fruit is known as sweet cherry (Russell K., 2003).

Prunus is the botanical name for a large group of deciduous and evergreen trees and shrubs. These popular plants are greatly valued for their delicious, edible fruits, gorgeous spring blossoms and some, for their colorful foliage. They are among the most beautiful trees and shrubs.. Most of the species need to be grown in temperate regions.

Sweet cherries are especially rich in anthocyanin, quercetin, melatonin etc. which are responsible for antioxidant activity of cherries.

Quercetin also acts as a free radical scavenger suggests it could play a beneficial role in reducing reactive oxygen species (ROS) (i.e. hydrogen peroxide, superoxide anion) associated with chronic diseases such as cardiovascular disease and cancer (Johnson, 2000; Wilms, 2005).

Melatonin supplementation appears to be effective in reducing jet lag (Herxheimer, 2002; Suhner, 2001). Cherries help in curing and reduction of diseases like cardiovascular disease (Corder, 2006), Alzheimer's disease (Yoshimura, 2003; Heo, 2004), Cancer Diabetes etc.

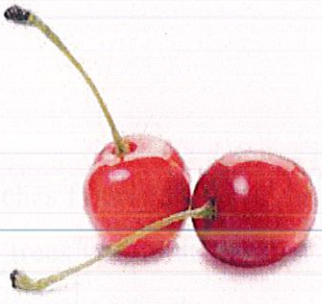


Fig.1.1 Cherry Fruit

Apricot (*P. armniaca*)

The Apricot is native to Asia. These trees are grown for their beautiful, fragrant blossoms and their delicious, velvety soft fruits, which resemble small Peaches. They are widely grownin California where the fruit is dried, canned and sold as fresh fruit.

Cherry (*P. avium* - sweet / *P. cerasus* - sour)

Cherries are originally from Transcaucasia, Asia Minor and Persia. The Cherry is a very beautiful tree that provides excellent fruit. There are two kinds of Cherry trees - Sweet Cherries, which are cultivars of P. avium, and Tart or Sour Cherries, which are cultivars of P. cerasus.. From mid- to late spring, clusters of pure white, cup-shaped flowers are produced.

Peach (*P. persica*) :

Peach trees are moderately small, with dense, erect growth unless pruned to encourage spreading growth. The long, glossy bright green leaves are from 4 to 9 inches long with finely serrated edges. These trees, when grown on their own roots, will grow from 8 to 20 feet high.

Plum (*P. domestica*) :

The Plum is widely cultivated throughout the U.S., since there are varieties suitable for growing in every state. Plums are extensively grown for commerce in Oregon, Washington, California and Idaho. The fruits are oval or

round having smooth, thin skin. The flesh may be purple, blue, red, green or yellow, also depending on the variety.

Japanese plums *(P. salicina):*

These plums grow well where peaches flourish, tolerate heat and need only a short period of winter dormancy. However, these trees bloom at the first touch of warm weather, making them susceptible to damage from late spring frosts.

European plums (*P. domestica*) :

Are harder than Japanese plums. European varieties include the late-season prune plums, which are great for drying and eating fresh, and the Gage types, small, greenish fruits valued for their light sweetness.

1.2 India: Cherry growth gains popularity in Himachal

Cherry cultivation is gaining popularity in Himachal Pradesh as a profitable alternative to other cash crops. *Prunus avium*, our common cherry, is a delicious fruit, rich in protein, sugar and minerals and has more calorific value than apple. It is grown in areas between 2,000 and 2,700 meters above sea-level, requiring 1,000-1,500 hours of chilling period during winters and the climate of Himachal Pradesh is most suited for its growth.

Himachal Pradesh that has emerged as the second largest cherry producing state in the country after Jammu and Kashmir. Of the total, about 420 tonne was grown in Shimla region alone in the cherry belts of Rampur, Narkanda, Rohru ,Kandayli, Kotgarh and Kotkhai. Himachal Pradesh produced 698 tonne of cherry in 2007, but its production slumped to 455 tonne in 2008 due to dry weather. This year, cherry production is expected to cross the 700-tonne mark in view of favorable weather conditions

1.3 Types of DNA Markers:

Molecular markers, unlike morphological markers are stable and have been found to be vey useful in population studies. (Aitkin *et al.,* 1994; Lakshmi *et al.,* 1997)

The most commonly used types of DNA markers include restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD),simple sequence repeat (SSR), amplified fragment length polymorphism (AFLP). PCR-based markers are generally preferred due to their technical simplicity for generation and are relatively cheap. SSR's are PCR based markers and extremely versatile, since they are used for basic and applied research.

1.4 Properties desirable for ideal DNA markers:

- Highly polymorphic nature
- Codominant inheritance (determination of homozygous and heterozygous states of diploid organisms)
- Frequent occurrence in genome
- Selective neutral behaviour (the DNA sequences of any organism are neutral to environmental conditions or management practices)
- Easy access (availability) and fast assay.
- High reproducibility

Table 1.1: Characteristics of commonly used types of DNA markers.

	RAPD	SSR	RFLP	AFLP
Feature Of Method	Random amplified markers usually generated by 10-mer arbitrary primers.	Primers designed from DNA sequences flanking SSR repeats.	Restriction digestion of genomic DNA and detection with cDNA or genomic DNA probes.	Genomic DNA restriction digestion followed by adaptor ligation and PCR.
Reliability	Low	High	High	high
Cost	Low	Low	High	High
Dominance	Dominant	Codominant	Codominant	dominant
Reproducibility	Low to medium	Medium to high	High to very high	Medium to high

In case of PCR based markers, primers of known sequences and length are used to amplify genomic and cDNA sequences which are visualize by gel electrophoresis. In our study, we have used two PCR based markers RAPD (Random Amplified Polymorphic DNA) and SSR (Simple Sequence Repeats).

1.5 RAPD as Molecular Markers :

RAPD markers have recently caught the fancy of many individuals in the field of applied plant breeding. This molecular marker is based on the PCR amplification of random locations in the genome of the plant. With this technique, a single oligonucleotide is used to prime the amplification of genomic DNA. Because these primers are 10 nucleotides long, they have the possibility of annealing at a number of locations in the genome. The number of amplification products is directly related to the number and orientation of the sequences that are complementary to the primer in the genome.

Advantages of RAPD (Lisa Monna *et al.*, 1994):

1. Cost effectiveness.
2. The fact no sequence information of template DNA or synthesizing of specific primers is required.
3. Relatively low amount or purity of template DNA can be used,
4. Rapidity and technical ease, and
5. Ability to generate markers in the regions containing repetitive sequences

Disadvantages of RAPD (Lisa Monna *et al.*, 1994):

1. Relatively low accuracy of linkage analysis because of its dominant nature.
2. High sensitivity to PCR conditions
3. Difficulty of direct use for screening YAC or cosmid libraries

1.6 SSR As Molecular Markers:

The repeating sequences of 1-6 base pairs of DNA have been termed as Simple Sequence Repeats (SSRs) (Jacob *et al.*, 1991),microsatellite (Litt and Luty, 1989) or Short Tandem Repeat (SSR) (Edwards *et al.* , 1991).

