Studies of genetic diversity in the natural population of *Cassia fistula* L. using RAPD and ISSR markers

Dissertation submitted to Thapar University in Partial Fulfillment

for the award of

Master of Science in Biotechnology

By

Kajal Verma

301501006

Under the Guidance of

Dr. Anil Kumar

Associate professor

Thapar University, Patiala

July, 2017

DECLARATION

This thesis is a presentation of my original research work. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature, and acknowledgement of collaborative research and discussions.

The work was done under the guidance of Associate Professor **Dr. Anil Kumar** at Thapar University of Engineering and Technology, Patiala.

Kajal Verma

Kajal Verma

CERTIFICATE

This is to certify that the dissertation entitled **'Studies of genetic diversity in the natural populations of *Cassia fistula* L. using RAPD and ISSR primers'** is a bonafide record of independent research work done by **Kajal Verma** (Reg. No. 301501006) under my supervision and submitted to **Thapar University**, Patiala in partial fulfillment for the award of the Degree of Master of Science fin Biotechnology.

It is also certify that this thesis any other part of this thesis has never been submitted, neither in part nor in full to this university or any other university for the award of any degree.

Candidate sign:

Kajal Verma

Dr. Anil Kumar

Associate Professor

Department of Biotechnology

Thapar University, Patiala

ACKNOWLEDGEMENT

I would first like to thank my thesis supervisor **Dr. Anil Kumar**, Associate Professor, Department of Biotechnology, **Thapar University**, Patiala for his consistent valuable guidance, support, discussions and moral encouragement whenever I required.

I am thankful to **Dr. M. Ghosh**, Haed and Professor, Department of Biotechnology, **Thapar University** Patiala for her support whenever required. I am also thankful to Department of Biotechnology and TIFAC-CORE for providing infrastructure to do my experimental work required for thesis completion.

I would also like to thank **Ms. Amanpreet Kaur** research scholar in DBT and **Ms. Saloni Sharma** Phd. Scholar for their passionate participation and moral support during my research work.

Finally, its time to express my profound gratititude to my parents and friends for providing their unfailing support and continuous encouragement throughout my years of study and completing my project work on time. This accomplishment would not have been possible without them.

Kajal Verma

Date: July, 2017

Table of contents

Abstract

Cassia fistula L., family Fabaceae, is an important ornamental as well as medicinal plant. Morphologically populations show a great deal of variations. Therefore, the present study was conducted to study the genetic diversity among 12 accessions of *Cassia fistula* selected from population growing at Thapar University campus. Plants were selected on the basis of variation in flowering and morphological characters. PCR based molecular markers (RAPD and ISSR) were used to study the genetic diversity. Total 40 primers were screened of both the RAPD and ISSR markers out of which 11 primers (6 of RAPD and 5 of ISSR) were used for the analysis. According to the combined results of both the markers, total 40 marker bands were scored, out of which 27 were found to be polymorphic. According to this, high genetic diversity was recorded between the plants with distinct morphological and flowering characters. Individually ISSR revealed higher diversity (76%) than RAPD markers (67%). Thus in case of *Cassia fistula* ISSR were more useful markers than RAPD. *Cassia fistula* sample 12 (CF12) was found to be distantly related with all the other plant samples. The high level of diversity observed may be due to the mutations or changes occurred in the plant as a phenomena of adaptation to new location. It was noteworthy that genetic makeup of the plants plays an important role in deciding phenotypic traits such as flowering patterns and morphology of the plants. This study is a step to document the genetic diversity of this important medicinal plant.

List of Tables

List of figures

List of Abbreviations

M	Meter
Cm	Centimeter
Mm	Millimeter
Gm	Grams
Ml	Milliliter
µl	Microliter
DNA	Deoxyribonucleic acid
PCR	Polymerase Chain Reaction
CTAB	Cetyltrimethyl ammonium bromide
TE	Tris –EDTA
M	Molar
mM	Millimolar
w/v	weight per volume
Sec	Seconds
Ng	Nanogram
Nmole	Nanomole
µmole	Micromole
DNTP	Deoxynucleotide triphosphate
Taq	Thermus Aquaticus
min.	Minutes
ºC	degree Celsius
MVSP	Multivariate Statistical Package
UPGMA	Unweighted Pair-group Method using Arithmetic Averages
Ft	Feet
bp	base pair
Fig.	Figure
RAPD	Random amplified Polymorphic DNA,

SSR	Simple Sequence Repeats
STS	Sequence Tagged Sites
ISSR	Inter Simple Sequence Repeat
RFLP	Restriction Fragment Length Polymorphism
AFLP	Amplified Fragment Length Polymorphism

Chapter 1

Introduction

Cassia (genus), family Leguminosae (now Fabaceae) and sub family Caesalpinioideae is one of the twenty five largest genera of the world (Laxmikanta et al. 2010). *Cassia* species are distributed all over the world but is native to the adjacent regions of Southeast Asia and Indian subcontinent (Miraj 2016). It grows in wasteland as rainy season weed and is found in hills upto 1,200 meters in Himalaya. Till date, near about 600 species of *Cassia* has been identified (Tripathi and goswami, 2011). Since times of Ayurveda, species like *Cassia alata, Cassia tora, Cassia sophera* are used for the treatment of many diseases such as syphllis, malaria, tumour, skin diseases, diabetes, cardiac problems, leprosy etc. (Abirami et al. 2016, Rao and Suresh, 2015). Some wild species of *Cassia* such as *C .grandis, C. nodosa, Cassia tora* etc are also used as fodder legume. One important species of this genus, *Cassia fistula* (Amaltas) is well known for its therapeutic value and beautiful flowers (Choudhary, 2013). Common name of *Cassia fistula* is Indian laburnum. *C. fistula* holds drought tolerance as well as salt tolerance upto some extent. It is used as firewood at many places because of high biomass production rate. Flower of *C. fistula* is a state flower of Kerala having great importance in Vishnu festival. *C. fistula* grows well in tropical countries like Pakistan, West - China and Bangladesh (Bhalerao and Kelkar, 2012). It is commonly grown as ornamental garden plants. *C. fistula* also known as 'golden shower tree' is a fast growing medium size tree having height of about 10- 20 m and width of 5 – 10 m. Its leaves are pinnate with 4 – 8 pairs of leaflets having size of about 7 to 15 cm long and 2 – 7 cm broad. At the time of beginning of flowering, leaves remain absent (Joselin et al. 2014)).

Most of the leaves are shed in the month of April before blooming. Flowering starts in the month of April and blooms till the month of July. Flowers of *C. fistula* contain five petals having bright yellow color and length of 20 - 40 cm arranged in drooping racemes. Fruit is cylindrical pod of brown color about 40 – 70 cm long and 20 –27 mm in diameter containing black colored seeds. *C. fistula* has great pharmaceutical importance because every part of this plant is used in Ayurveda. Due to its therapeutic value, *C. fistula* is named as aragvadha (disease killer) since ancient times. The amount of protein and energy value in the pod pulp is higher than many canned, fresh and dried fruits. Souci et al. (1986) reported that nutrients like iron, manganese, zinc, calcium and potassium are present in fruit of *C. fistula* in much greater content than other fruits like apple, orange pear etc.

Classification

Kingdom	**Plantae**
Subkingdom	*Tracheobinota*
Superdivision	*Spermatophyta*
Division	*Mangoliophyta*
Class	*Magnoliopsida*
Subclass	*Rosidae*
Order	*Fabales*
Family	*Fabacae*
Genus	*Cassia*
Species	*fistula*
Domain	*Eukaryota*

Vernacular names

Urdu	*Amaltaas*
Bengali	*Soniyaar*
Oriya	*Sunaari*
Hindi	*Amultus, Sonhali*
English	*Golden shower*
Sanskrit	*Nripadruma*
Gujarati	*Garmala*
Arab	*Khyarsambhar*
Marathi	*Bahawa*
Kannada	*Kakkemara*
Punjabi	*Amaltaas, Kaniyaar, Girdnaale*

Figure 1: Morphological characters of *C. fistula* A) Mature golden shower tree in the month of May B) pinnate leaf C) Pentamerous yellow coloured racemeas of flowers

Along with the ornamental use, *C. fistula* has great importance as medicinal plant. The tree possesses various pharmacological properties and is used as antifungal, anti- inflammatory, antibacterial, anti-pyretic, anti-itching, purgative and laxative agent.

These activities are due to the presence of:

- anthraquinone derivatives (Kaji et al. 1968, Mukhupadhyay et al. 1998),
- polyphenolics containing flavonoids (Narayanan and Sheshadri, 1972; Gupta et al. 2000)
- a triterpene derivative (Misra et al. 1997),
- alkaloids (Asseleih et al. 1990),
- catechins and proanthocyanidins (Narayanan and Sheshadri, 1972; Gupta et al. 2000; Morimoto et al. 1988; Kashiwada et al. 1996).

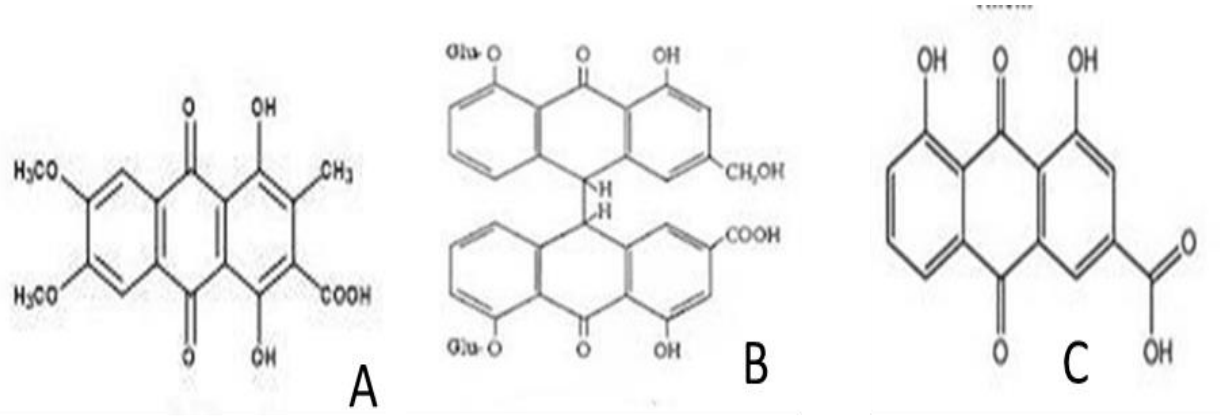

Figure 2: Structure of some anthraquinone derivatives present in *Cassia fistula* such as A) Fistulic acid B) Sennoside A, C) Rhein

All the parts of *C .fistula* possess some special properties and are used in treatment of many diseases. Medicinal value of tree parts are listed below:

1) Leaf: Leaves of *C. fistula* are used in the treatment of skin diseases, fever, dry cough and wound healing (Bhalerao and kelkar, 2012)

2) Bark: It is used in the treatment of diabetes, cardiac problems and leprosy.

