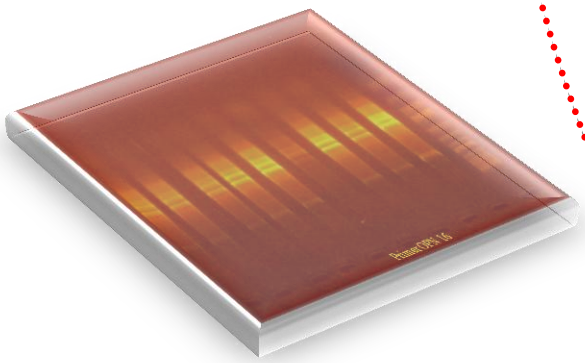

Final Report

On

Establishment of advance laboratory for molecular characterization and chemoprofiling of *Commiphora wightii* plant

Submitted to

Madhya Pradesh Biotechnology Council, Bhopal

Submitted by

Forest Genetics Plant Propagation & Biotechnology Division

STATE FOREST RESEARCH INSTITUTE
JABALPUR (M.P.) 482008

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(S.K. Tiwari)
Principal Investigator

Project Profile

Title of the project	:	Establishment of advance laboratory for molecular characterization and chemoprofiling of *Commiphora wightii*
Funding agency	:	Madhya Pradesh Biotechnology Council, Bhopal
Sanctioned	:	Oct. 2010 to October 2014
Project cost	:	Rs.10.85 lakhs
Executive Authority	:	Dr. G. Krishnamurthy, IFS, Director , State Forest Research Institute, Jabalpur (M.P.)
Principal Investigator	:	Dr. S.K.Tiwari, Scientist & Head Forest Genetics Plant Propagation & Biotechnology Division , State Forest Research Institute, Jabalpur (M.P.)
Co-investigator	:	Amit Pandey, Sr. Research Officer, Forest Genetics Plant Propagation & Biotechnology Division , State Forest Research Institute, Jabalpur (M.P.)
Equipment purchased	:	Gel Documentation System Geneview 645C, (Genetix)
Project Staff	:	nil

List of Abbreviations

µ	micron
µl	mililiter
^{0}C	Degree Celsius
cm	Centimetre
DNA	Dioxy Ribo Nucleic Acid
et al	et al (and others)
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
IAA	Indole acetic acid
IBA	Indole butyric acid
IUCN	International Union for Conservation Natural Resources
mg	milligram
ml	millilitre
mMol	milimole
mv	milli volt
nm	nanometre
pmols	picomole
RT	Retention Time

CHAPTER 1

INTRODUCTION

Introduction:

Uses of plants as a source of medicine has been an ancient practice and is an important component of the health care system in India. In the Indian systems of medicine, most practitioners formulate and dispense their own recipes, hence this requires proper documentation and research. In west also the use of herbal medicines is growing with approximately 40 per cent of population reporting use of herb to treat medical diseases within the past year. General Public, academic and government interest in traditional medicines is growing rapidly due to the increase side effects of the adverse drug reactions and cost factor of the modern system of medicine.

There are about 45,000 medicinal plant species in India, with concentrated spots in the region of Eastern Himalayas, Western Ghats and Andaman & Nicobar Island. The officially documented plants with medicinal potential are 3000 but traditional practitioners use more than 6000. India is the largest producer of medicinal herbs and is called the botanical garden of the world. There are currently about 250000 registered medical practitioners of the Ayurvedic system ,as compared to about 700,000 of the modern medicine system. In rural India, 70 per cent of the population depends on the traditional type of medicine, the Ayurveda. In India, many forms of alternative medicines are available for those who do not want conventional medicine or who cannot be helped by conventional medicine. Ayurveda and Kabiraji (herbal medicine) are two important forms of alternative medicine that is widely available in India.

Ayurvedic form of medicine is believed to be existent in India for thousands of years. With the scripts in the Atharva Veda, we have evidence of a traditional use of medicinal plants that is more than 3000 years old. It is estimated that about 80,000 species of plants are utilized in some form or other by the different systems of Indian medicine.

Out of thousands of plants used medicinally, some of them are recognized and classified as Rare, Endangered and Threatened (RET) medicinal plants depending on their threat status. The RET medicinal plants are rare, endangered and threatened forest species, that are grown and multiplied naturally in restricted areas. These RET medicinal plant species play a very important role as folk remedies against many diseases. Globally, about 10 per cent of the known flowering plants are threatened with extinction. Similar estimates have been made for India also. A study made on

Peninsular India, has assigned various threat levels for about 108 species of plants (Gowda et al., 1997). International Union for Conservation of Nature and Natural Resources (IUCN) headquartered at Gland, Switzerland categorizes different species depending on the amount of threat they are facing. The species which is work out under this project is also comes under the threat category.