It is believed that when DNA is being replicated, errors occur in the process and extra sets of these repeat sequences are added to the strand. Overtime, these repeat sequences vary in length between one cultivar and another. These markers present a high level of polymorphism, particularly when tandem repeats number ten or greater. The repeat sequence is often simple, consisting of two, three or four nucleotides and can be repeated 10 to 100 times.

Advantages of SSR's:

1. Co-dominance (more informative when dealing with heterozygote's)
2. Highly variable (important for species with narrow gene pools)
3. Widely used.
4. Excellent for use in marker assisted selection, fingerprinting and marker assisted backcrossing.

Disadvantages of SSR's:

1. They typically require polyacrylamide gel electrophoresis and generally give information only about a single locus per assay, although multiplexing of several markers is possible.
2. SSR markers also require a substantial investment of time and money to develop.

1.7 Objectives:

- In silico analysis of frequency and distribution of SSR's in ESTs of *prunus* species
- To study the genetic diversity of *Prunus* species in Himachal Pradesh using RAPD and SSR markers.

Chapter 2

MATERIAL AND METHODS

Plant materials

Nine *Prunus* sp were collected from the NBPGR Shimla (Table 2.1). The leaves were collected from individual plant tree and stored in laboratory at –80° C until further analysis.

Table 2.1: List of *Prunus* sp. Acquired From NBPGR, Shimla

Sample No.	**Name**
Sample 1	*P.mira*
Sample 2,9	*P.armnaica*
Sample 3	*P.nepulensis*
Sample 4,5	*P.persica*
Sample 7	*P.domestica*
Sample 8	*P.salicinia*
Sample 10	*P.cerasifera*
Sample 6,11,12,13	*P.avium*
Sample 14, 15	*P.cerasoides*

2.1 Methodology:

DNA profiling of important *Prunus* sp. of Himachal Pradesh using RAPD and SSR molecular markers was done.

RAPD ANALYSIS:

- DNA was isolated from the samples and then the analysis was done.
- DNA was amplified by Polymerase chain reaction using universal primers.(Table2.2)
- Polymorphism was observed among the plant samples.

Table 2.2: List of Universal Primers Used for RAPD Analysis

ID	Sequence
OPN-16	AAGCGACCTG
OPG-18	GGCTCATGTG
OPL-20	TGGTGGACCA
OPT-17	CCAACGTCGT
OPT-20	GACCAATGCC
OPU-13	GGCTGGTTCC
OPF-14	TGCTGCAGGT
OPX-03	TGGCGCAGTG
OPF-5	CCGAATTCCC
OPX-20	CCCAGCTAGA

SSR ANAYSIS:

- EST sequences were downloaded for all the species from an EST database through NCBI (National Center for Biotechnology Information). (http://www.ncbi.nlm.nih.gov/).
- Microsatellites were identified using MISA (microsatellite identification tool).

- SSR Primer's were designed based upon the statistics obtained from the MISA software through WEBSAT. (http://wsmartins.net/websat/).
- PCR amplification using *denovo* SSR primers.
- Results obtained.

2.2 Microsatellite Search:

EST sequences were downloaded from NCBI (http://www.ncbi.nlm.nih.gov/) for each of the *prunus sp.* The Perl script Microsatellite MISA was used to identify SSR's in all the *prunus sp.* sequences. The parameters of the SSR search were defined as follows.

The size of the motifs was defined as 2 to 6 nucleotide.

1. Download the sequences of all chromosomes of *prunus* sp.
2. Microsatellites were identified using MISA (Microsatellite identification tool).
3. 2 files were obtained in (a) .misa and (b) .statistics format.

2.3 Primer Designing For SSR Primers:

7 Primers flanking different motif and of variable size, were designed based on the EST sequence from the different species of *prunus* based on the following criteria.

Procedure:

1. Maximum length sequences of the various species were taken. Raw DNA sequence or a sequence (or multiple sequences) in FASTA format were entered in WEBSAT (http://wsmartins.net/websat/).
2. Minimum repeat length for motifs was decided.
3. Primers were selected who had Tm more than 57 and min. GC% to be 40%
4. All the primers that have been designed were custom synthesized from IDT Pvt. Ltd. and used for PCR amplification.

Table 2.3: List of Novel Primers Designed from the Flanking Regions of SSR Repeats present in *prunus sp.*

ID	SEQUENCE (FORWARD)	SEQUENCE (REVERSE)	TM (^{0}C)	PRODUCT SIZE	Motif type/size
AVIUM 1	TGCTCAAGAAGC TGTGAAGAAG	GCCAATCAATCCT TACAAAACC	50	387	$(TGG)_4$
AVIUM 2	GCCAATCAATCCT TACAAAACC	TGCTCAAGAAGCT GTGAAGAAG	50	387	$(CCA)_4$
ARMNAICA 1	TACTGTGAGGCTG TTCGTGAAT	AGGATAGATGACA GGCGTGAAG	52	359	$(ACT)_4$
ARMNAICA 2	CAAACTTGGACCT CACAAATCA	GGATGAGACCGCA GCTAAATAC	51	282	$(A)_{10}$
PERSICA	TCCTGCCTTTTAA GTTTGTTGC	TCCATGTCCTCTCT CTCACTCA	51	328	$(TGA)_4$
PRUNUS 1	ACACACCCAAAG CTCCAATCT	AGAGCCCATTTCT AAACCACAA	49.5	342	$(CT)_6$
PRUNUS 2	CTTTCAACTGTCA AGGTCCGAT	CGTAAGCAGAGTT ATCCCTTCG	52	206	$(CCT)_4$

2.4 Isolation Of Genomic DNA:

DNA was extracted from the sample's according to the method described by A.M. Torres, N.F. Weeden *et al.* (1993).

- About 100 mg. of tissue was crushed in 1 ml. of liquid N_2.
- Before this tissue is thawed 1 ml. of CTAB buffer containing 0.4% of 2-b mercaptoethanol and 0.5 M sodium bisulphite was added and the grinding briefly was continued.
- Approximately, 0.5 ml. of this aqueous slurry was poured into a 1.5ml. Eppendorf micro centrifuge tube containing 100ul. Of 24:1 chloroform isoamyl alcohol.
- The tube was briefly shaken and incubated at 60 degrees for 30-45 min.
- The solution was allowed to cool to room temperature and sufficient 24:1 chloroform isoamyl alcohol was added to nearly fill the tube.
- The mixture was shaken vigorously to form an emulsion then centrifuged at 5 degrees to 4 min. (7000g) to separate the phases.
- The aqueous phase was transferred to clean micro centrifuge tube.
- An equal volume of cold (-20 degrees) 95% ethanol was added to precipitate the DNA which was spooled out.
- Washed in 76% ethanol 0.2M sodium acetate for 5 min. and dissolved in 100-200 ul. of TE buffer , pH =8.0.