3) Flowers: It is used in the treatment of general debility (lack of strength in the body), intermittent fever and cardiac problems (Kumar et al. 2017)

4) Pods : Pods of *C. fistula* are used to extract natural dye that is used for dyeing the mordant wool with iron, alum and copper salts (Khan et al. 2004). The coloring agent was named fistulic acid later. Also they contain a natural electrolyte that is used as coagulant to clarify the turbidity from industrial waste water.

C. fistula can also be used as a biosorbent (Hanif et al. 2007), potential source of food (Adebayo et al. 2004) and commercial source of gum (Kapoor and Farooqi 1993). The presence of these chemicals makes *C. fistula,* an important plant from medical point of view. Therapeutic and industrial properties of the tree are due to presence of various biochemical such as lupeol, glycosides, fistulic acid etc.

Few important chemical constituents present in various parts of *C. fistula* and these are listed below (Table 1)

Table 1: The chemical constituents present in various parts of *Cassia fistula*

Part	Chemical constituents
Stem bark	Lupeol, B - sitosterol, hexacosanol tannin
Leaves	Rhein and its glycoside sennosides A and B
Flowers	Ceryl alcohol, fistulin, rhein dianthroquinone glucoside
Seeds	Galactomannam composed of D - galactose and D – mannose
Plant	Seven bioflavanoids and two triflavanoids
Pod	Rhein glycoside and fistulic acid
Sap wood	Leucoanthicyanidin – 5, 4' – dihydroxy flavan
Bark and heart wood	Barbaloin and rhein, fistucacidin
Fruit pulp	Proteins, carbohydrates, arginine, leucine, methionine,phenylalanine

Source: (Choudhari, 2013)

C. fistula thought to be originated from India and Sri Lanka but is now pantropical (Bosch, 2007). Its propagation occurs naturally by seeds. In 1911, Troup conducted an experiment and found that golden jackals help in dispersal of seeds by feeding on them. In *C. fistula* pollination occurs through various pollinators such as bees and flies, due to which these plants are distributed all over the world. Plants when shifted from one place to another they have to adapt to the new habitat.

This leads to the change in genetic makeup of the population. If the gene pool is diverse there are more differences between the individuals within population. Mutation is the main cause of genetic diversity which helps the population to evolve and survive through adaptation to the changing environment. So, there is need to study the genetic diversity within and between populations.

Diversity can be studied through biochemical markers, morphological markers and molecular markers (Mohanty et al. 2006; Arya et al. 2011; Okusanya et al. 2016). But the use of biochemical and morphological markers is limited because they are affected by changing environmental conditions. Thus, molecular markers are mostly used as a promising tool to study the relationship between the individuals (Tripathi et al. 2012).

Several advantages of molecular markers are:

1) highlights the polymorphism in the nucleic acid sequences among different individuals.
2) are applicable to all parts of genome (exons, introns , and regulation regions)
3) do not exhibit pleotropic effects
4) some of them are codominant
5) ability to find polymorphism which do not produce phenotypic variation.

There are many types of molecular markers used to detect genotype identification, genetic diversity and genetic mapping such as Random amplified Polymorphic DNA (RAPD), Simple Sequence Repeats (SSRs microsatellites), Sequence Tagged Sites (STS), Inter Simple Sequence Repeat (ISSR), Restriction Fragment Length Polymorphism (RFLP), and Amplified Fragment Length Polymorphism (AFLP). Out of these, RAPD and ISSR are most preferred for studying population diversity because of low cost, quick and easier technique, not influenced by environmental conditions and requires no previous genomic information (Bansal et al. 2014; Tilwari e al. 2016)

RAPD stands for Random Amplified Polymorphic DNA. RAPD is based on PCR amplification and the DNA segments are amplified using random decamer primer. In this technique knowledge of the DNA sequence of target genome is not required. Depending on the positions complementary to the primers, amplification of DNA segments is carried out. No PCR product will be formed if mutation has occurred on the site that was previously complementary to the primer and gives a different pattern of amplified segments of DNA on the gel (Kumar et al. 2007).

ISSR stands for Inter Simple Sequence Repeat. It is based on amplification of DNA fragments between simple sequences repeats (SSR). Simple sequence repeats are found in eukaryotic genome and are repeating sequences. These repeating sequences contain short motifs about 2–6 base pairs long. For generating ISSR markers, microsatellites are used because these are highly variable and evenly distributed across the genome. In short, ISSR is based on variation in the DNA regions between satellites. ISSR includes PCR amplification of DNA fragments present between two adjacent or identical microsatellites having opposite orientation. Multiple fragments of different size that are distributed in genome randomly are amplified using microsatellite sequence as single primer (Mondini et al.2009).

In the present study RAPD and ISSR markers were used to study the intra population diversity among the plants of *Cassia fistula* L from the populations growing at Thapar university, Patiala.

Objectives

- ✓ To study genetic diversity in population of *Cassia fistula* L. at Thapar university using molecular markers.

- ✓ To establish the phylogenetic relations among the plants of *Cassia fistula* L. population growing at Thapar University campus, Patiala.

Chapter 2

Review of literature

Cassia fistula L. is a species belonging to the genus *Cassia* and family Fabaceae (Bhalerao and Kelkar, 2012). In addition to the ornamental importance of the tree, it has many medicinal properties (Tilwari et al. 2016). This makes *C. fistula* an important plant for industrial applications. In *Cassia* species propagation occurs through seed by golden jackals (Troup, 1911). Because of seed dispersal and pollination by pollinators these species grows at different locations. Whenever the plants evolve and adapt to geographic locations it becomes necessary to study genetic variations for the conservation. *C. fistula* was first described by Linneaus in 1737 and confirmed in 1955 by DeWitt (Irwin and Barneby, 1982). Name "*Cassia fistula"* was originated from Greek name *Casia* or *Kassia* for a fragrant and aromatic plant (Irwin and Barneby, 1982). Linneaus used the term *Cassia* as generic term in which all plants were included having same type of herbal and medicinal uses (Royal Botanic Gardens Kew, 2014). Due to non-distinguishable features of some plants between closely related species in the same genus, problems like misidentification and misinterpretation arises (Linneaus, 1753). Specially this problem appears in the large genera like *Cassia* where wide range of similarities are present in the individuals of the same species. De Candolle (1816) wrote a monograph of *Cassia* in which generic delimitations of *Cassia* were interpreted. After that Bentham (1871) introduced more comprehensive revision of *Cassia* genus, DeWitt (1955) followed the concept of Bentham for the plants of Malaysia.

A more detailed classification was given by Irwin and Barneby (1981, 1982) because they realized complexity and diversity between the species of *Cassia*. Irwin and Barneby (1982) also raised the level of classification by introducing the sub tribe *Cassinae* which contains three genera i.e *Senna, Chamaecrista* and *Cassia*. This concept of Irwin and Barneby got wide acceptance in the world specially in Africa (Lock, 1989) and Malaysia (Larsen and Hou, 1996). The very first work was done by Baker. In the book "Hooker's Flora of British India" 20 species of *Cassia* are enumerated. Afterwards from the year 1903 to 1929, numerous species of *Cassia* present in upper Gangetic plains were enlisted. There are many reports on regional floras in which the species of *Cassia genus* are listed from different parts of country. But in India not much work has been reported on taxonomy of species of *Cassia* genus. In the book "Legumes of India" Sanjappa (1992) has compiled the species of genus *Cassia*.

Studies carried out on diversity are important to the taxonomists in classifying the closely related plants. In *Cassia*, diversity studies has been carried out by using morphological markers (Okusanya et al. 2016), biochemical markers (Mohanty et al. 2006) and molecular markers (Mohanty et al. 2010; Rao and Suresh, 2015; Tilwari et al. 2016). RAPD and ISSR markers are mostly used to study the genetic variations in many plants including *Cassia* species (Hamza et al.. 2009; Kaur et al. 2017). These techniques are preferred due to less requirement of information about genome sequence and cost effectiveness (Abirami et al. 2016). Apart from molecular variations due to geographic locations, it has been reported that morphological behavior of the plant also correlate with genetic variations (Okusanya et al. 2016).

Genetic diversity studies have been carried out on different species of *Cassia* by various researchers (Kumar et al. 2007; Tilwari et al. 2016; Cunningham et al. 2002).

Describing the studies on genetic diversity, Raj et al. (2011) carried out a study on 5 different accessions of *Cassia angustifolia* in Tirunelveli district of Tamil Nadu using RAPD markers. It was reported that for calculating intra and inter-population diversity of *Cassia angustifolia*, RAPD technique was quite useful. RAPD markers revealed high degree of polymorphism between these accessions even by using limited number of primers.

Jimoh et al. (2013) did a study on the genus *Senna*, to check diversity in six accessions collected from Nigeria using RAPD markers. In this study closeness of species was revealed and high genetic diversity was reported between these accessions. In genus *Senna*, this study proves RAPD a powerful tool to check genetic diversity.

The use of RAPD markers in studying the genetic diversity is well described (Raj et al. 2011; Jimoh et al. 2013). Cunningham et al. (2002) carried out a study on *Cassia brewsteri* alongwith some other species of *Cassia* to check intraspecific and interspecific diversity between accessions using RAPD markers. In total 35 accessions were collected from different locations of Australia to perform study and results revealed high level of variation at genome level. It was reported that diversity increases with the geographic distances. Also rich interspecific diversity between all the accessions of *Cassia brewsteri* was found.

The study was followed by Kumar et al. (2007) where genetic diversity between 19 accessions of *Cassia fistula* collected from Uttar Pradesh and Uttaranchal were evaluated using RAPD markers. Wide range of diversity was exhibited by these accessions. There could be two reasons for variations; 1) these species have large mutation rate and large effective population size due to longer generation time. 2) or may be due to interspecific gene flow between other species of *Cassia*.

Another study was carried out by Mohanty et al. (2010) to check genetic diversity in 28 wild species of fodder legume belonging to *Cassia (C. grandis, C.nodosa, C.tora)* using RAPD, ISSR and SSR markers. In this study it was reported that there is a correlation between molecular markers and geographical origin of plants. It was reported that there is variation in the species which are closely related and also inter-specific phylogenetic relations were derived. It was further reported that these variations can be a result of mutations that originated when species were adapting to the changing environment for survival. This leads to the physiological polymorphism and diversity in the characteristics of species e.g. flowers, growth conditions, leaf, pod etc.