The Alkaloids: Alkaloids are a group of naturally occurring chemical compounds, that contain mostly basic nitrogen atoms. This group also includes some related compounds with neutral and even weakly acidic properties. Some synthetic compounds of similar structure are also attributed to alkaloids. In addition to carbon, hydrogen and nitrogen, alkaloids may also contain oxygen, sulphur and more rarely other elements such aschlorine, bromine, and phosphorus. Alkaloids are produced by a large variety of organisms, including bacteria, fungi, plants, and animals, and are part of the group of natural products(also called secondary metabolites). Many alkaloids can be purified from crude extracts by acid-base extraction. Many alkaloids are toxic to other organisms. They often have pharmacological effects and are used as medications, as recreational drugs, or in entheogenic rituals. Examples are the local anesthetic and stimulant cocaine; the psychedelic psilocin the stimulant caffeine, nicotine the analgesic morphine, the antibacterial berberine, the anticancer compound vincristine, the antihypertension agent reserpine; the cholinomimeric galantamine, the spasmolysis agent, the vasodilator vincamine, the anti-arhythmia compound , the anti-asthma therapeutic ephedrine, and the antimalarial drugquinine. Although alkaloids act on a diversity of metabolic systems in humans and other animals, they almost uniformly invoke a bitter taste. The boundary between alkaloids and other nitrogen-containing natural compounds is not clear-cut. Compounds like amino acid peptides, proteins, nucleotides, nucleic acid, amines, and antibiotics are usually not called alkaloids. Natural compounds containing nitrogen in three xocyclic position (mescaline, serotonin, dopamine, etc.) are usually attributed to amines rather than alkaloids. Some authors, however, consider alkaloids a special case of amines. Guggulsterone E and Z are the predominant alkaloids in *Commiphora wightii*.

Chemoprofiling

As already pointed out, the chemical composition of plants may vary to some extent and needs to be standardized to guarantee comparable therapeutic effects. A number of chromatographic fingerprinting analyses are known to disclose the detectable ingredients composition and concentration distribution. Standard analytical techniques include thin-layer chromatography, high-performance liquid chromatography and capillary electrophoresis are now available for detecting the active compound. Recently, novel technological developments became available for chemoprofiling, such as infrared spectroscopy, metabolic fingerprinting and quantitative determinations based on nuclear magnetic resonance spectra.

The scientific basis of evidence-based medicine is still poor in herbal medicine. For the integration of herbal medicine into western medicine, internationally accepted standards are necessitated, including quality control of herbal products as well as preclinical and clinical evidence of safety and efficacy. In recent years, thriving innovative technologies emerged in phytotherapy research, e.g. DNA-based technologies for the authentication of plant species, good practice guidelines for standardized experimentations. High-quality herbal materials should undergo rigorous examination by analytical techniques for chemoprofiling of medicinal herbs, as well as toxicological methods to detect contaminations. The state of the art, which is necessary for the pharmaceutical use of medicinal herbs, is documented in monographs of national and international pharmacopeias. The thriving advances in molecular biology also affect phytotherapy research, which benefits from novel technologies of systems biology. Herbal recipes are frequently comprised of complex mixtures of different plant species, and even within one and the same species, the composition and amount of chemical compounds can considerably vary, depending on exogenous (e.g. climate, soil composition and altitude) and endogenous factors (e.g. genetics, epigenetics). Selection of chemical markers is crucial for the quality control of herbal medicines, including authentication of genuine species, harvesting the best quality raw materials.

Several methods are available for the identification of chemomarkers such as HPLC , HPTLC etc. The contents of these and other biologically active compounds may vary depending upon place to place due to variation in agroclimatic zones, environment, genotype, time of collection of plant material, etc.

Importance of genetic diversity assessment:

Collection and conservation of germplasm is gaining importance all over the world with an aim to utilize these genetic resources in the ongoing as well as future plant improvement programmes. A number of techniques are now available for germplasm characterization, depending on the need, type and nature of the species. A number of molecular marker systems are now available for germplasm evaluation and classification. Use of DNA markers in germplasm characterization has now been well established and these markers are commonly used in a wide variety of species which can be used for assessing the genetic diversity within and between the populations (Williams *et.al*., 1990). PCR (Polymerase Chain Reaction) technique usually refers to amplification of specific regions of genomic DNA. Utility of RAPD (Randomly amplified polymorphic DNA) markers for genetic mapping has been clearly shown in a number of plant species (Carlson *et.al*. 1991). After scoring of the DNA bands the population/s/genotype/s which shows greater degree of polymorphism can be used for further genetic improvement programme.

About the species

***Commiphora wightii* (Guggul)**

***Commiphora wightii* (Arnott) Bhandari** is an important medicinal plant of herbal heritage of India. In Indian languages, it is known by various names like guggul in Hindi, gukkulu and maishakshi in Tamil, guggulu in Sanskrit and Indian bdellium in English. This plant is distributed in arid areas of India, Bangladesh and Pakistan. In India it is found in arid, rocky tracts of Rajasthan Gujarat and Madhya Pradesh.

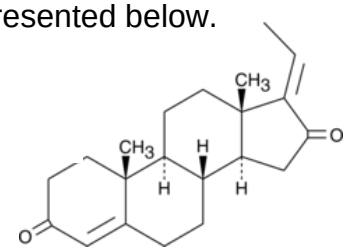
Systematic position of *C. wightii*

Kingdom	Plantae
Phylum	Tracheophyta
Class	Magnoliopsida
Order	Sapindales
Family	Burseraceae
Genus	*Commiphora*
Species	*wightii*
Species Authority	(Arnott) Bhandari