2.5 Quantification of Purified DNA :

Preparation of 1.5% agarose gel:

- 1.05 gm. of agarose was weighed and 70ml. of 1 X TAE was added and heated in a microwave oven for about 1-2 min.
- This solution was cooled and 3 ul. of EtBr was added into it.
- This solution was poured onto a casting tray, comb was inserted and the gel was left for about 20-30 min. for solidification.
- After the gel was solidified comb was removed.

- Gel was placed in the electrophoresis tank and 1X TAE was poured into it. Samples were loaded in the wells with the help of a 6X loading dye (3 ul. dye and 5 ul. sample).
- Voltage was applied to the tank and the gel was allowed to run for about 1 hr.
- Gel was visualized in GelDoc.

50X TAE Composition:

In 1000ml.

Tris (hydroxymethyl) aminomethane	242.28 gm.
Acetic acid (glacial)	57.1 ml.
0.5M EDTA (pH 8.0)	100ml. (50 mM.)

For 1X: 10 ml. of 50X TAE buffer and 490 ml. distilled water.

1.5% agarose

1.05 gm. agarose in 70ml. 1X TAE buffer.

2.6 PCR Amplification :

RAPD Amplification:

- The primers were dissolved with 200ul. of autoclaved distilled water by vortexing for 30 sec. The primer solutions were stored at 4 degrees overnight and then stored at -20 degrees till their use.
- The stock solutions of primer was diluted to a concentration of 50 pM for PCR amplification.
- 10 universal primers were used for the RAPD study of genetic diversity among the *prunus* species.

- Amplification reactions were performed in volumes of 25ul. containing 18.25ul. autoclaved distilled water , 1ul. primer , 0.5ul. dNTP's (3 U/ul.), 2.5 ul.PCR buffer (10 X), 0.25ul. Taq Polymerase (5U/ul.) and 1ul. of DNA.
- DNA amplification was performed in a thermal cycler.
- Conditions for the amplification:

  94 ^{0}C --- 3 min.

  94 ^{0}C --- 30 sec.
  37.5^{0}C—1 min.
  72^{0}C --- 2 min.
) X (40 cycles)

  72^{0}C ---7 min.

  4^{0}C ---storage

- The amplification products were resolved in 1.5% agarose gel (1X TAE Buffer) and visualized in Gel Documentation System.

SSR Amplification :

- The primers were dissolved with 200ul. of autoclaved distilled water by vortexing for 30 sec. The primer solutions were stored at 4 degrees overnight and then stored at -20 degrees till their use.
- The stock solutions of primer was diluted to a concentration of 50 pM for PCR amplification.
- 7 primers were used for the study of genetic diversity among the *prunus* species.
- Amplification reactions were performed in volumes of 25ul. containing 18.25ul. autoclaved distilled water , 1.25ul forward and reverse primers each , 0.5ul. dNTP's (3U/ul.), 2.5 ul. PCR buffer (10 X), 0.25ul. Taq Polymerase (5U/ul.) and 1ul. of DNA.

- DNA amplification was performed in a thermal cycler.
- Conditions for the amplification were as follows:

94 ^{0}C --- 4 min.

94 ^{0}C--- 30 sec.
50-53 ^{0}C—1 min.
72^{0}C --- 2 min.
} X (40 cycles)

72^{0}C---7 min.

4^{0}C---storage

- The amplification products were resolved in 1.5% agarose gel (1X TAE Buffer) and visualized in Gel Documentation system

2.7 Data Collection and Analysis :

The banding patterns obtained from gene specific primers were scored as present (1) or absent (0), each of which was treated as an independent character. Dice coefficient was used to calculate the similarity between pairs of accessions. The similarity matrix was subjected to cluster analysis by unweighted pair group method with arithmetic means (UPGMA) and a dendogram was generated using the program NTSYS pc (Rohlf FJ, 1992) POPGENE software was used to calculate Nei's unbiased genetic distance among different genotypes with all markers. Data for observed number of alleles (Na), effective number of alleles (Ne), Nei's genetic diversity (H), Shannon's information index (I), number of polymorphic loci (NPL) and percentage polymorphic loci (PPL) across all the three populations were also analyzed. (Zhao WG *et al.*, 2006) Within species diversity (Hs) and total genetic diversity (Ht) (Nei M, 1978) were calculated within the species and within three major groups (as per their collection site) using POPGENE software.

Chapter 3

RESULTS AND DISCUSSION

3.1 Insilco analysis of SSR:

Prunus armnaica

Highest number of SSR motifs in this species were observed in monomers followed by dimer and trimer. Maximum repeating units was found to be A/T then that of dimer AG/CT and AT/AT.

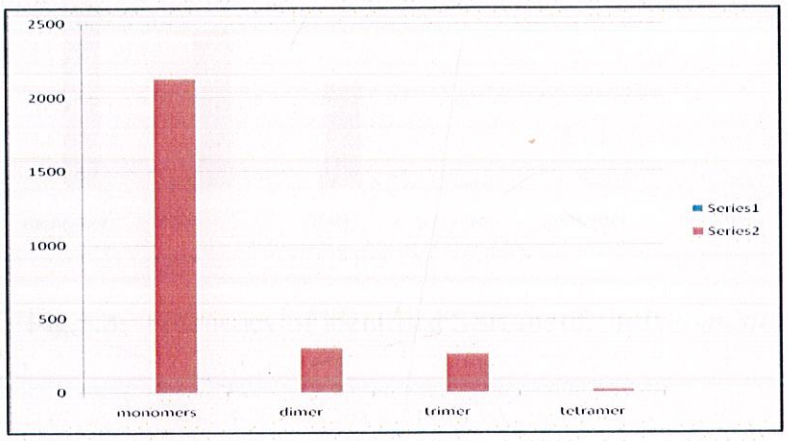


Fig. 3.1: Frequency of identified SSR motifs in *P. armnaica*

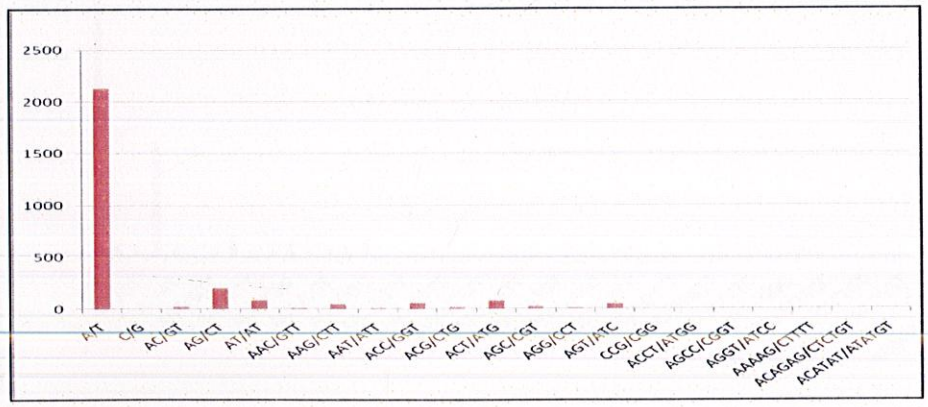


Fig.3.2 Frequency of classified repeat types (considering sequence complementary) in *P. armnaica*

Prunus domestica

Highest number of SSR motifs in this species were observed in monomers followed by dimer's and trimer's . Maximum repeating units was found to be A/T then that of dimer AT/AT followed by trimer AAT/ATT.