A study establishing the relationship between geographic location and molecular patterns of plant was published in 2011 (Arya et al. 2011) In this study different accessions of *Cassia occidentalis* were used to check intra-specific genetic diversity using RAPD markers. Accessions were taken from different districts of Haryana to study genetic diversity. Results revealed that there was close relation between genetic diversity of plants and their geographic distribution.

This analysis showed extreme genetic diversity in the species of *C. occidentalis* present in different environment and low diversity in same or adjacent regions. It was reported that as geographic distances increases diversity also increases.

Rao and Suresh (2015) also reported the genetic variations of few edible *Cassia* species (*C. tora* and *C. sophera*) from Telangana and Karnatka states by the use of RAPD markers. The plants that were taken from different states and they were found to exhibit physical distances and intraspecific genetic variability. This may be helpful in suitable characterization of plants. It was revealed that plants having similarity in their morphology exhibited genetic variation.

In contrary to above mentioned reports, a study in 2016 showed that there is no co-relation between geographic location and molecular variation (Tilwari et al. 2016). Recently Tilwari et al. (2016) conducted study to check genetic diversity between 13 different accessions of *Cassia tora* Accessions were collected from different geographic regions of Madhya Pradesh and RAPD markers were used for studying genetic variations. This analysis revealed that there is no correlation between the clusters obtained and geographical origin which favors the theory of common origin of all the species of *Cassia tora*. Also high level of diversity was observed between all the accessions. But accessions of Harda and Indore of Madhya Pradesh were found to be more diverse from all of the accessions.

Similar study was carried out by Abirami et al. (2016) using 29 different accessions of *Cassia fistula*. In addition to geographic locations, morphological features of the plant are also known to be affected by genetic variations (Okusanya et al. 2016). Genetic diversity was studied using flowering phenology by Okusanya et al. (2016). In this study various accessions of *Cassia fistula* growing in Nigeria were observed for three years to study the flowering behavior. It was reported that there was variation in the time of flowering between all the accessions and this indicates significant diversity and also effect of environmental factors mainly temperature and rainfall on the flowering patterns. The cause of difference in the flowering pattern is not well known. Less genetic diversity was observed between the accessions which shows that the accessions were planted at the same time or the accessions were from the same source.

A similar study to the present study was carried out to study the diversity among 15 *B. monerri* accessions using ISSR and RAPD markers. (Tripathi et al. 2012; Bansal et al. 2014). Also morphological characters were studied to establish a link between molecular and morphological data. The results revealed high level of diversity individually by both the analyses. Also it was reported that morphological analysis more or less follow molecular data and morphological characterization and is not a useful tool to find genetic diversity. Molecular markers proved to be a useful tool over morphological markers.

Apart from using molecular markers and morphological markers genetic diversity can also be studied using chromosome and 4C nuclear DNA analysis. Mohanty et al. (2006) carried out a study to find intraspecific genetic diversity in 15 accessions of *Cassia fistula* using chromosome and 4C nuclear DNA analysis (C = is a constant representing amount of DNA contained within a haploid nucleus of eukaryotic organisms). Analyzing the Karyotype of all the accessions intraspecific genetic variability in the number and positions of secondary constrictions was observed. It was found that all the species have symmetric karyotype but different in chromosome size. In annual species of *C. fistula* total chromosome length is greater than perennial species which implies that annual species of *C. fistula* might be originated from perennial species.

Chapter 3

Material and methods

Plant material

Cassia fistula trees growing at Thapar University campus were selected randomly for study. Selected trees were labeled (1 – 12) by cutting a small portion of bark. The location data of these trees is mentioned in the Table 2.

Genomic DNA isolation

Genomic DNA was isolated from freshly collected leaf samples of 12 selected plants of *Cassia fistula* using CTAB method (Doyle and Doyle 1990). According to the method, 1gm of sample was crushed in 10 ml CTAB buffer and incubated at 60^0C for 1hour after adding 200µl β-mercaptoethanol. After incubation equal volume of Chloroform:Isoamyl alcohol (24:1) was added to slurry by gently shaking. Supernatant was transferred to fresh tube and centrifuged (10,000xg; 10 min). Then DNA was precipitated by adding 0.66 volume of isopropanol and incubated for 1 hour at -20^0C. After centrifugation (10,000 xg; 15 min) supernatant was discarded and pellet was dissolved in 1 ml TE buffer. Then samples were treated with 2µl RNAse (preheated) followed by incubation at 37^0C for 1 hour. Equal volume of Phenol:Chloroform was added to the vial and after centrifugation aqueous phase was retained. Then added 0.3 volume of 3M sodium acetate and 0.6 volume of Isopropanol and incubated for 1 hour at -20^0C. After centrifugation at 10,000 rpm pellet was retained and after washing with 70% ethanol it was dissolved in TE buffer and stored at -20^0C for further use.

Composition of CTAB buffer :

2% CTAB	20 gm CTAB
20mM	40 ml EDTA stock (0.5M)
100mM TRIS-Hcl	100 ml Tris-Hcl stock (1 M)
1.4 M Nacl	280 ml Nacl stock (5M)

Make upto 1 litre with water. pH-7.5 – 8.0, and autoclave + 0.2% Mercaptoethanol.

Quantification and Quality check of DNA

Isolated genomic DNA was subjected to separation on ethidium bromide stained agarose gel (0.8 % w/v) to check the purity of sample. Purified samples were quantified using Nanodrop 1000 Spectrophotometer (Thermo Scientific).

Standardization of amplification

For standardization, temperature and time was optimized by varying the annealing temperature from 35^0C to 45^0C for RAPD and from 55^0C to 60^0 for ISSR. Also the time was optimized by varying time from 45sec to 55sec. The temperature at which maximum amplification was observed was selected for the amplification.

Testing of primers

In order to select primers for RAPD / ISSR yielding maximum no. of bands in *C. fistula*, DNA from sample no.1 was used as template for amplification with 20 ISSR primers and 20 RAPD primers.

Amplification was performed using 50ng DNA, 10nmole primer, 100 µmole DNTP, 1U Taq polymerase.

Study of genetic diversity using RAPD / ISSR markers

Amplification of 12 DNA sample of *Cassia* was carried out using PCR - Veriti 96 well Thermocycler (Applied Biosystems). The selected primers are used for amplification each of RAPD and ISSR using the standardized amplification protocol.

Standardized amplification protocol followed is given below:-

Initial denaturation = 94^0C for 4 min.

Denaturation (41 cycles) = 94^0C for 1 min

Annealing = 40^0C for RAPD and 55^0C for ISSR for 45 sec.

Extension = 72^0C for 1.30 min

Final extension = 72^0C for 5 min.

Amplified fragments were visualized on 1% (w/v) agarose gel stained with ethidium bromide and results were documented under UV trans-illuminator (Gel Doc Mega; Biosystematica, USA).

Data scoring and analysis

From Gel doc system pictures of gels were used to score the data. The bands of amplified fragments were changed into binary characters (1) to show the presence and (0) to show the absence of bands and a matrix was made. The matrix was used to find similarity between plants using Jaccard's coefficient (Bansal et al. 2014)

This coefficient gives value in the range of 0 to 1. The value close to 1 shows greater similarity. Formula of Jaccard's coefficient :-

$$\text{Jaccard's coefficient} = \frac{a}{a + b + c}$$

Where, a = bands present in both the samples (A and B)

b = bands present in sample A only

c = bands present in sample B only

Construction of phylogenetic tree

After data scoring and analysis phylogenetic tree was constructed using the UPGMA (unweighted pair-group method using arithmetic averages) method in the MVSP software. On the basis of data entered, Multivariate Statistical Package 3.2.1 (MVSP; Kovach Computing Services, Anglesey, Wales) makes the dendrogram. This dendrogram gives information about phylogenetic relationships and diversity between all the accessions of *Cassia fistula*.

Comparison between similarity index and flowering behavior

All the accessions of *Cassia fistula* were observed daily in the month of May to note the flowering characteristics. Also other parameters like height, date of flowering initiation, leaves, fruit and geographical location were also recorded by observing all the accessions. The flowering behavior of all the accessions of *Cassia fistula* was compared with the data obtained from molecular markers.

Chapter 4

Results

Different plants of *Cassia fistula* were selected on the basis of difference in the morphology of the tree and flowering patterns. The plants that seemed to be distinct from other plants were selected. Flowering was observed daily till the blooming. Also other parameters like fruits, leaves, bark, height at the time of flowering were observed. It was observed that the selected plants showed flowering at different times i.e in one plant there was initiation of flowering, while in other plant not even a single flower bud was present. It was also observed that most of the plants flower in the absence of leaves. This indicates rich diversity between the plants on the basis of recorded data. Also high diversity was observed on the basis of other parameters that were recorded at the time of flowering e.g. height, bark, fruit etc. The selected plants were screened using molecular markers for genetic diversity. After that similarity index was calculated on the basis of molecular marker data. Similarity index was calculated using Jaccard's coefficient to find similarity between sample sets. It was calculated in the range of 0 to 1. More the value closer to 1 more is the similarity between sets. If the value is closer to 0 it shows that the two sets are unrelated. Then data obtained from the flowering pattern (Table.2) was compared with the results of the molecular marker analysis and it was concluded that genetic makeup of the plants plays an important role in deciding the morphology and flowering patterns of the plants.