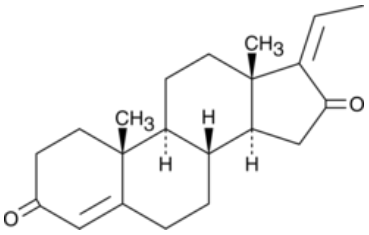
C. wightii is a small tree or shrub. It is a slow growing plant and takes 8 to 10 years to reach to a height of 3 to 3.5 meters. The plant is dimorphic, one having bisexual and male flowers and the other having female flowers with staminodes. A third category of plant with only male flowers has also been reported (Gupta *et al*., 1996). The fruits are green berry like drupe. Size of the fruit varies from 6 to 8 mm in diameter. Fruit parts exposed to sun develop pinkish tinge. Fruits remain on the plant for several months. Seeds show polyembryonic nature. The use of plants in the treatment of diseases occupies an important place in Ayurveda, the traditional medicine system of India. The Atharva Veda, one of the four wellknown Holy Scriptures (Vedas) of the Hindus, is the earliest reference to the medicinal and therapeutic properties of guggul. Sushruta Samhita (600 B.C.), a well-known Ayurvedic medical text, describes the usefulness of the gum resin from the tree *C. wightii* in the treatment of a number of ailments, including obesity and disorders of lipid metabolism. The plant *C. wightii* provides oleogum resin mentioned by Sushruta (3000 year ago) as being a valuable drug. The oleo-gum resin commonly known as “gum guggul” or “Indian myrrh” is the

economically important product of Indian bdellium. The oleo-gum is collected as exudates from woody stem.

Guggulsterone is a plant steroid found in the resin of the guggul plant, *Commiphora wighttii.*. Guggulsterone can exist as either of two stereoisomers, E-guggulsterone and Z-guggulsterone. In humans, it acts as an antagonist of the farnesoid X receptor, which was once believed to result in decreased cholesterol synthesis in the liver. Several studies have been published that indicate no overall reduction in total cholesterol occurs using various dosages of guggulsterone, and levels of low-density lipoprotein ("bad cholesterol") increased in many people. Z-guggulsterone, a constituent of Indian Ayurvedic medicinal plant *Commiphora mukul*, inhibits the growth of human prostate cancer cells by causing apoptosis. Their ring structure of Guggulsterone E&Z is presented below.



(E)-Guggulsterone



(Z)-Guggulsterone

The plant generally takes 5 to6 years to reach tapping maturity under the dry climatic conditions. The thick branches are incised during the winter to extract the oleo-gum resin. Guggul gum is a mixture of 61% resins and 29.3 % gum, in addition to 6.1% water, 0.6 % volatile oil and 3.2 % foreign matter. Unfortunately the plant *Commiphora wightii* has become endangered because of its slow growing nature, poor seed setting, lack of cultivation, poor seed germination rate and excessive and unscientific tapping for its gum resin by the pharmaceutical industries and religious prophets. This plant is incorporated in Data Deficient category of IUCN's Red Data list.

Guggul plant growing in natural habitat at Rajasthan Gujarat & MP

CHAPTER-2

OBJECTIVES

Objectives of the project:

The broad goals and objectives of proposed work were:

1. Survey and collection of wide range of germplasm from wild and their maintenance in mist chamber/green house for further investigation.
2. Chemoprofiling of collected germplasm for the quantitative determination of guggulsterone.
3. Assessment of genetic diversity through molecular markers technology

CHAPTER-3

MATERIAL & METHODS

Material and Methods

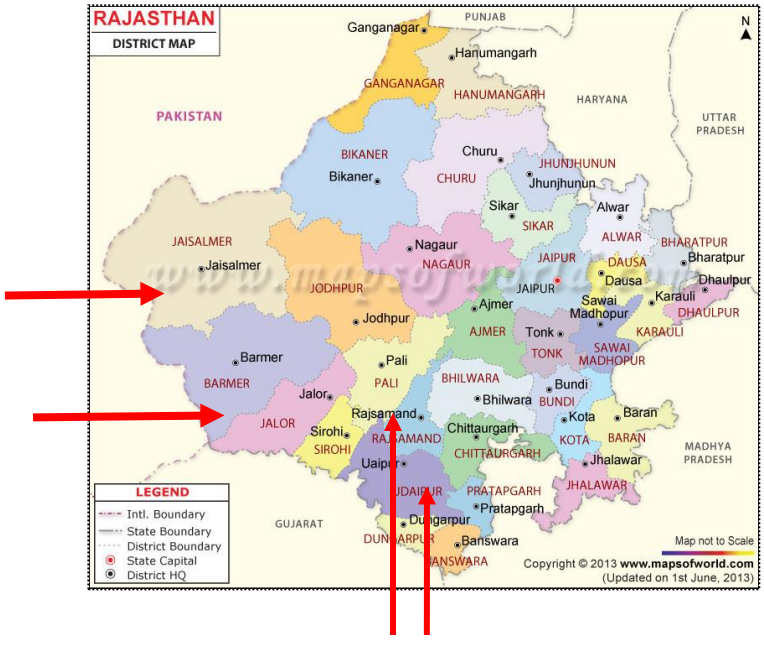
This chapter describes the material and methods used for various experimental procedures followed and the different methods adopted during the study as follows-

1. Germplasm exploration:

Area surveyed under the project: As per the available literature regarding its natural occurrence from three states viz. Rajasthan, Gujarat and Madhya Pradesh were surveyed for the identification of potential pockets and collection of wild germpalsm from these states. For species potentiality in these states the information was collected from the forest officials and with the help of local people. From each site 10 individual plants/genotypes were selected randomly and 25 numbers of vegetative cuttings were prepared from each genotype. The state wise germplams were collected from the following areas:

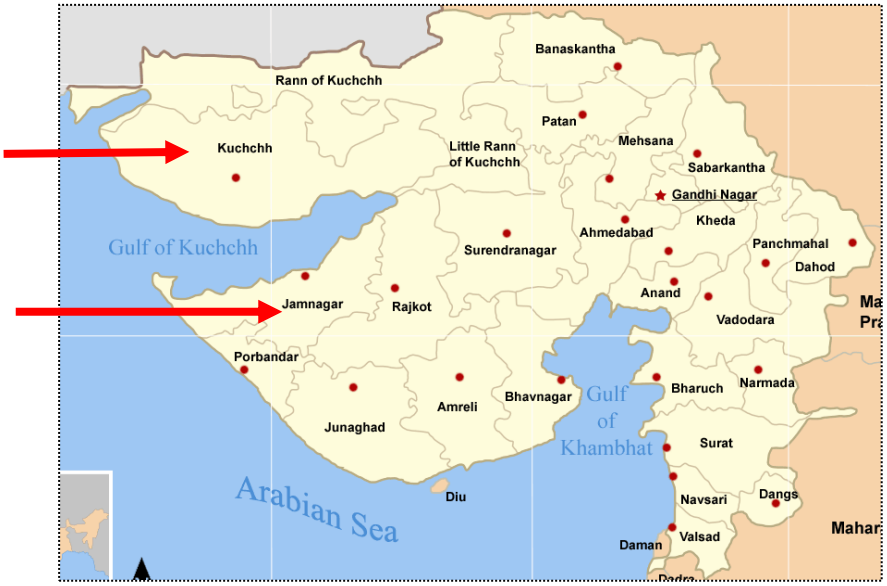
a. State of Rajasthan:

District /provenance	Range/village	Accession number
Udaypur	Sajjangadh wild life sanctuary	CW-5
	Thurmagra	CW-10
	Chirvaghat	CW-2
Rajsamand	Haldighati	CW -1
Badmar	Kiradu (historical guggle in India)	CW-4
Jasalmare	Akool Wood Fossil Park	CW-6



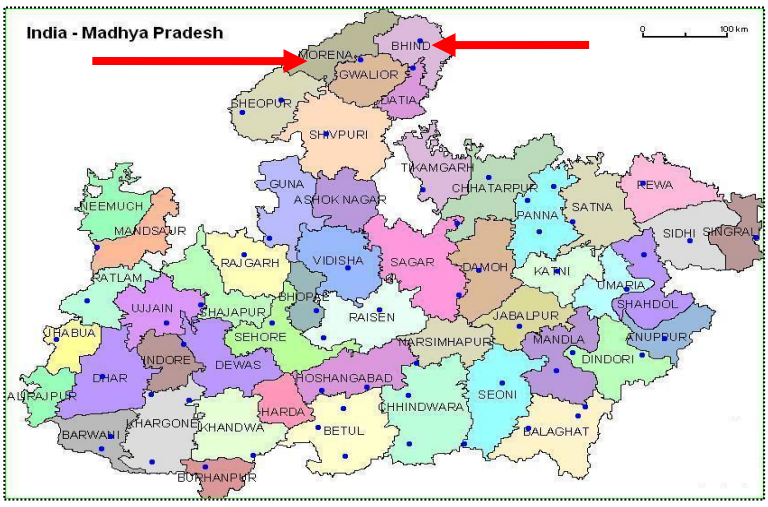
b. State of Gujarat:

District /provenance	Range/village	Accession number
Bhuj	Daselpur Round (Badhai village)	CW-11
	Nakhatrana Round	CW-13
	Mathal nursery	CW-8
Jamnagar	Dwarka Range, Goringa	CW-12
	Poshitra	CW-7



c. State of Madhya Pradesh

District /provenance	Range/village	Accession number
Murena	Kemera	CW-9
Bhind	Bhind	CW-3



2. Methods for standardizing clonal propagation techniques:

The collected stem branch cuttings were maintained in mist chamber for the induction of roots. Different categories of stem branch cuttings were prepared. Treated with different concentrations of root promoting hormones like IBA and NAA Ranges from 1000, 2000, 3000, 4000, and 5000 PPM. Cuttings placed in sand bad under mist chamber, RH 85% with intermittent misting system along with 35±5^{0}C. Maximum rooting 65% recorded in medium size cuttings (Length 6-10 inch & Width 5-7mm) treated with 2000 ppm of IBA as shown (fig-1). Other conc. of IBA showed poor to moderate rooting response. The preliminary observations are mentioned below. The rooted stacklings were shifted in 1:1:1 FYM in polythenebags for hardening (fig-2).

Table- 1 Induction of roots from stem branch cuttings of *C. wightii* with different hormonal treatments

S.No.	Hormonal concentration (ppm)	Hormonal treatment	Rooting response (%)
1.	1000	**IBA**	52
	2000		**65**
	3000		60
	4000		43
	5000		34
2.	1000	NAA	34
	2000		45
	3000		36
	4000		22
	5000		10

Figure 1 .Induction of roots from stem branch cuttings

Figure 2 . Hardened plants

3. Phytochemical analysis

The bark/gum of the plant is used for the treatment of various diseases. For the collection of bark/gum from mature trees having minimum GBH 15- 20cm were selected. To standardize best analytical method for quantitative determination of active ingredients present in the species it is necessary to search out and analyze all the factors affecting the analysis. These factors can be categorized into moisture content in the plant, temperature of drying, isolation techniques, method of extraction including solvents and different polarities of the solvents as well as different mixture of solvents having different ratios and HPLC analysis with different parameters. Table 2 & 3 provides the details of solvents reagent and instruments used to perform the study.