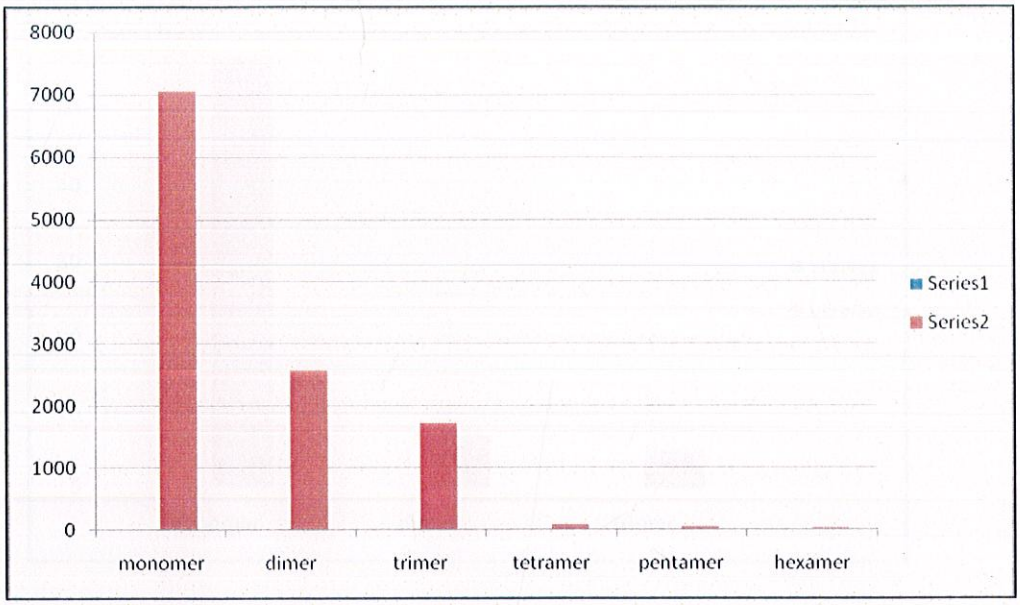


Fig.3.3: Frequency of identified SSR motifs in *P.domestica.*

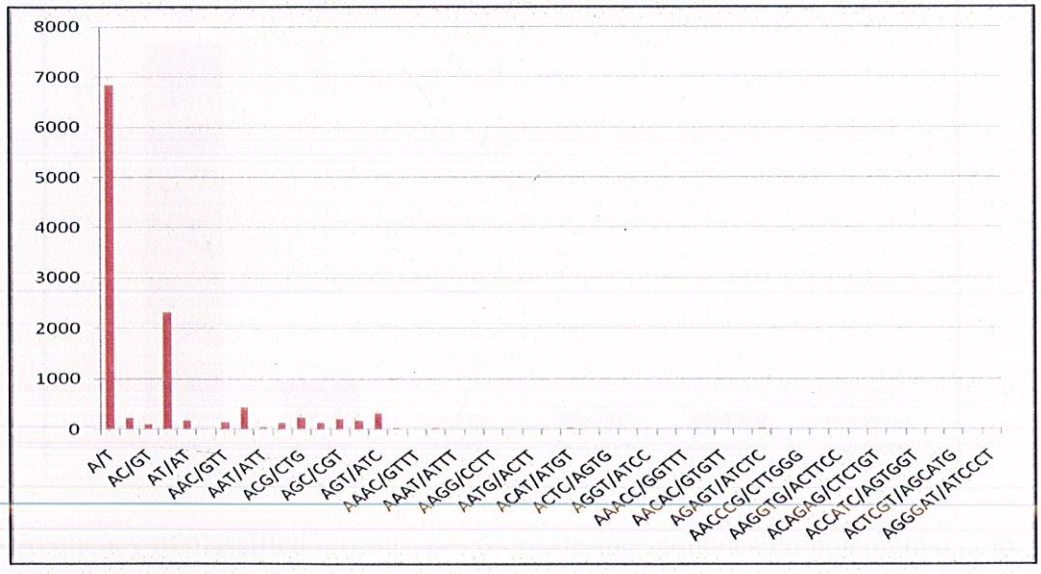


Fig.3.4 : Frequency of classified repeat types (considering sequence complementary) in *P.domestica*

Prunus avium

Highest number of SSR motifs in this species were observed in monomers followed by dimer's and trimer's .Maximum repeating units was found to be A/T then that of dimer AG/CT followed by trimer's AAG/CTT and ACC/GGT.