Table 2: Variation in pattern of flowering and morphology of selected trees of *C. fistula*

Sample no.	Date of flowering initiation	No. of fruit(pods)on each plant	Leaves at the time of beginning of flowering	Plant height	latitude	Longitude
CF1	8-5-17	1 pod	new leaves were appearing	upto 10 ft	$30^0$21’13’’N	$76^0$21’58’’E
CF2	3-5-17	No	less leaves	upto 15 ft	$30^0$21’09’’N	$76^0$21’59’’E
CF3	3/5/17	No	less leaves and starting of leaves	30 ft approx.	$30^0$21’19’’N	$76^0$21’58’’E
CF4	5-5-17	No	Many	12 ft approx.	$30^0$21’15’’N	$76^0$21’57’’E
CF5	5-5-17	2-3 pods	Less (new appearing)	upto 10 ft	$30^0$21’5’’N	$76^0$21’51’’E
CF6	5-5-17	No	less	upto 15 ft	$30^0$21’09’’N	$76^0$22’10’’E
CF7	1-5-17	4 pods	less leaves	upto 10 ft	$30^0$21’09’’N	$76^0$22’11’’E
CF8	1-5-17	Many pods	less amt. of leaves	15 ft. approx.	$30^0$21’08’’N	$76^0$22’10’’E
CF9	6-5-17	8-10 pods	Many leaves	upto 15 ft	$30^0$21’05’’N	$76^0$22’12’’E
CF10	3-5-17	No	3-4 leaflets	15 ft. approx.	$30^0$21’04’’N	$76^0$22’14’’E
CF11	6-5-17	No	less leaves	10 ft approx.	$30^0$21’04’’N	$76^0$22’14’’E
CF12	28-4-17	6 pods	2-3 leaflets	upto 15 ft	$30^0$21’04’’N	$76^0$22’14’’E

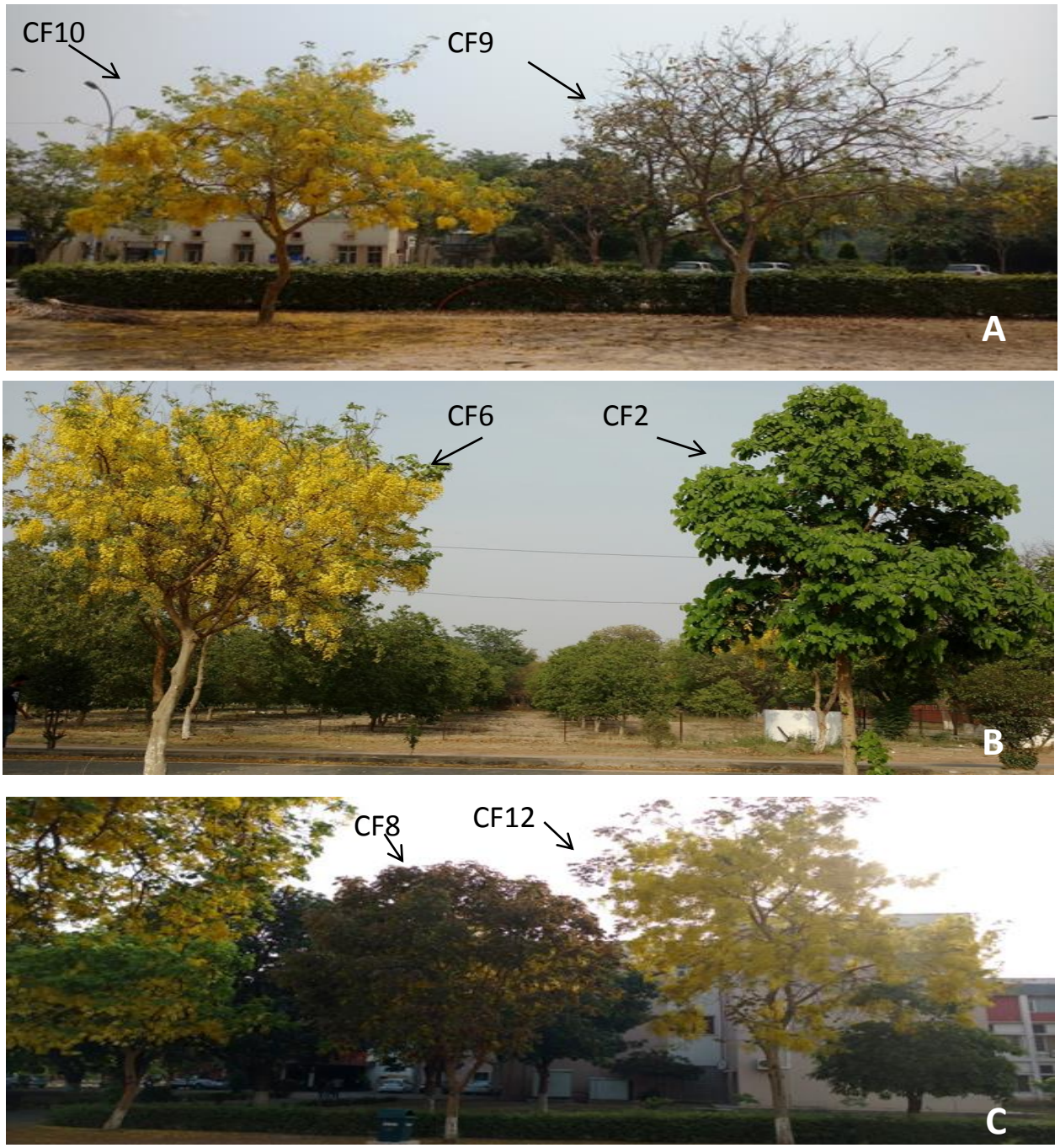

Figure 3: Variation in flowering and other morphological features of selected accessions of *Cassia fistula* (A) accession CF 9 and CF10 (B) accession CF6 and CF2 (C) accession CF8 and CF12

Table 3: List of RAPD and ISSR primers used for genetic diversity study of *Cassia fistula* samples along with sequence and degree of polymorphism

S. no	Primer code	Sequence of Primer	Amplicon size (bp)	No. of amplified bands	No. of polymorphic bands	% polymorphism
1	ISSR 2	$(GA)_8CG$	250 – 750	5	3	60
2	ISSR 3	$(GA)_8$ TC	400 – 700	3	3	100
3	ISSR 4	$(AC)_8GCGC$	100 – 450	4	3	75
4	ISSR 7	$(CA)_8GC$	100 – 500	3	2	66.7
5	ISSR 8	$(GA)_8$ TA	100 – 750	3	2	66.7
6	RAPD 3	AGCGCCATTG	1000	2	1	50
7	RAPD 12	GTGTGCCCCA	450 – 1000	4	3	75
8	RAPD 18	GAGAGCCAAC	300 - 1000	4	3	75
9	RAPD 29	GGGTAACGCC	500 – 1000	3	2	66.7
10	RAPD 30	GTGATCGCAG	300 – 800	4	4	100
11	RAPD 33	CAGCACCCAC	300 – 1000	5	2	40

*DNA for diversity study was isolated from selected samples showing high degree of variation in flowering pattern

RAPD fingerprinting

Total 20 primers of RAPD were screened out of which 6 primers were selected to find diversity between 12 accessions of *C. fistula*. All the six primers produced a total of 22 marker bands out of which 15 marker bands were polymorphic. The size range of amplified bands was in range of 250 bp to 1050 bp. Maximum number of bands (5 and 4) were observed with RAPD primer 33 and RAPD primer12. The Jaccard's similarity coefficient based on RAPD revealed that similarity value among accessions ranges from 0.312 to 0.882, inferring that these are genetically similar at moderate level. Maximum value of similarity was recorded between accessions CF1 and CF2. Dendrogram was made from RAPD data (Fig. 4). In the dendrogram all the accessions of *C. fistula* were grouped into two major cluster that were further divided into small sub clusters. In one of the cluster, there were only two accessions CF6 and CF7 that shows these are more similar, and in another cluster there were four sub clusters of all the other remaining accessions which shows diversity between these accessions. CF6 and CF9 were found to be highly diverse exhibiting the lowest value of similarity (0.312). CF7 was also found to be diverse from CF9. But the accessions CF11 and CF3 were showing the maximum value of similarity which is 1 which shows that these are genetically same having no diversity.

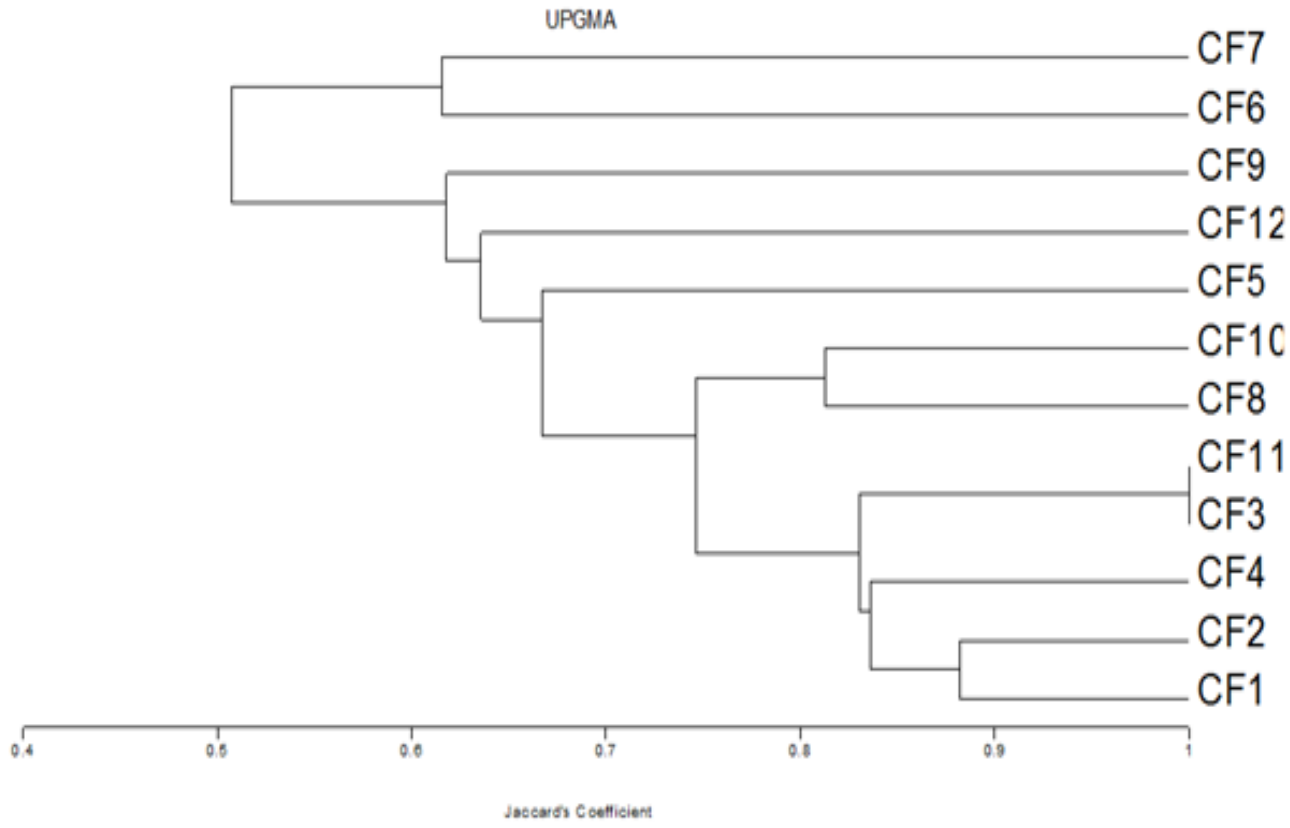

Figure 4: Unweighted pair group method with average (UPGMA) cluster based on Jaccard's coefficient calculated from RAPD data of various accessions of *Cassia fistula*

Table 4: Similarity relationship between different selected samples of *Cassia fistula* calculated using Jaccard's Coefficient from RAPD molecular marker data