Solvents and reagents:

Table- 2 Chemicals and reagents used in the extraction process

S No	Name of the chemical	Specification of the chemical
1	Methanol	Acronym CH_3OH Specific density 20^0C Percentage purity 99.9% Manufacture E-Merck, India
2	Acetonitrile	Acronym CH_3CN Specific density 20^0C Percentage purity 99.9% Manufacture E-Merck, India
3	HPLC grade water	Acronym HOH Specific density 20^0C Percentage purity 99.9% Manufacture E-Merck, India
4	Hexane	Acronym Specific density 20^0C Percentage purity 99.9% Manufacture E-Merck, India

5	Hydrocholric acid	Acronym HCl Specific density 20^0C Percentage purity 99.9% Manufacture E-Merck, India
6	Ammonia	Acronym NH_3 Specific density 20^0C Percentage purity 99.9% Manufacture E-Merck, India
7	Chloroform	Acronym CH_3CN Specific density 20^0C Percentage purity 99.9% Manufacture E-Merck, India
8	Sodium sulphate	Acronym Na_2SO_4 Specific density 20^0C Percentage purity 99.9% Manufacture E-Merck, India
9	Dragon droff's reagent	Acronym Specific density 20^0C Percentage purity 99.9% Manufacture CDH

Chromatographic conditions:

A chromatography Instrument Company (CIC, Baroda, India) modular HPLC system was used. Analysis was performed on a reverse phase C-18 ODS-2 column having particle size 400 Á.

Instruments

Table -3 Instruments involved in analysis

S No	Name of the instrument	Specifications
1	Soxhlet (Plate-I)	Manufacturer E-Merck, India
2	Rota vapour	Manufacturer Popular India Pvt Ltd.
3	Millipore filtration unit	Manufacturer Instrument Company, Bangalore Pore size of filter paper 0.45µm
4	Ultra sonicater	Manufacturer Flexit, Pune
5	HPLC	Manufacturer Chromatography and Instrument Company, Baroda Column length C-18 Column's pore size 40 Å
6	UV Detector	Manufacturer Linear
7	Syringe	Manufacturer Knaver, Hegauerweg, Berlin
8	Electronic weighing balance	Manufacturer Sartorius
9	pH meter	Manufacturer Naina, Solaris

The analysis of collected samples were performed with the help of above mentioned instruments and chemicals followed by given parameters-

Collected samples were dried at different temperatures under hot air oven for temperature treatment as well as under the sun light to avoid fungal infection as mention in table- 4

Temperature for drying-

Different methods of drying were applied on the gum after its harvesting and left for drying by spreading on filter paper under shade in aerated room and under oven at different temperatures (table -4).

Table-4 Drying temperatures and time period of drying

S No	Method of drying	Time taken for drying
1	Spreading material on filter paper and dry at room temperature	15 days
2	Drying in oven at 40^0C	4 days
3	Drying in oven at 45^0C	3 days

Sample preparation for quantitative determination: For quantitative determination of Guggulsterone through chemoprofiling the collected gum samples were dried as mentioned in table-4 and were powered through pestle mortar. The dusts of the bark sample were thoroughly filtered with fine mesh for obtaining very fine dust of the bark sample.

Extraction process

Soxhlet extraction

2 gm of powered material with 20 ml of solvent mixture was taken in soxhlet apparatus and refluxed for 10 hours. It was then loaded on Rotor-vapour and heated approximately till their boiling point. The remaining concentrated material with some impurities defatted with hexane 3-4 times to remove fatty acids. The hexane extract was discarded and the aqueous portion was washed 3-4 times with 3% HCl solution. The solution was filtered, heated in water bath and 25% NH_3 solution was added, pH of the solution was adjusted to 7.0-7.5. The solution was extracted with $CHCl_3$ through separatory funnel 3-4 times. The dark portion was discarded and the combined aqueous extract was transferred in a conical flask. Anhydrous Sodium Sulphate was added to this extract then filtered and washed with chloroform. Extracted Guggulsterone were

confirmed by Dragon Droff's reagent and then 20 ml appropriate solvent or solvent mixture was added and filtered with Millipore. (Table –5)

Table- 5 List of solvents and their mixtures tried for sample preparation

S No	Solvent and their mixtures
1	Pure Methanol
2	90% Methanol
3	80% Methanol
4	70% Methanol
5	60% Methanol
6	Pure Acetonitrile
7	90% Acetonitrile
8	80% Acetonitril
9	70% Acetonitrile
10	60% Acetonitrile

2.0ml standard of Guggulsterone E&Z (Natural Remedies Pvt. Ltd) was accurately dissolved in 5 ml solvent used for samples preparations to obtain concentrated stock solution in 10.0 ml volumetric flask (Borosil). Various concentration ranges between 0.1-5.0 ml were prepared from the stock solution and stored at 2-8°C and brought to room temperature before use. 5.0µl from each standard solution was injected in six replicates.

4. Genetic Diversity Assessment: Genetic diversity stands for all living things on earth. It refers to the range of variations among a set of entities and is commonly used to describe variety and variability of plant in terms of genetic diversity, biodiversity, species diversity and ecological diversity. In simple terms, Genetic diversity is the vast variety of natural plant existing in any region. Molecular markers work by highlighting differences (polymorphism) within a nucleic sequence between different individuals. These differences include inseration, deletion, translocations, duplications and point mutation. Random Amplified Polymorphic DNA marker (RAPD) was the first PCR-based molecular marker to employ in genetic variation analyses. In this studies Random amplified polymorphic DNA markar was used because these marker shows high polymorphism. The germplasm of *C. wightii* was collected from six populations from Rajsthan (Sajjangadh wild life sanctuary, Thurmagra, Chirvaghat Haldighati, Kiradu (historical guggle in India) Akoolwood Fossil Park), five populations from Gujrat (Daselpur Round (Badhai village), Nakhatrana Round, Mathal nursery, Dwarka Range,

Goringa, Poshitra) and Two populations from Madhya Pradesh (Kemera, Bhind). Overall 13 populations were collected from different localities of three states.