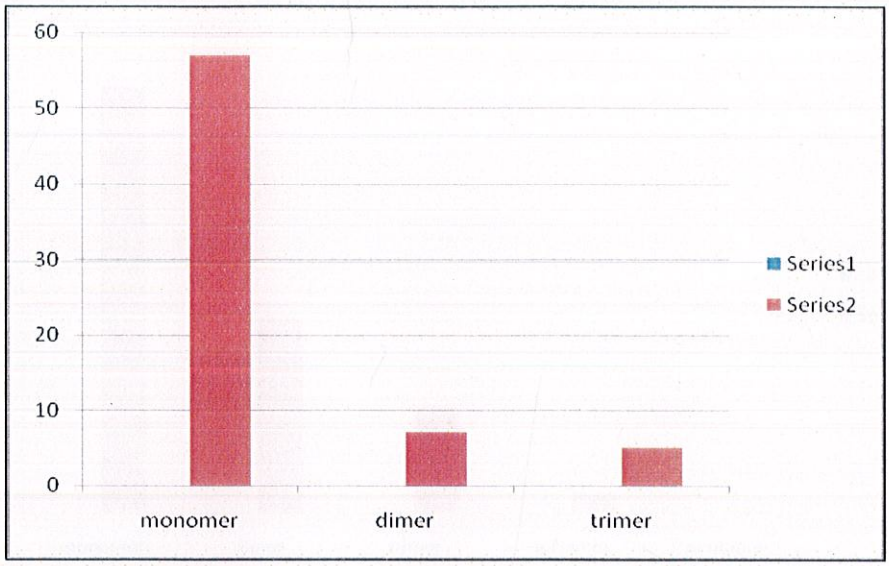


Fig.3.5 : . Frequency of identified SSR motifs *P.avium*

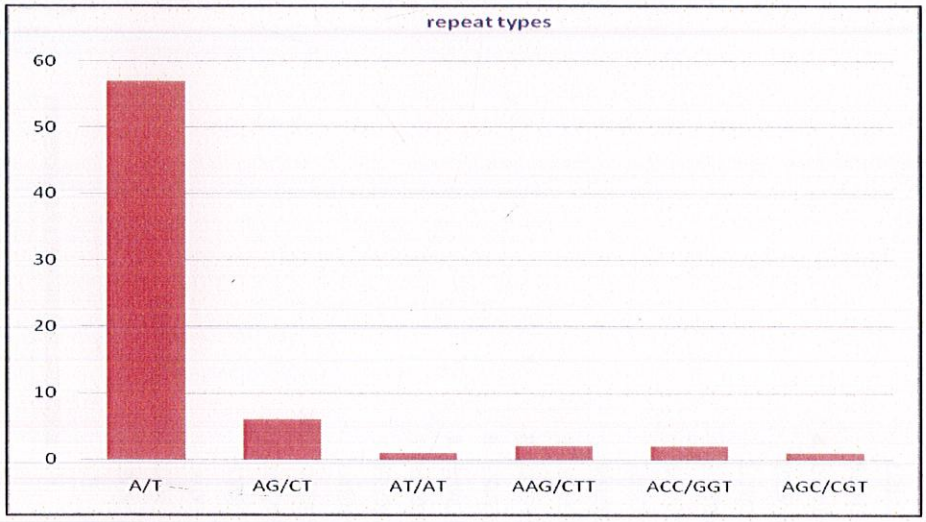


Fig. 3.6 : Frequency of classified repeat types (considering sequence complementary) in *P.avium*

Prunus persica

Highest number of SSR motifs in this species were observed in monomers followed by dimer's then trimer's and finally tetramer's. Maximum repeating units was found to be A/T then that of dimer AG/CT followed by trimer's AAG/CTT.

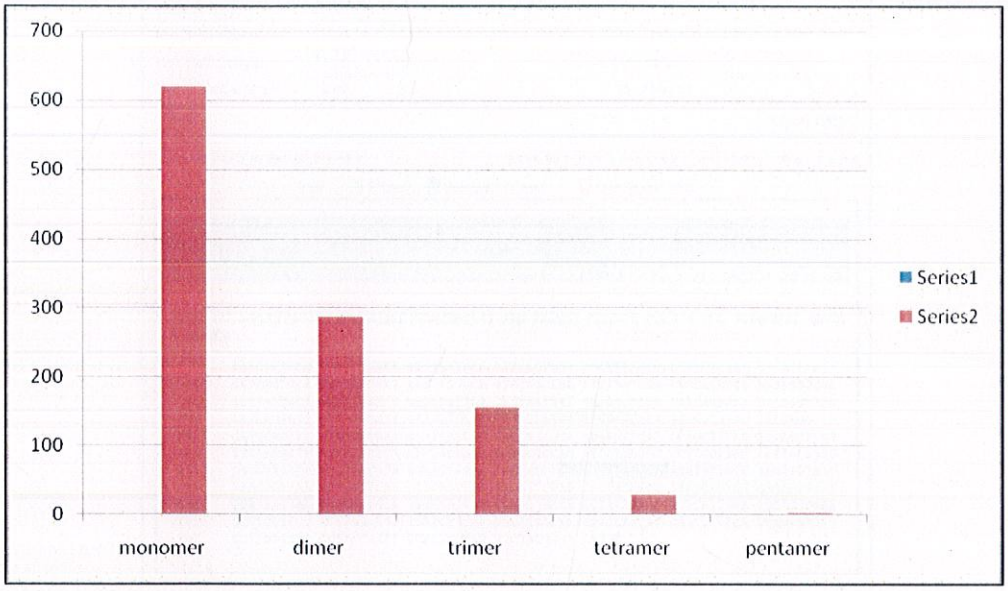


Fig.3.7: Frequency of identified SSR motifs *P.persica*

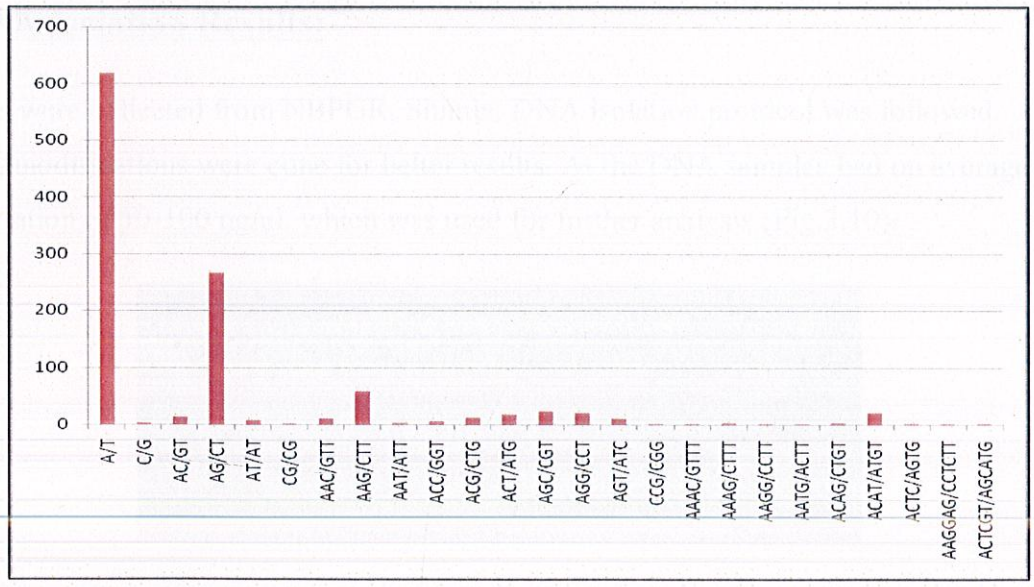
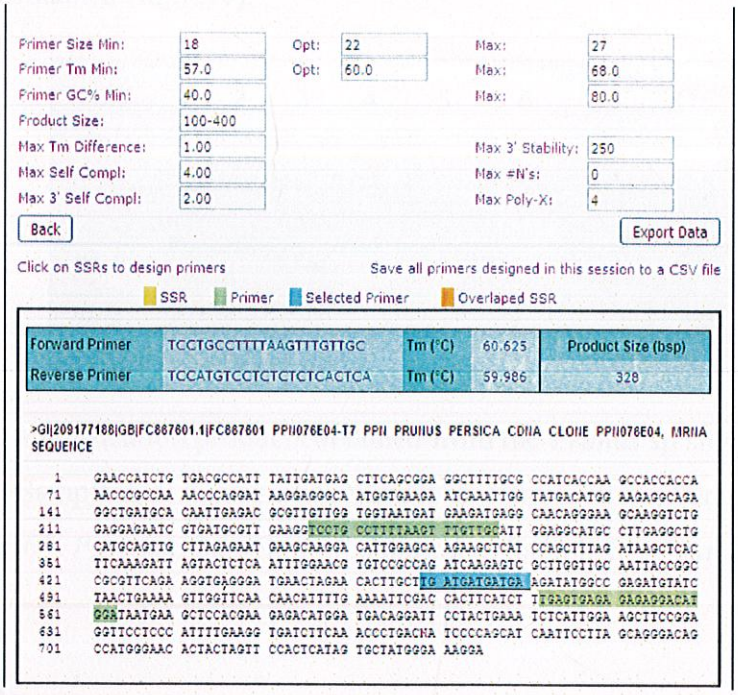