	CF1	CF2	CF3	CF4	CF5	CF6	CF7	CF8	CF9	CF10	CF11	CF12
CF1	1.000											
CF2	0.882	1.000										
CF3	0.823	0.882	1.000									
CF4	0.875	0.833	0.834	1.000								
CF5	0.75	0.823	0.631	0.684	1.000							
CF6	0.534	0.470	0.470	0.470	0.534	1.000						
CF7	0.647	0.667	0.647	0.612	0.647	0.615	1.000					
CF8	0.705	0.65	0.764	0.667	0.612	0.467	0.625	1.000				
CF9	0.647	0.611	0.612	0.631	0.588	0.312	0.368	0.687	1.000			
CF10	0.764	0.778	0.823	0.667	0.667	0.437	0.556	0.812	0.647	1.000		
CF11	0.823	0.882	1	0.834	0.667	0.411	0.612	0.764	0.612	0.823	1.000	
CF12	0.667	0.684	0.723	0.65	0.5	0.476	0.473	0.526	0.556	0.647	0.723	1.000

*CF1-12 refers to *cassia fistula* sample 1 to 12

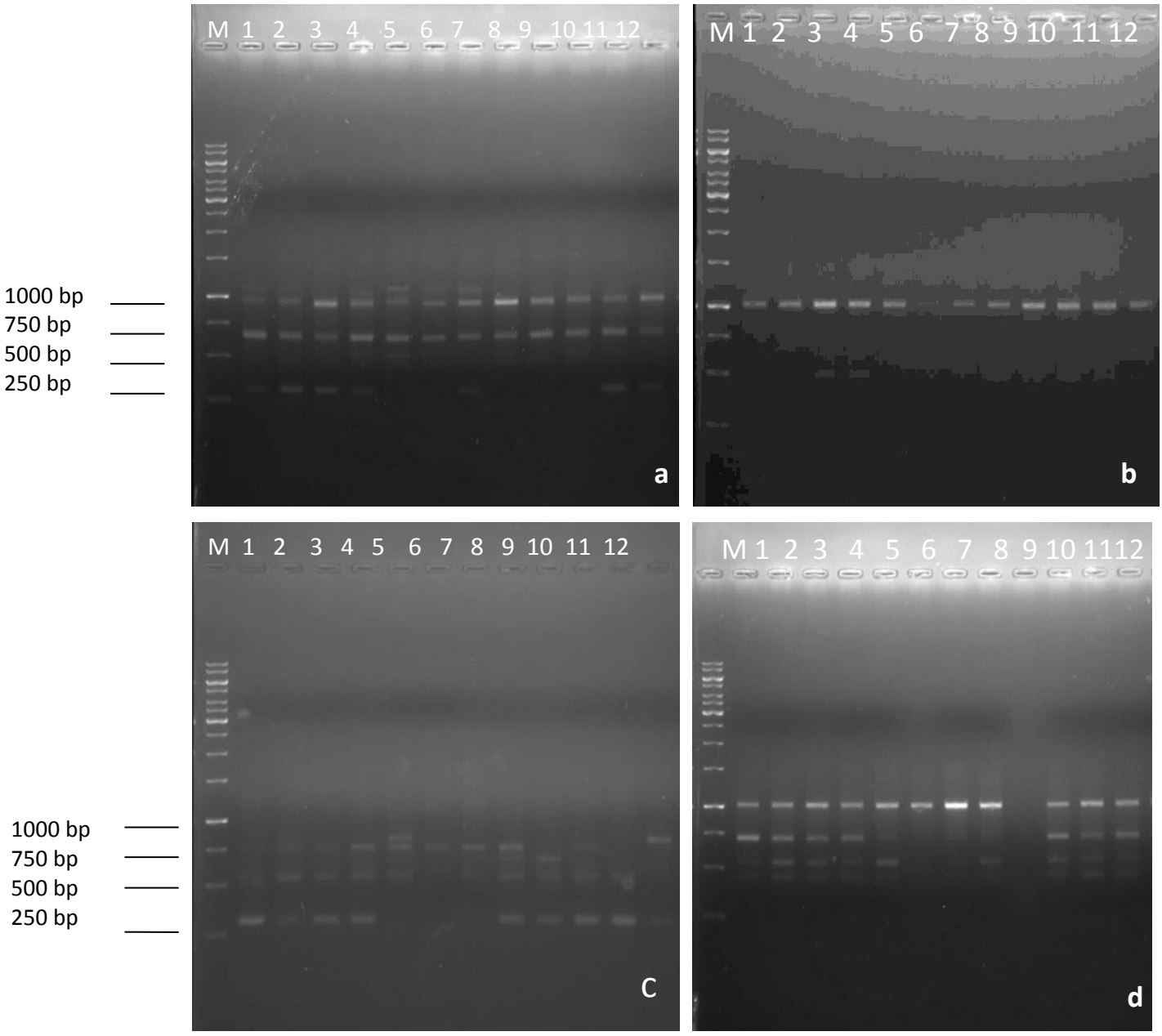

Figure 5: RAPD analysis of 12 accessions of *Cassia fistula* revealing diversity in 12 selected samples growing at Thapar University campus. Diversity was studied on the basis of bands amplified using primers (a) primer 33 (b) using primer 29 (c) using primer 30 (d) using primer 12 (Lanes: M: Ladder of 1 kb, 1-12: Selected samples of *Cassia fistula*

ISSR fingerprinting

A total of 18 marker bands were produced from 5 primers that were selected for amplification of 12 accessions of *Cassia fistula* out of which 13 marker bands were polymorphic. The size range of marker bands varies from 100 bp to 750 bp. Maximum no. of bands (5 and 4) were observed in ISSR primer 2 and ISSR primer 4. The Jaccard's coefficient value of 12 accessions of *Cassia fistula* obtained from ISSR data revealed that the similarity value ranges from 0.266 to 0.937 inferring the high genetic similarity between these accessions. Maximum value of similarity (0.937) was recorded between accession CF4 and CF10. Dendrogram was made from ISSR data (Fig.6). All the accessions were divided mainly into two groups, one group having three small clusters of all the accessions and the other group consists only one accession (CF12). This indicates that CF12 is more diverse from all accessions. But high level of diversity was observed between CF2 and CF12 (0.266). After CF2, CF3 and then CF1 was found to be diverse from CF12 which indicates that there was high level of similarity between these accessions. This shows that ISSR proves to be more informative tool than RAPD for assessing genetic diversity in *C. fistula*.

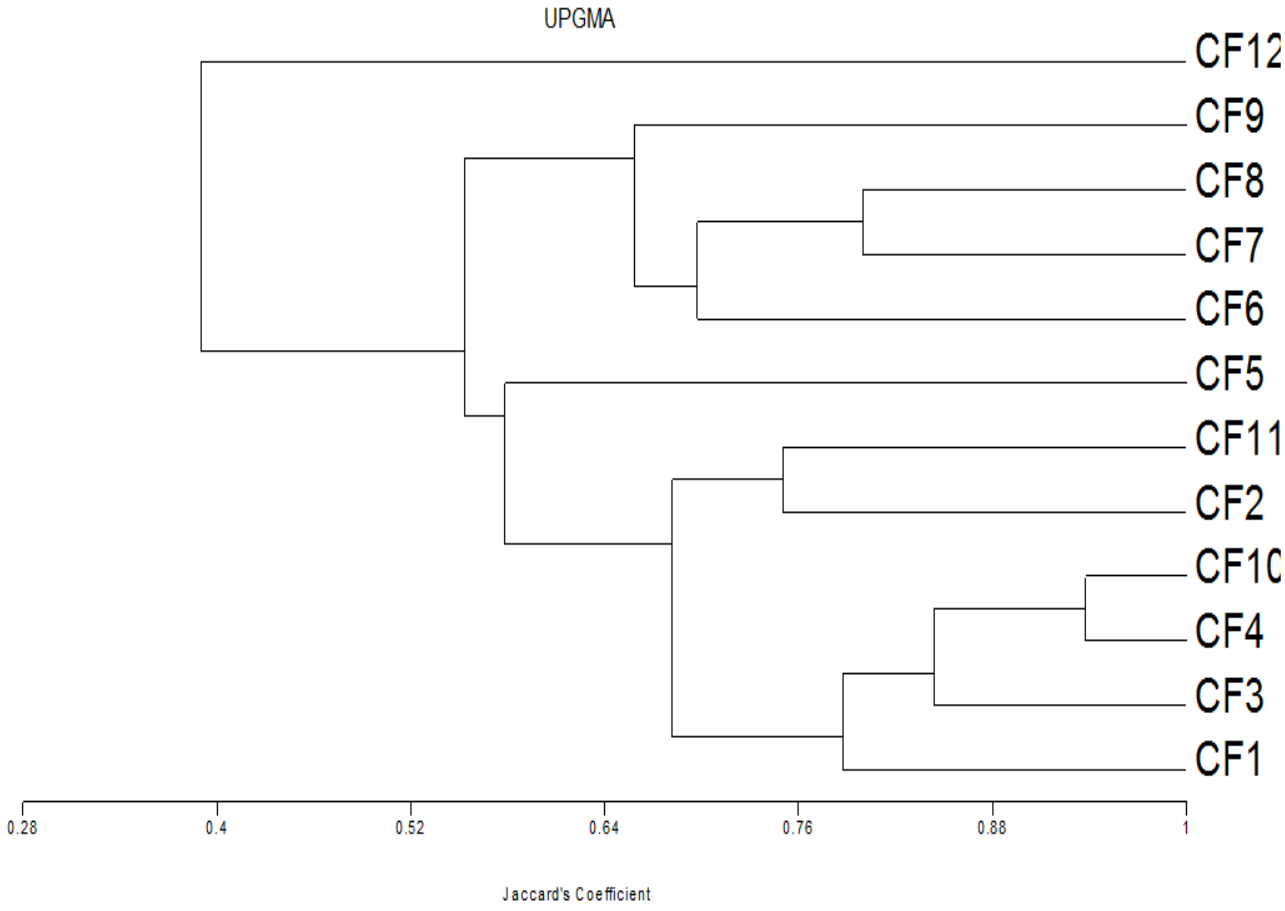

Figure 6: Dendrogram obtained from ISSR profiles from different species of *Cassia fistula*

Table 5: Similarity relationship between different selected samples of *Cassia fistula* calculated using Jaccard's coefficient from ISSR molecular marker data