Maintenance of germplasm- The population wise stem branch cuttings were placed in mist chamber for obtaining sprouts and young leaves for DNA extraction.

DNA Isolation protocol used for *Commiphora wightii* -

Young leaves (1g wt.) grinded in Pestle *&* Mortar using liquid nitrogen (LN_2) to convert the leaves into fine powdered form. The powder transferred in 1.0 ml of CTAB buffer containing 100mM Tris (pH 8.0), 20mM EDTA (pH 8.0), 1.4 M NaCl, 2.5% CTAB (w/v), (Promega) .In this solution 1% PVP (Calbiochem) and 10mM B-mercaptoethanol (Merck) added freshly. It was mixed vigorously by vortexer and incubated at 60±5 ^{0}C for 30 minutes followed by treatment with equal volume of chloroform: isoamylalcohol (24:1) (Amresco). This mixture was centrifuged (Eppendorf, AG Germany) at 5125x g for 15 minutes at room temperature. After centrifugation the upper phase (supernatant) was transferred to a fresh autoclaved centrifuge tube and then 1/10 volume of 3M sodium acetate (pH 5.2) and ½ volume of 5M NaCl (Promega) was added to it. DNA was precipitated using 0.6 volume chilled isopropanol (Promega) and pelleted by centrifugation at 5125X g for 10 minutes at 4^{0}C. The supernatant was decanted and the DNA pellet was washed with 70% ethanol (Merck). The crude DNA pellet was air dried and suspended in 500µl of 0.5ml high salt TE buffer (10mM Tris pH 8.0, 1mM EDTA, 1M NaCl) (Promega).

DNA Verification: The isolated genomic DNA was verified using 0.8% Agarose gel (Promega) through electrophoresis (Genetix).

Amplification of isolated DNA : The isolated genomic DNA was amplified through PCR reaction which were carried in 0.2ml Polypropylene PCR tubes (Axiva) using thermal cycler EP gradient Master Cycler (Eppendorf, AG Germany). Each 20 µl reaction mixture contain, 1X Taq buffer (100mM Tris-Cl in pH 9, 500mM KCl, 15mM $MgCl_2$ and 0.1% gelatin (Promega), 2.5 mM $MgCl_2$, 0.2 µl dNTPs (Promega), 20 pmols Oligonucleotide primers (IDT Avantor),1U Taq DNA polymerase (Promega) and 20 ng template DNA.

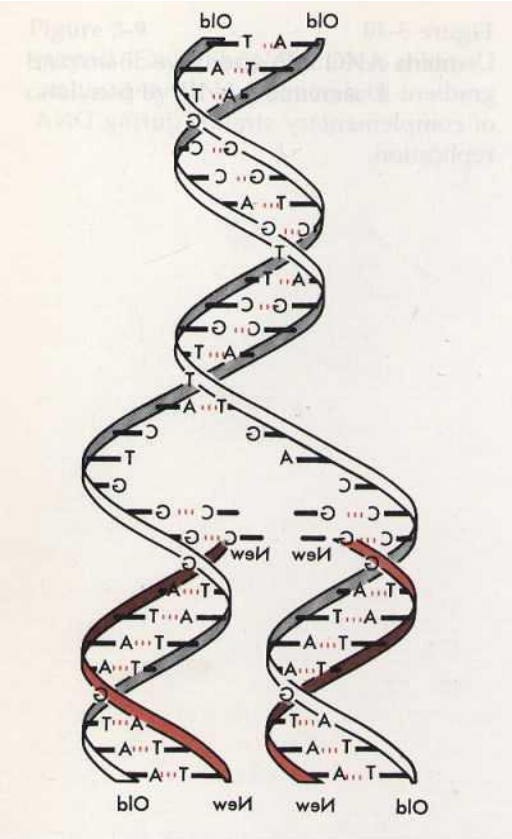
This reaction mixture was subjected to the three final PCR steps through (denaturation, annealing and extension) as initial denaturation at 94^{0}C for 5 minutes. followed by 45 amplification cycles, each consisting of 30seconds at 94^{0}C (denaturation step), 1 min at 37^{0}C (annealing step) and 2min at 72^{0}C (extension step) with final

extension of 10 min. at 72^{0}C. The amplification products were separated on 1.5% w/v agarose gel (Promega) and stained with 0.7 µg/ml Ethidium bromide solution (Promega). DNA ladders of 1 kbp (Promega) were mixed and used as mol wt. marker for comparison of amplified product. Gels were photographed through Gel Documentation System Geneview 645C, (Genetix). All reactions were repeated thrice to confirm the results.

Scoring of amplified DNA fragments: The DNA fragments, obtained from different populations using RAPD marker were manually scored for their presence and absence. The data generated subjected to statistical analysis following the method of Jaccard's similarity coefficient. The various populations were utilized for the construction of dendrogram with the help of UPGMA and NT-sys software.