Fig.3.8 : Frequency of classified repeat types (considering sequence complementary) in *P.persica*

3.2 WEBSAT Result:

Primers were designed using WEBSAT tool. (fig. 3.9)



Primer Size Min:	18	Opt:	22	Max:	27
Primer Tm Min:	57.0	Opt:	60.0	Max:	68.0
Primer GC% Min:	40.0			Max:	80.0
Product Size:	100-400				
Max Tm Difference:	1.00			Max 3' Stability:	250
Max Self Compl:	4.00			Max #N's:	0
Max 3' Self Compl:	2.00			Max Poly-X:	4

Back | Export Data

Click on SSRs to design primers | Save all primers designed in this session to a CSV file

SSR | Primer | Selected Primer | Overlaped SSR

Forward Primer	TCCTGCCTTTTAAGTTTGTTGC	Tm (°C)	60.625	Product Size (bsp)
Reverse Primer	TCCATGTCCTCTCTCTCACTCA	Tm (°C)	59.986	328

>GI|209177188|GB|FC867601.1|FC867601 PPII076E04-T7 PPII PRUNUS PERSICA CDNA CLONE PPII076E04, MRNA SEQUENCE

```
   1   GAACCATCTG TGACGCCATT TATTGATGAG CTTCAGCGGA GGCTTTTGCG CCATCACCAA GCCACCACCA
  71   AACCCGCCAA AACCCAGGAT AAGGAGGGCA ATGGTGAAGA ATCAAATTGG TATGACATGG AAGAGGCAGA
 141   GGCTGATGCA CAATTGAGAC GCGTTGTTGG TGGTAATGAT GAAGATGAGG CAACAGGGAA GCAAGGTCTG
 211   GAGGAGAATC GTGATGCGTT GAAGGTCCTG CCTTTTAAGT TTGTTGCATT GGAGGCATGC CTTGAGGCTG
 281   CATGCAGTTG CTTAGAGAAT GAAGCAAGGA CATTGGAGCA AGAAGCTCAT CCAGCTTTAG ATAAGCTCAC
 351   TTCAAAGATT AGTACTCTCA ATTTGGAACG TGTCCGCCAG ATCAAGAGTC GCTTGGTTGC AATTACCGGC
 421   CGCGTTCAGA AGGTGAGGGA TGAACTAGAA CACTTGCTTG ATGATGATGA AGATATGGCC GAGATGTATC
 491   TAACTGAAAA GTTGGTTCAA CAACATTTTG AAAATTCGAC CACTTCATCT TTGAGTGAGA GAGAGGACAT
 561   GGATAATGAA GCTCCACGAA GAGACATGGA TGACAGGATT CCTACTGAAA TCTCATTGGA AGCTTCCGGA
 631   GGTTCCTCCC ATTTTGAAGG TGATCTTCAA ACCCTGACNA TCCCCAGCAT CAATTCCTTA GCAGGGACAG
 701   CCATGGGAAC ACTACTAGTT CCACTCATAG TGCTATGGGA AAGGA
```

Fig. 3.9 : *P.persica* WEBSAT result; primer designed

3.3 DNA Isolation Results:

Samples were collected from NBPGR, Shimla. DNA isolation protocol was followed. Further, modifications were cone for better results. At the DNA samples had on average a concentration of 50-100 ng/ul. which was used for further analysis (Fig.3.10).

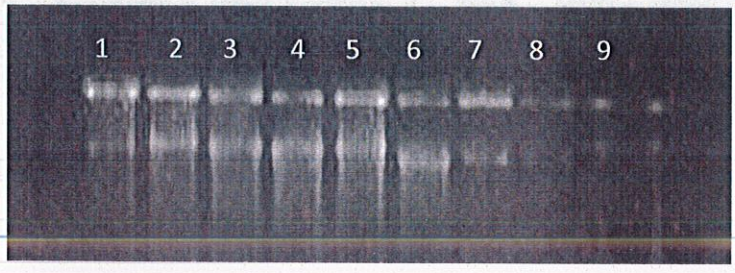


Fig.3.10 : Agrose gel electrophoresis showing purified high molecular weight *Prunus* sp. genomic DNA Lane 1 to 9; are the genomic DNA extracted from Sample 1- *P.mira* , Sample 2,9- *P.armnaica*, Sample 3- *P.nepulensis* , Sample 4,5 – *P.persica*,Sample 6 – *P.avium,* Sample 7- *P.domestica*, Sample 8 – *P.cerasifera.*

3.4 RAPD Result :

10 Primers were tested for RAPD study. Polymorphism was observed among the samples from the result so obtained (fig.3.11).

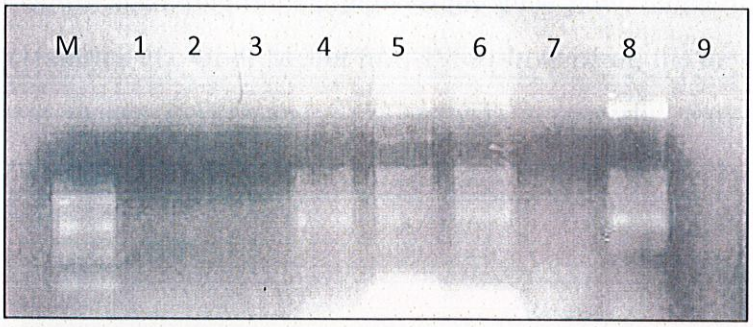


Fig. 3.11 : RAPD amplification products obtained from the *Prunus* sp studied. L1 to L10, Sample 1- *P.mira*, Sample 2,9- *P.armnaica*, Sample 3- *P.nepulensis* , Sample 4,5 – *P.persica* , Sample 6 – *P.avium,* Sample 7- *P.domestica*, Sample 8 – *P.salicinia.* M = 100bp λ DNA ladder.

3.5 SSR Result:

7 Primers were tested for SSR analysis. Monomorphism was observed among the samples from the result so obtained (Fig. 3.12).