	CF1	CF2	CF3	CF4	CF5	CF6	CF7	CF8	CF9	CF10	CF11	CF12
CF1	1.000											
CF2	0.714	1.000										
CF3	0.800	0.785	1.000									
CF4	0.812	0.687	0.875	1.000								
CF5	0.555	0.444	0.647	0.722	1.000							
CF6	0.600	0.375	0.470	0.529	0.529	1.000						
CF7	0.666	0.615	0.625	0.647	0.470	0.833	1.000					
CF8	0.647	0.647	0.705	0.736	0.631	0.667	0.800	1.000				
CF9	0.437	0.400	0.600	0.529	0.533	0.583	0.667	0.667	1.000			
CF10	0.750	0.733	0.812	0.937	0.666	0.583	0.588	0.75	0.500	1.000		
CF11	0.642	0.750	0.600	0.625	0.470	0.400	0.533	0.470	0.428	0.666	1.000	
CF12	0.333	0.266	0.312	0.352	0.467	0.500	0.461	0.428	0.416	0.375	0.416	1.000

*CF1-12 refers to *cassia fistula* sample 1 to 12

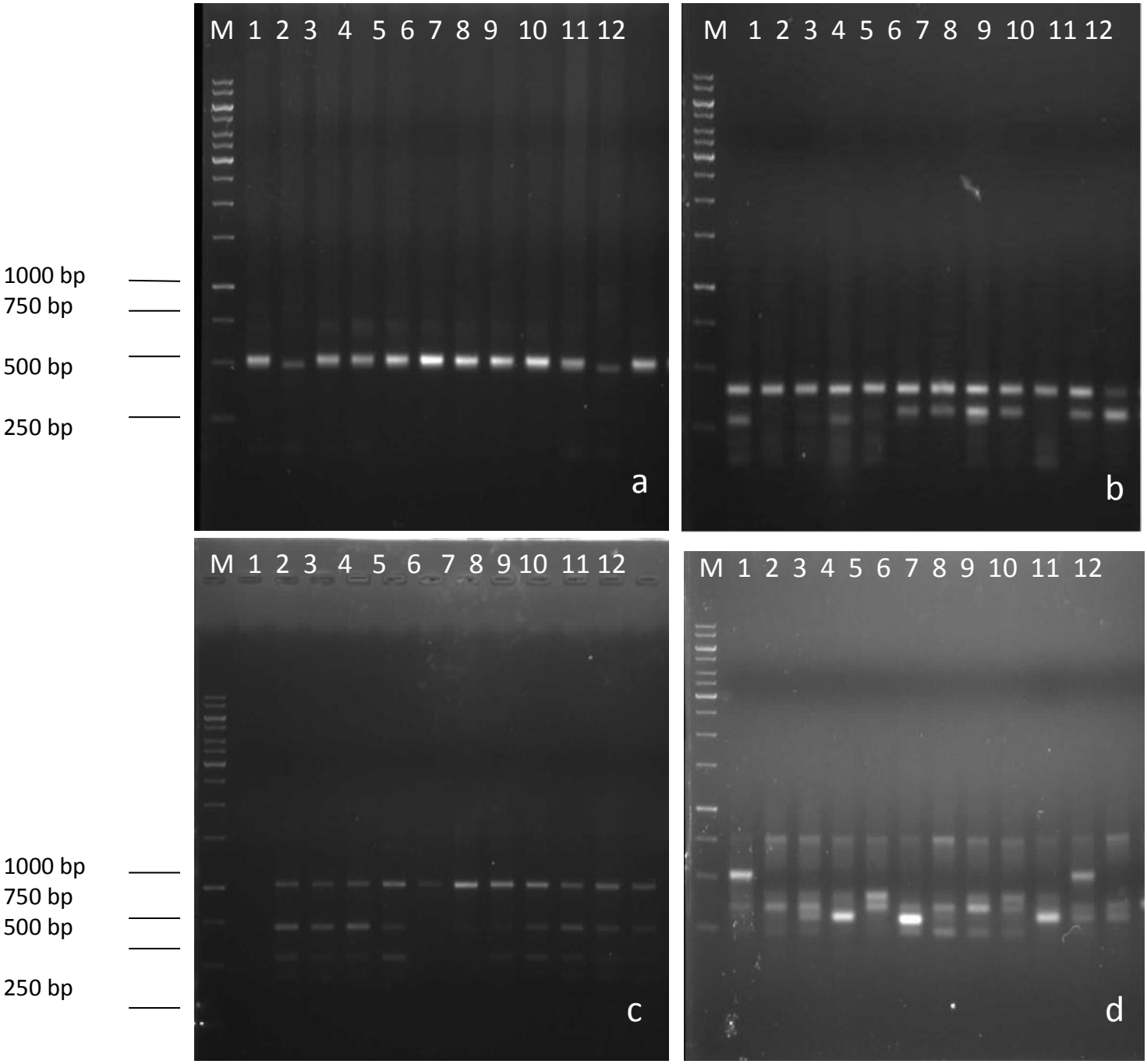

Figure 7: ISSR analysis of 12 accessions of *Cassia fistula* revealing diversity in 12 selected samples growing at Thapar University campus. Diversity was studied on the basis of bands amplified using primers (a) primer 7 (b) using primer 4 (c) using primer 8 (d) using primer 2 (Lanes: M: Ladder of 1 kb, 1-12: Selected samples of *Cassia fistula)*

Combined results of RAPD and ISSR fingerprinting

RAPD and ISSR molecular markers were used for amplification of 12 accessions of *Cassia fistula*. Amplification was performed using all the DNA samples and amplified fragments were visualized on agarose gel alongwith marker. The size of the fragments was measured using the gene ruler and the bands produced were found to be polymorphic. Bands produced by the genome of one individual but absent in other individual shows polymorphism. RAPD and ISSR bands were scored as 0 (absence of band) and 1(Presence of band) and this data was placed in rectangular matrix. This matrix was used to calculate similarity index. Then cluster analysis was performed using the UPGMA (unweighted pair-group method using arithmetic averages) method in the MVSP software. Using the combined data of both ISSR and RAPD, total 40 primers were screened – 20 primers of both RAPD and ISSR. Primers were screened using DNA sample of one of the accession of *C. fistula*. Total 11 primers were selected because these primers gave amplification and sequence of the selected 11 primers is given in the (Table 3). On the basis of amplification these eleven selected primers from which 5 primers were of ISSR markers and 6 of RAPD markers were used for analysis. RAPD and ISSR produces total of 40 marker bands from which 27 were polymorphic. The size of bands ranges from 100 bp to 1050 bp. Maximum no. of bands (5) were observed in RAPD Primer 33 and ISSR Primer 2. Jaccard's coefficient was calculated to find the similarity value among all the accessions. Jaccard's coefficient was calculated between all the accessions. If the value of Jaccard‟s coefficient comes higher it represents accessions are highly similar and if value of coefficient is low that represents the accessions are less similar to one another. Maximum value of similarity (0.852) is recorded between accession CF3

and CF4 which shows that these are more similar to each other. The Jaccard‟s coefficient value ranges from 0.406 to 0.852 (Table 6) Combined results of RAPD and ISSR are shown in dendrogram (Fig.8). In the dendrogram CF12 forms an outgroup separately that means it was more diverse than all the accessions of *Cassia fistula*. The accessions CF11 and CF6 are found to be more diverse as they are showing lowest value of similarity (0.406). After CF11, CF2 is showing diversity from CF6. Then small clusters were formed between all the accessions which shows diversity between these accessions. Also similarity was observed between the accessions. CF4 and CF3 were found more similar and CF1 was similar to these two accessions. Small clusters between CF11 and CF2, CF6 and CF7 shows that these accessions are also very much similar. As CF12 is diverse from all the accessions by forming an outgroup but is attached to all the sub clusters which means that CF12 is unrelated with all the accessions. All the other accessions are related with each other which shows that all these plants belongs to common ancestors.

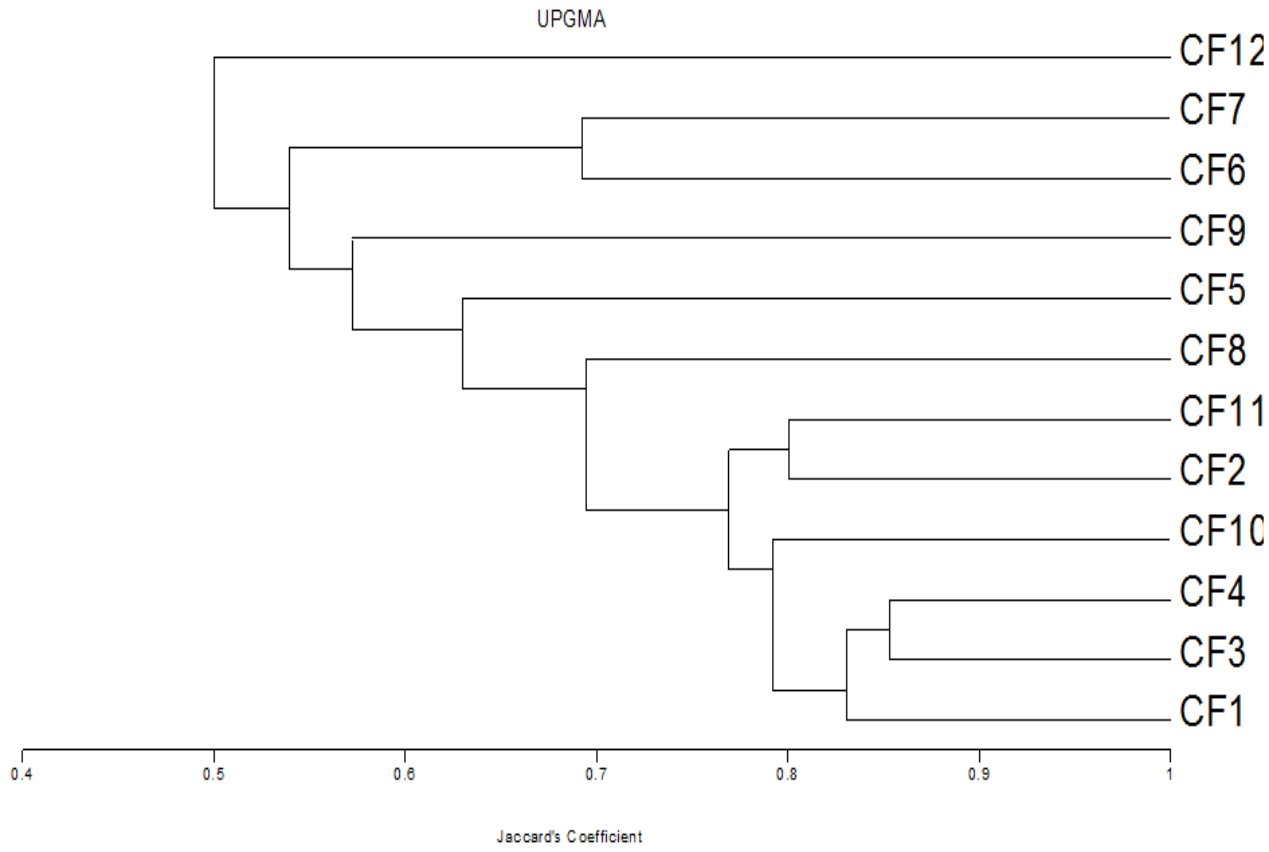

Figure 8: Dendrogram obtained from ISSR and RAPD combined data from different species of *Cassia fistula*