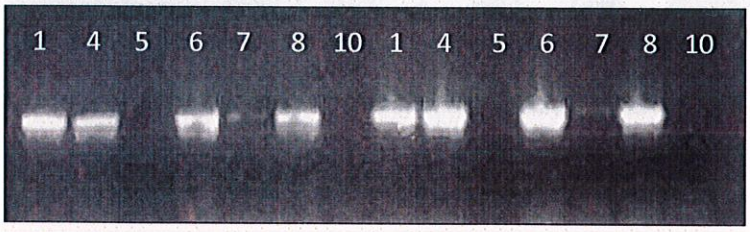
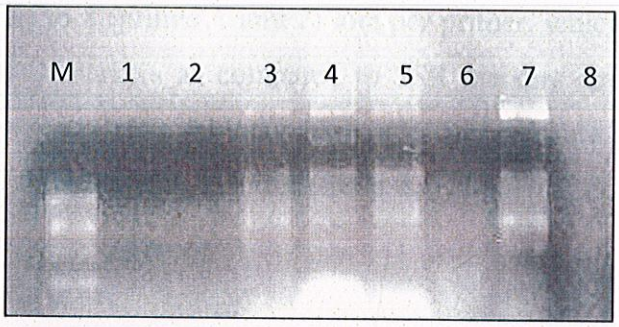


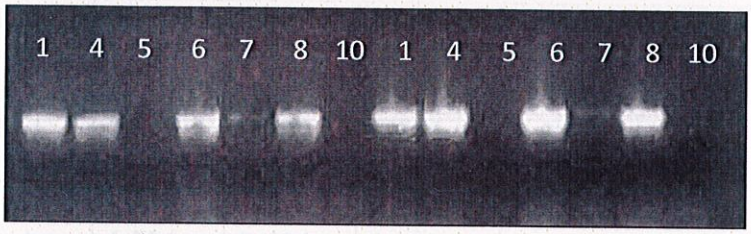
Fig.3.12 : SSR amplification products obtained from the *Prunus* sp. studied. L1 to L10, L1- *P.mira* , L 4,5 – *P.persica* , L6 – *P.avium,* L7- *P.domestica*, L8 – *P.salicinia* , L10- *P.cerasifera.*

3.6 Analysis of Genetic Variability

This study is the first application of SSR and RAPD markers to the assessment of genetic diversity in *Prunus sp.* Since the population genetic structure of a species is affected by multiple evolutionary factors including the mating system, gene flow, mode of reproduction and natural selection (Hamrick JL, Godt M J *et al.* , 1989) In general, the detection of high levels of polymorphism makes RAPD and SSR analysis a powerful tool for assessing genetic diversity in *Prunus sp.*



(a)



(b)

Fig. 3.13: Representative agarose gels showing different levels of polymorphism for coding regions in 7 *Prunus sp.* for primer OPX-20 for (a) and primers Avium 1 and Avium 2 for fig (b).

(a) RAPD loci and (b) SSR . The numerals represent the *prunus* species where Sample 1- *P.mira,* Sample 2,9 – *P.armnaica* , Sample 3- *P.nepulensis* Sample 4,5 – *P.persica* , Sample 6 – *P.avium,* Sample 7- *P.domestica*, Sample 8 – *P.salicinia,* Sample 10-*P.cerasifera.*
M: Lambda 100bp+ ladder.

Of 10 paralog eSSR primers tested on 7 *Prunus* species 7 were polymorphic, for all the polymorphic primers the number of amplified fragments ranged from 04 to 07 and the size of the amplified product ranged between 200-390 bp which yielded 52 fragments. The 7 polymorphic primers flanking SSR generated a total of 14 polymorphic loci with 2 loc1 per primer. The observed high proportion of polymorphic loci (93.33%) suggests that there is high genetic variation in *Prunus sp.* In contrast to the level of polymorphism observed with SSR marker, the 10 primer tested for RAPD analysis on the 7 *Prunus* species showed high degree of polymorphism (100%) and total of 58 polymorphic loci with allele frequency ranging from 3(primer 6) to 8 (primer 1 and 2) loci per primer, which may be attributed to the randomness of RAPD markers as compared to SSR markers that are developed from expressed part as expressed portion is under high selection pressure and highly conserved regions as compared to the intergenic regions (Scott KD, Eggler P *et al.* , 2000; Rungis D, Berube Y *et al.*,2004).

Table 3.1 : Summary of genetic variation statistics for all loci using RAPD, SSR, RAPD+SSR (combined) primers among the *prunus species*.

	Na	Ne	H	I	Ht	Polymorphic loci(PPL)	Gst
RAPD	**2.00 ± 0.0**	**1.686 ± 0.27**	**0.389 ± 0.10**	**0.573 ± 0.11**	**0.3899 ± 0.01**	**58 (100%)**	
SSR	**2.0 ± 0.0**	**1.721 ± 0.16**	**0.414 ± 0.05**	**0.603 ± 0.66**	**0.414 ± 0.00**	**14 (93.33%)**	
Combined	**2.0 ± 0.0**	**1.693 ± 0.25**	**0.394 ± 0.09**	**0.5789 ± 0.11**	**0.3946 ± 0.009**	**72(98.6%)**	**0.4476**

Na = Observed number of alleles; Ne = Effective number of alleles; H = Nei's gene diversity; I = Shannon's Information index; Ht = Total genetic diversity; Gst = Genetic diversity between population; PPL = percentage of Polymorphic Loci.

The respective details of Na, Ne, H, I, Ht, and PPL were studied for 7 *Prunus* species and were observed to be higher for species analyzed with SSR marker indicating more variability (Table 2). The Gst value 0.44 indicated that 55.24% of the genetic diversity resided within the population (Table 2). The overall percentage of polymorphic loci was 98.6 percent. In population genetics, a value of gene differentiation (Gst) >0.25 is generally regarded as the threshold quantity beyond which significant population differentiation occurs (Slatin M,1987).

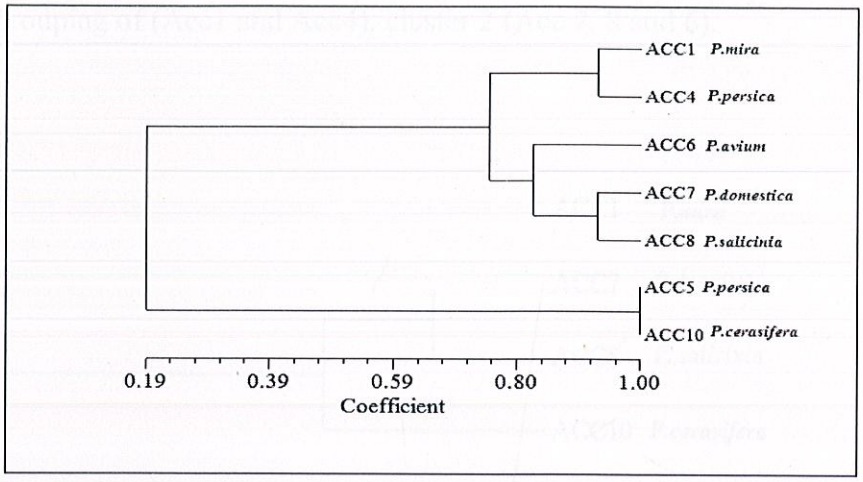


Fig. 3.14: Dendrogram illustrating genetic relationships among 7 genotypes of *Prunus* Sp. The dendrogram was generated by UPGMA cluster analysis calculated from 160 RAPD bands produced by 10 primers.