Table 6: Similarity relationship between different selected samples of *Cassia fistula* calculated using Jaccard's coefficient from ISSR and RAPD combined molecular marker data

	CF1	CF2	CF3	CF4	CF5	CF6	CF7	CF8	CF9	CF10	CF11	CF12
CF1	1.000											
CF2	0.806	1.000										
CF3	0.800	0.806	1.000									
CF4	0.848	0.742	0.852	1.000								
CF5	0.611	0.600	0.621	0.702	1.000							
CF6	0.586	0.424	0.441	0.515	0.531	1.000						
CF7	0.677	0.645	0.617	0.687	0.571	0.692	1.000					
CF8	0.676	0.647	0.735	0.702	0.657	0.500	0.709	1.000				
CF9	0.563	0.531	0.606	0.600	0.575	0.464	0.500	0.700	1.000			
CF10	0.781	0.806	0.818	0.781	0.657	0.428	0.576	0.787	0.558	1.000		
CF11	0.741	0.827	0.806	0.735	0.556	0.406	0.593	0.606	0.531	0.774	1.000	
CF12	0.500	0.515	0.545	0.527	0.485	0.484	0.468	0.457	0.531	0.500	0.600	1.000

*CF1-12 refers to *cassia fistula* sample 1 to 12

Chapter 6

Discussion

Cassia fistula is highly important medicinal plant in ayurveda. So it is important to maintain genetic diversity and to conserve the plants *in situ*. To preserve the genetic diversity of natural population, conservation of medicinal plants is very important (Karthikeyan et al. 2011). As the level of genetic diversity in plants is mainly related with the life history of species and breeding system. So, for the management and conservation of plants there is need to know about the genetic structure (Zanella et al. 2011). For this purpose this study was performed to find genetic diversity between accessions of *C. fistula*. In this study, 12 accessions of *Cassia fistula* on the basis of morphological differences were collected from different locations of Thapar university. Different morphological parameters like height, bark, fruit of the tree, flowering initiation were recorded. On the basis of data obtained from morphological characteristics, it was observed that all the accessions exhibited high variability among the morphological characters. As there was difference in the date of flowering initiation, also there was significant variation in the height, bark, fruit, etc. It was also observed that some plants have many leaves at the start of flowering but some plants do not have even a single leaf, also some plants were still shedding their leaves at the time of flowering. But it is clear that most of the plants started flowering during leafless period. This difference represents the diversity in the plants since they all belong to common ancestors. This may be due to mutations occurred in the plants when these were brought to the present location. Due to change in location, plants have to adapt according to the changing environment for survival. This could be one of the reason for observed diversity amongst accessions.

To find genetic diversity selected plants were screened using PCR- based molecular markers. RAPD and ISSR markers were used for this purpose. It has been proved earlier that these markers are more useful for accessing genetic diversity in different species of *Cassia* genus.(Arya et al. 2011; Bansal et al. 2014; Tilwari et al. 2016; Jimoh et al. 2013).

In the present study, RAPD and ISSR primers produced 27 polymorphic bands that grouped 12 accessions of *Cassia fistula* into small clusters. In ISSR fingerprinting (Fig.6) CF12 is placed separately which shows that it is more diverse than all the accessions. All the remaining accessions are divided into three main clusters which are further divided into small sub clusters. CF4 and CF10 are more similar and these are similar to CF3 and and also to CF1. But CF12 is more diverse from all the accessions. High level of diversity is observed in ISSR analysis.

In RAPD analysis there are two main clusters in which CF6 and CF7 are joined together and forms an outgroup. It means CF6 and CF7 are more closer but diverse from all the accessions that is why placed as an outgroup. This cluster is related to CF9 and then to CF12, CF5 and to small clusters. CF11 and CF3 are joined together which shows that both are closely related and there is no diversity between these two accessions. But in combined data of both markers again CF12 is placed separately which shows it is diverse from all accessions and this is related to four main clusters. Also high level of diversity is observed in combined data of both the markers (Fig.8).

In the present study there is high level of genetic diversity observed between all the accessions which may be due to mutations that are common in species and also due to environmental factors (Rodriquez et al. 1999, Alam et al. 2009). This molecular analysis reveals common origin for all the accessions of *Cassia fistula* because all the accessions are joined together which shows that all the accessions are related to each other. This high level of diversity indicates adoption of the plant to the new environment where it can be conserved and propagated easily. The diversity calculated by ISSR (76%) is much higher than diversity detected by RAPD data (64%). This difference is because of the reason that these markers target different parts of the genome (Gajera et al. 2010). This study proves ISSR more powerful tool than RAPD for accessing genetic diversity as ISSR reveals more diversity than RAPD.

The present study reveals very high genetic diversity than the earlier reports on *Cassia fistula* and other species of *Cassia* genus (Kumar et al. 2007, Mohanty et al. 2010). In earlier reports RAPD is proved as a more useful tool for assessment of genetic variations (Kumar et al. 2007, Raj et al. 2011, Jimoh et al. 2013). But contrary to these studies, present study proves ISSR a useful tool for calculating genetic variations in *C. fistula* because ISSR reveals very high genetic diversity (76%) than RAPD markers. In this study very high polymorphism (more than 50%) was observed among the accessions of *C. fistula*, indicating diverse genetic base of all the accessions collected randomly from different locations in Thapar university campus.

The results of the present study are in agreement with the earlier reports on *C. fistula* or different species of *Cassia* genus which shows significant variation among the different species of *Cassia* genus (Arya et al. 2011, Kumar et al. 2007, Jimoh et al. 2013). Various studies on assessing the genetic variation in plants using molecular markers had given the co-relation between geographical distance and genetic similarity (Islam 2004, Arya et al. 2011). Recently, Tilwari et al. (2016) carried out a study on medicinal plant of same genus (*Cassia tora*) and study reveals no co- relation between the geographical distance and genetic similarity. But, no such type of co- relation is observed in this study.

The results of the molecular fingerprinting were compared with the results of flowering data. According to the Table 2 of flowering data accession CF7 and CF8 are showing flowering initiation on the same date (1/5/17) and according to molecular marker data accession CF7 and CF8 (Fig. 7) are also showing more similarity. Then accession CF2, CF3 and CF10 are showing initiation of flowering at the same date (3/5/17) that means they are more similar with one another. These three plants are also showing more similarity with one another (Fig.8) according to the molecular marker analysis. Here some relation is established between data of both the flowering and molecular markers. After that the accession CF4, CF5 and CF6 are showing same date of flowering initiation. By comparing with data from molecular markers it is clear that accession CF5 and CF6 are closer but sample no. 4 is showing less similarity to accession CF5and CF6.

Here some divergence is seen between both the data but relation is also established to more extent. Then in accession CF9 and CF11 blooming occurs on the same data which means these two are more similar. Comparing with markers data accession CF9 and CF11 are showing similarity at moderate level. Sample CF1 is flowering on particular date (8/5/17) which means it is not similar to any other accession. Contrary to flowering data, in molecular data accession CF1 is showing similarity to all the accessions. Here no relation is established between both the data. Accession CF12 showed flowering on (28/4/17) which shows it is diverse from all the accessions and same is shown in molecular marker dendrogram as CF12 is forming an outgroup. Inspite of some divergence between both the markers and flowering data, wide range of similarity is observed between flowering data and molecular markers data. This concludes that genetic makeup of plants is responsible for deciding the morphology and flowering patterns. This study seems to be first study to establish the relation between flowering patterns and molecular markers in *Cassia fistula*. Similar study was carried out on *B. monerri* in which co-relation was found between the morphological data and molecular data (Tripathi et al. 2012). But there was very low co-relation found which infers that morphological analysis more or less follow molecular analysis. Same results were found in olive and almond cultivars (Mirali and Nabulsi 2003, Hagidimitriou et al. 2005). Contrary to these reports, in present study high co-relation was found between molecular data and morphological data. This implies that morphological characters are also useful tool for the assessment of genetic diversity in *Cassia fistula*.

This study was an attempt to find genetic diversity between the accessions of *Cassia fistula* selected randomly at Thapar university campus.

Chapter 7
Conclusion

The present study shows that RAPD and ISSR molecular markers are very useful to find diversity between *Cassia fistula* accessions which is important for the identification and conservation of species. Level of variations recorded by ISSR markers is higher as compared to RAPD markers. This proves ISSR a more informative tool than RAPD to find diversity between these accessions.

Higher level of diversity is observed in accessions of *Cassia fistula* that would be helpful in further detailed research to understand the reason of this diversity. The high level of diversity seen may be due to mutations, change in genetic structure, genetic drift, gene flow and mating system.

By comparing the data of molecular markers and flowering patterns a link was established between both the data and it was concluded that genetic makeup of the plants plays an important role in deciding phenotypic traits such as flowering patterns and morphology of the plants. Also very high diversity was observed between the accessions by analyzing all the other parameters of plants like height, bark, fruit etc.

It was also found that morphological characterization proved a useful tool for assessing genetic diversity in *Cassia fistula*.

Chapter 8

References

1. Abirami K, Singh DR, Simhachalam P, Baskaran V (2016) Genetic Diversity Analysis of Medicinal Plant *Cassia alata* in Andaman Island through ISSR Markers. Journal of Experimental Agriculture International Article no. 29275, 14:1-6
2. Adebayo OT, Fagbenro OA, Jagede T (2004) Evaluation of *Cassia fistula* meal as a replacement for soyabean meals in practical diets of *Oreochromis niloticus* fingerlings. Aquaculture Nutrition 10:99-104
3. Alam N, Shim MJ, Lee MW, Shin PG, Yoo YB, Lee TS (2009) Phylogenetic relationship in different commercial strains of *Pleurotus nebrodensis* based on ITS sequence and RAPD. Mycobiology 37:183-188
4. Arya V, Yadav S, Yadav JP (2011) Intra-specific Genetic Diversity of Different Accessions of *Cassia occidentalis* by RAPD Markers. Genetic Engineering and Biotechnology Journal 22:1-8
5. Asseleih LMC, Hernandez OH, Sanchez JR (1990) Seasonal variation in the content of sennosides in leaves and pods of two *Cassia fistula* populations. Phytochemistry 29: 3095–3099
6. Bansal M, Kumar A, Reddy MS (2014) Diversity among wild accessions of *Bacopa monnieri* (L.) Wettst. and their morphogenetic potential. Acta Physiol Plant 36:1177–1186
7. Bentham G (1871). A revision of the genus *Cassia.* Transactions of the Linnean Society London 27:503-593

8. Bhalerao S.A. and Kelkar T.S (2012) Traditional medicinal uses, phytochemical profile and pharmacological activities of *Cassia fistula* Linn. International Research Journal of Biological Sciences 1:79-84

9. Bosch CH, 2007. Cassia fistula L. Prota, 11(1): Medicinal plants/Plantes médicinales 1 [ed. by Schmelzer, G. H. \Gurib-Fakim, A.]. Wageningen, Netherlands: PROTA. http://database.prota.org/PROTAhtml/Cassia%20fistula_En.htm

10. Chaudhari MV (2013) Aragvadha (*Cassia fistula* L.):A phyto-pharmacological review. Journal of Ayurveda and Holistic Medicine 1:22-29

11. Cunningham CD, Walsh BK , Anderson RE, B, Harrison KD, Carroll JB (2002) Analysis of genetic diversity in *Cassia brewsteri* with Randomly Amplified DNA Fingerprints (RAFs). Australian Systematic Botany 15:237–244

12. De Wit HCD (1955) A revision of the genus *Cassia* (Caesalpinioideae) as occurring in Malaysia. Webbia 11:197-292

13. De Candolle (1816) ESSAI sur les proprieties medicales des plantes compares avecleurs extrieures et leur classification naturelle

14. Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissues. Focus 12:13–15

15. Gajera BB, Kumar N, Singh AS, Punvar BS, Ravikiran R, Subhash N, Jadeja GC (2010) Assessment of genetic diversity in Castor (*Ricinus communis* L.) using RAPD and ISSR markers. Industrial Crops and Products 32:491–498

16. Gupta M, Mazumder UK, Rath N, Mukhopadhyay DK (2000) Antitumour activity of methanolic extract of *Cassia fistula* L. seed against Ehrlich ascites carcinoma. Journal of Ethnopharmacoogy 72:151-156

17. Hagidimitriou M, Katsiotis A, Menexes G, Pontikis C, Loukas M (2005) Genetic diversity of major Greek olive cultivars using molecular (AFLPs and RAPDs) markers and morphological traits. Journal of the American Soceity for Horticultural Science 130:211–217

18. Hamza H, Rejili M, Elbekkay M, Fercichi A (2009) New approach for the morphological identification of date palm (*Phoenix dactylifera L*.) cultivars from Tunisia. Pakistan Journal of Botany 41: 2671–2681

19. Hanif MA, Bhatti NH, Nadeem R, Ziya K, Ali AM (2007). *Cassia fistula* (Golden shower): A Multipurpose Ornamental tree. Floriculture and Ornamental Biotechnology 1:20-26

20. Irwin HS, Barneby RC (1981) Recent advantages in legume systematics. Royal Botanic Garden, Kew 1:97-106.

21. Irwin HS, Barneby RC (1982) The American Cassiinae: a synoptical revision of Leguminosae tribe Cassieae subtribe Cassiinae in the New World. Memoirs of the New York Botanical Gardens 35:1-918

22. Islam A (2004) Genetic diversity of the genus *Curcuma* in Bangladesh and further biotechnological approaches for *in vitro* regeneration and long-term conservation of *C. longa* germplasm PhD. Thesis, University of Hannover

23. Jimoh, MA., Edeoga, HO, Omosun G (2013) DNA Fingerprinting of Six *Senna* Species and the Taxonomic Implication. International Journal of Advanced Research in Biotechnology 1:022-026

24. Joselin J, Florence AR, Brintha TSS, Jeeva S (2014) Secondary metabolites from ornamental flowers: A study of common avenue trees of the family *Caesalpiniaceae*. Journal of Chemical and Pharmaceutical Research 6:2089-2096

25. Kaji NN, Khorana ML, Sanghavi MM (1968) Studies on *Cassia fistula* Linn. Indian Journal of Pharmceutical Sciences 30:8-11

26. Kapoor VP, Faarooqi MIH (1993) *Cassia fistula* (Amaltas) seed a potential source of commercial gum. Research and Industry 38:3-7

27. Karthikeyan A, Madhanraj A, Pandian SK, Ramesh M (2011) Genetic variation among highly endangered *Bacopa monnieri* (L.) Pennell from Southern India as detected using RAPD analysis. Genetic Resources and Crop Evolution 58:769–782

28. Kashiwada Y, Toshika K, Chen R, Nonaka G, Nishioka I (1996) Tannins and related compounds XCIII occurrence of enantiomeric proanthocyanidins in the Leguminosae plants, *Cassia fistula* L.; *Cassia Javanica* L. Chemical and Pharmaceutical Bulletin 38: 888−893

29. Kaur A, Reddy MS, Kumar A (2017) Efficient, one step and cultivar independent shoot organogenesis of potato. Physiol Mol Biol Plants 23:461–469

30. Khan M, Khan MA, Srivastava PK, Mohammad F (2004) Natural dyeing on wool with Tesu (flame of the forest), Dolu (Indian rhubarb) and Amaltas (*Cassia fistula*). Colourage 51:33-38

31. Kumar A, Tripathi V and Pushpangadan P (2007) DNA marker-based genetic variation in *Cassia fistula* L. Indian Journal of Genetics and plant breeding 67:173-176

32. Kumar KA, Satish S, Sayeed I, Hedge K (2017) Therapeutic Uses of *Cassia fistula:* Review. International Journal of Pharma And Chemical Research 3:38-43

33. Larsen K, Hou D (1996) Caesalpiniaceae. Foundation of Flora Malesiana, 12:556-570, 673-691

34. Laxmikanta A, Mukherji AK, Pratap CP (2010) Validation of genetic status of different taxa in the subtribe cassinae (Leguminosae: Caesalpinoidae) using RAPD, ISSR and AFLP markers. International Journal of Plant Physiology and Biochemistry 2:18-28

35. Linnaeus C (1753) Species plantarum vol.1 Laurentii. Holmiae

36. Lock J.M (1988*) Cassia* senso lat. (Leguminosae-Caesalpinioideae) in Africa. Kew Bulletin 43:333-342

37. Lock J.M (1989) Legumes of Africa: a check-list. Royal Botanic Gardens

38. Miraj S (2016) *Cassia fistula* Linn: Evidence for pharmaceutical applications. Der Pharmacia Lettre 8:129-131

39. MirAli N, Nabulsi I (2003) Genetic diversity of almonds (*Prunus dulcis*) using RAPD technique. Scientia Horticulturae 98:461–471

40. Misra TR, Singh RS, Pandey HS, Singh BK (1997) A new diterpene from *Cassia fistula* pods. Fitoterapia LXVIII 58:375

41. Mohanty S, Das AB (2006) Interspecific Genetic diversity in 15 species of *Cassia* L. Evident by chromosome and 4C nuclear DNA analysis. Journal of Biological Sciences 6:664-670

42. Mohanty S, Das AB, Ghosh N, Panda BB, Smith WD (2010) Genetic diversity of 28 wild species of fodder legume *Cassia* using RAPD, ISSR and SSR markers: a novel breeding strategy. Journal of Biotech Research 2:44-55

43. Mondini L, Noorani A and Pagnotta MA (2009) Assessing Plant Genetic Diversity by Molecular Tools. Diversity 1:19-35

44. Morimoto S, Nonaka G, Chen R (1988) Tannins and related compounds. LXI. Isolation and structures of novel bi-and triflavonoids from the leaves of *Cassia fistula* L. Chemical and Pharmaceutical Bulletin 36:39−47

45. Mukhopadhyay M, Saha A, Dutta A, De B, Mukherjee A (1998) Genotoxicity of sennosides on the bone marrow cells of mice. Food and Chemical Toxicology 36:937–944

46. .Narayanan V, Seshadri TR (1972) Proanthocyanidins of *Cassia fistula*. Indian Journal of Chemistry 10:379–381

47 Okusanya OT. Shonubi OO. Bello, OA. and Bamidele J. F (2016) Variation in flowering phenology of *Cassia fistula* Linn. population in Ota, Ogun state, Nigeria. Ife Journal of Science 18:887-894

48 Raj TLS, A Britto A J, and Kumar PBJR (2011). RAPD – PCR fingerprint analysis of *Cassia angustifolia* Vahl. in Tirunelveli, International Journal of Biological Technology 2:17-20

49 Rao S, Suresh C (2015) Analysis of genetic differentiation of Few Edible *Cassia* Species Using RAPD Molecular Markers. International Journal of Pharmacy and Pharmaceutical Research 3:58-65

50 Rodriguez A, Oliver H, Zou H, Chen P, Wang X, Abrams JM (1999) Dark is a Drosophila homologue of Apaf-1/CED-4 and functions in an evolutionarily conserved death pathway. Nature Cell Biology 1:272-279

51 Royal Botanic Gardens Kew, 2014. Cassia L. genus. Kew Scientific Research & Data-Legumes of the World website. Richmond, UK: Royal Botanic Gardens Kew.

52 Sanjappa M (1992) Legumes of India. Publisher: Bishen Singh Mahendra Pal Singh, ISBN-8121100658

53 Souci SW, Fachmann W, Krant H, (1986) Food Composition and Nutrition, Tables 1986/1987. Wissens – chaftliche verlagsgesells chaft, Stuttgart, Germany, 1032 pp.

54 Tilwari A, Chauhan D, Sharma R and Singh RK (2016) Assessment of genetic variations among Medicinal plant *Cassia tora* from different geographic regions of Central India using RAPD Markers. Med Aromat Plants (Los Angel) 5:1-7

55 Tripathi N, Chouhan DS, Saini N, Tiwari S (2012) Assessment of genetic variations among highly endangered medicinal plant *Bacopa monnieri* (L.) from Central India using RAPD and ISSR analysis. 3 Biotech 2:327-336

56 Tripathi V, Goswami S (2011) Generic relationship among *Cassia L., Senna Mill* and *Chamaecrista Moench* using RAPD markers. International Journal of Biodiversity and Conservation 3: 92-100

57 Troup R.S (1911) Silviculture of Indian trees. Published under the authority of His Majesty's Secretary of State for India in Council, Oxford Clarendon Press

58 Zanella CM, Bruxel M, Paggi GM, Goetze M, Buttow MV, Cidade FW and Bered F (2011) Genetic structure and phenotypic variation in wild populations of the medicinal tetraploid species *Bromelia antiacantha* (Bromeliaceae). America n Journal of Botany 98:1511-1519