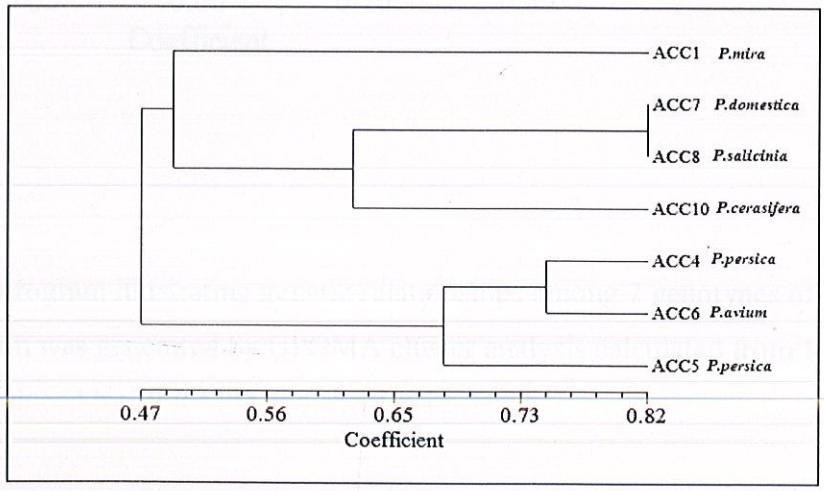


Fig. 3.15: Dendrogram illustrating genetic relationships among 7 genotypes of *Prunus* Sp. The dendrogram was generated by UPGMA cluster analysis calculated from 46 SSR bands produced by 10 eSSR primers.

3.7 Dendogram analysis for RAPD and SSR analysis

A dendogram based on UPGMA analysis grouped the 7 *Prunus sp.* into three main clusters with DICE similarity coefficient ranging from 0.45-0.86 (RAPD), 0.19-1.0 (SSR) and 0.47-0.82(RAPD+SSR) (Fig. 3.16). Using genetic data obtained from RAPD analysis a dendogram was generated and all the prunus species clustered in to 3 clusters where cluster 1 represents prunus sp (Acc1) while cluster 2 (Acc7,8 and 10) and cluster 3 (Acc 6,5 and4). In the case of SSR analysis the species again clustered in to 3 cluster where cluster 1 represented grouping of (Acc1 and Acc4), cluster 2 (Acc 7, 8 and 6).

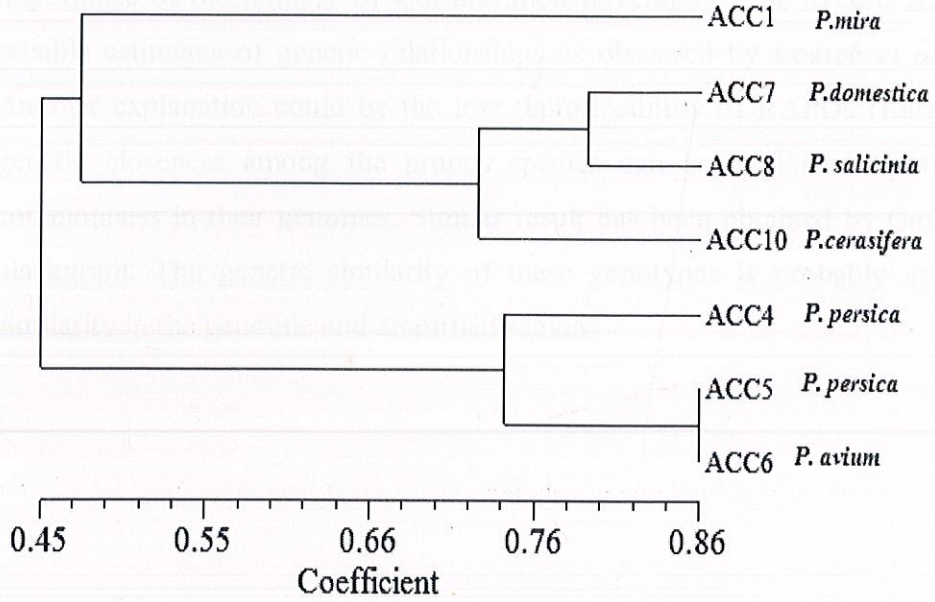


Fig.3.16: Dendrogram illustrating genetic relationships among 7 genotypes of *Prunus* sp. The dendrogram was generated by UPGMA cluster analysis calculated from 160 RAPD+46 SSR bands produced by 20 RAPD and eSSR primers.

3.8 RAPD and SSR Combined Data for Cluster Analysis

Based on combined data set of RAPD and SSR markers, the dendrogram obtained gave similar clustering pattern as obtained with RAPD analysis with Jaccard's similarity coefficient ranging from 0.47 to 0.82 (Fig. 3.16). Cluster I represents *prunus* species (Acc1) whereas cluster II and III represents (Acc7,8 and 10) and (Acc 6,5 and4) respectively. Other genetic variation studies were also performed on RAPD and ISSR combined data which are represented in different tables (Table 3.1). The differences found among the dendograms generated by RAPDs and ISSRs could be partially explained by the different number of amplified fragments analyzed (160 for RAPDs and 46 for SSRs) reinforcing again the importance of the number of loci and their coverage of the overall genome, in obtaining reliable estimates of genetic relationships as observed by Loarce *et al.* (1996) in barley. Another explanation could be the low reproducibility of RAPDs (Karp *et al.* 1997). The genetic closeness among the prunus species can be explained by the high degree of commonness in their genomes. Similar result has been obtained by Gaffor *et al.* (2001) in blackgram. The genetic similarity of these genotypes is probably associated with their similarity in the genomic and amplified region.

CONCLUSION

With this study, we can conclude that the molecular analyses of both RAPD and SSR markers were extremely useful for studying the genetic relationships of 9 *Prunus* sp collected from NBPGR, Himachal Pradesh region. The results indicates the presence of high genetic variability, which should be exploited for the breeding of *Prunus* from this region. Since no single, or even a few plants, will represent the whole genetic variability in *Prunus*, there appears to be a need to maintain sufficiently large populations in natural habitats to provide more information of species identification, matching hybrid combinations and genetic breeding. Based on polymorphic feature, genetic diversity and genetic similarity among the species of *Prunus* based on RAPD and SSR study, we recommend that any future breeding plans for this species should be specifically designed to include representative populations with the highest genetic variation for developing both high yielding and stable cultivars.

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