CHAPTER - 4

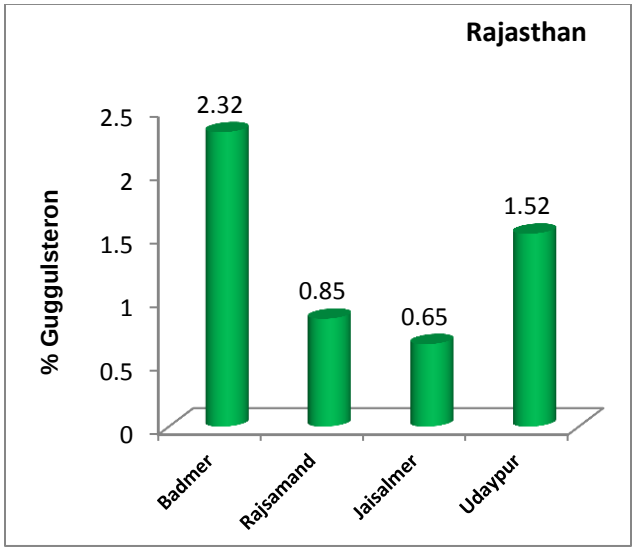
RESULT AND DISCUSSION

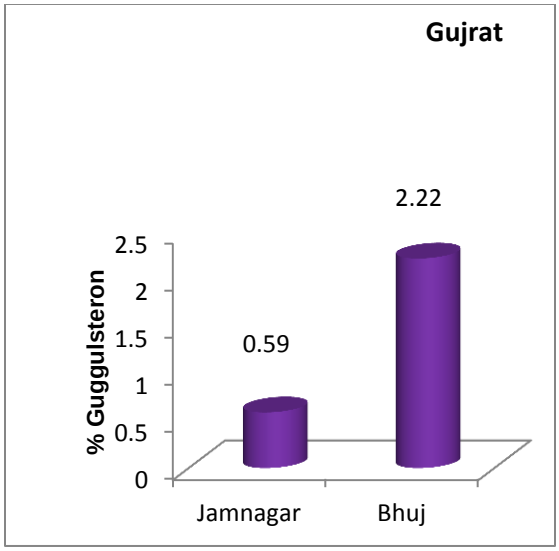


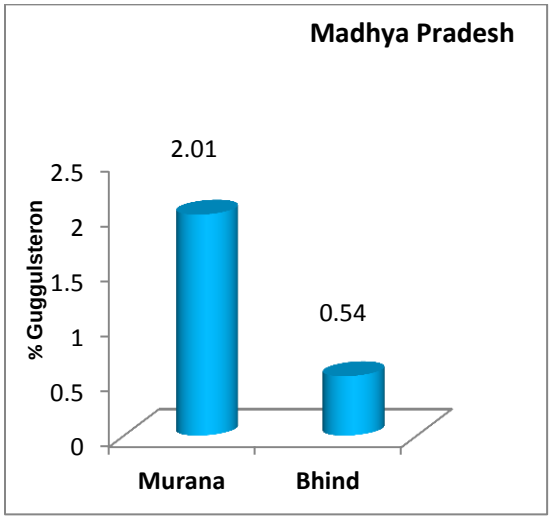
A. Chemoprofiling: The percent concentration of Guggulsterone in the accessions collected from different geographical locations of Rajasthan, Gujrat and MP are presented below:

a. Rajasthan: ranges from 0.65 % to 2.32%.
b. Gujarat: ranges from 0.59 % to 2.22%.
c. MP: ranges from 0.54 % to 2.01%.

From the HPLC analysis it is summarized that the population which were collected from Kiradu (historical guggle in India) and surrounding areas of Badmer District (Rajasthan) showed highest alkaloid concentration followed by Gujrat and Madhya Pradesh.

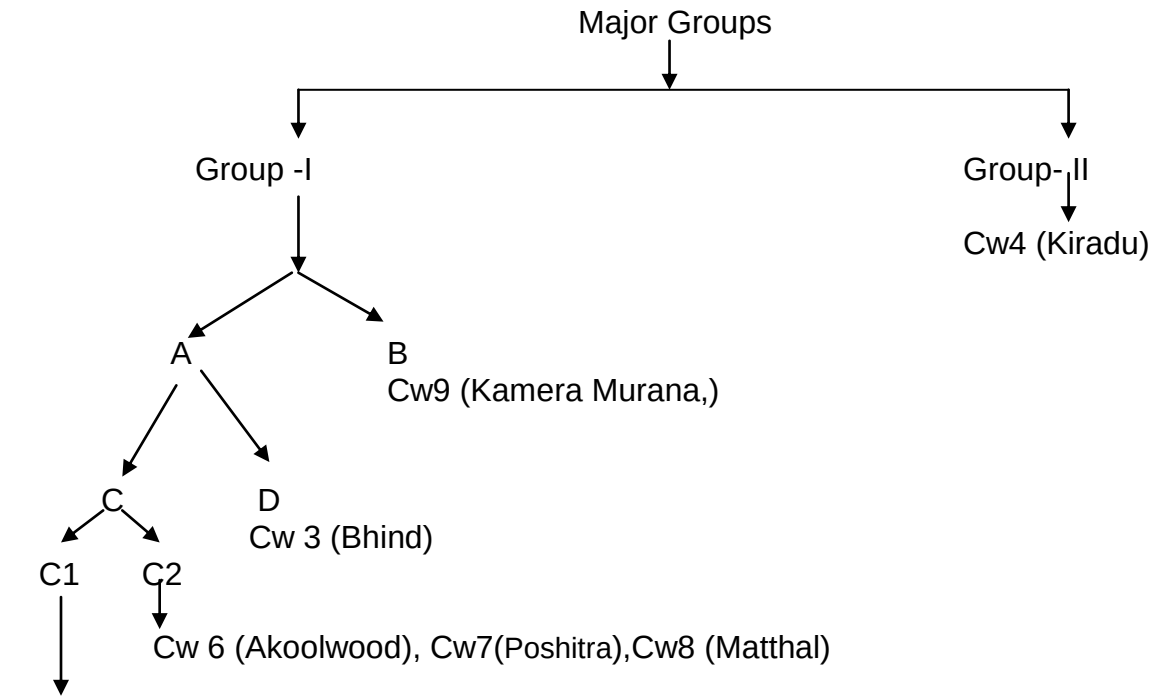






B. Genetic Diversity Assessment: The genetic diversity assessment was assessed within and between the populations of three states which is summarized below:

A dendrogram (Fig.-3) was generated based on RAPD marker analysis among 13 populations collected from three states, which showed genetic diversity within and between the states. Out of these 13 populations only the 9 populations showed genetic diversity and rest 4 populations could not responded with applied primers (Table-8). According to the dendrogram analysis, all the populations were divided into two major clusters which were further sub divided as:



Genetic diversity Assessment within the states: From the grouping/dendrogram (Fig-3,4 &5) and according to Jaccard's coefficient and cluster analysis it is summarized that as per the similarity index all the populations are placed in the range of 0.64 to 0.94. From this findings the population which were collected from Rajasthan, Cw1 (Haldighati) and Cw2 (Chirvaghat) showed highest genetic similarity index 0.92 and lowest genetic similarity was seen between Cw1 (Haldighati) Cw4 (Kiradu) (Table-6) that means these population are highly diversify with each other. While Cw4 (Kiradu) showed highly genetic diversify population (0.64) among the populations of Cw1(Haldighati), Cw2 (Chirvaghat) ,Cw5 (Sajjangarh) and Cw6(Akkalwood) . In case of Gujarat Cw8 (Matthal) population showed more diversify index (0.81) as compared

Cw7(Poshitra) (0.87).The populations which were collected from MP. Cw9 (Murana,Kamera) showed highly diversify index (0.69) as compared to Cw3 (Bhind) (0.80).

Table-6 Jaccard's similarity coefficient index

Commiphora *wightii*

Genotypes	Cw1	Cw2	Cw3	Cw4	Cw5	Cw6	Cw7	Cw8	Cw9
Cw1	1.0000000								
Cw2	0.9264706	1.0000000							
Cw3	0.7971014	0.8030303	1.0000000						
Cw4	0.5797100	0.6250000	0.6724138	1.0000000					
Cw5	0.8088235	0.8153846	0.6818182	0.6551724	1.0000000				
Cw6	0.8142857	0.8208955	0.7164179	0.6129032	0.8387097	1.0000000			
Cw7	0.7638889	0.7428571	0.6666667	0.6393443	0.7538462	0.8730159	1.0000000		
Cw8	0.7887324	0.7681159	0.7968750	0.6949153	0.7272727	0.8153846	0.8153846	1.0000000	
Cw9	0.6901408	0.6911765	0.6615385	0.6315789	0.6461538	0.734375	0.7076923	0.7903226	1.0000000

According to Jaccard's similarity coefficient, highest similarity was observed between Cw1 and Cw2 (0.92), while lowest similarity was seen between Cw1 and Cw4 (0.57).

Genetic diversity Assessment between the states: From the genetic analysis it is concluded that the populations of Cw4 (Kiradu) showed highly genetic diversify (0.64) among the populations of Gujarat and MP. No such populations were found more closes genetic affinity from these three states except Rajasthan. According to Jaccard's coefficient and cluster analysis five RAPD markers were found to be more polymorphic. According to RAPD Marker assay it was found that 42 % bands showed polymorphism while 35% bands showed monomorphism. All the primers showed 54.54% polymorphism. (Table -7).

Table-7 **RAPD Markers Assay**

S.№	Code	Total bands	Monomorphic bands	Polymorphic bands	Percentage of Polymorphism
1	OPA-08	3	1	2	66.60
2	OPC 10	4	2	2	50.00
3	OPA -07	8	3	5	75.00
4	OPC-15	8	2	6	50.00
5	OPA-05	6	3	3	50.00
6	OPA-20	8	5	3	63.00
7	OPA-11	8	4	4	50.00
8	OPA-06	6	3	3	40.00
9	OPA-09	3	2	1	25.00
10	OPA-04	5	1	4	80.00
11	OPN-16	5	3	2	40.00
12	MAP-08	7	2	5	71.00
13	MAP-13	6	4	2	33.00
	Total	77	35	42	54.54

Table-8 **List of RAPD primers and sequences**

S.№	Primer	Sequences 5'-3'	GC content
1	OPA-08	5'-GTGACGTAGG-3'	60
2	OPC 10	5'-GACGGATCAG-3'	60
3	OPA -07	5'-GAAACGGGTG-3'	60
4	OPC-15	5'-GACGGATCAG-3'	60
5	OPA-05	5'-AGGGGTCTTG-3'	60
6	OPA-20	5'-GTTGCGATCC-3'	60
7	OPA-11	5'-CAATCGCCGT-3'	60
8	OPA-06	5'-GGTCCCTGAC-3'	70
9	OPA-09	5'-GGGTAACGCC-3'	70
10	OPA-04	5'-AATCGGGCTG-3'	60
11	OPN-16	5'-AAGCGACCTG-3'	60
12	MAP-08	5'-CTATCGCCGC-3'	70
13	MAP-13	5'-GTGCAATGAG-3'	50

As per the above findings it is concluded that the population which were collected from Kiradu (historical guggle in India) and surrounding areas of Badmer District (Rajasthan) showed highest alkaloid concentration as well as in terms of genetic diversity is concern. This information can be used for its genetic improvement programmes either through breeding or through propagation of elite material for *Commiphora wightii*.

Fig-3- Dendrogram

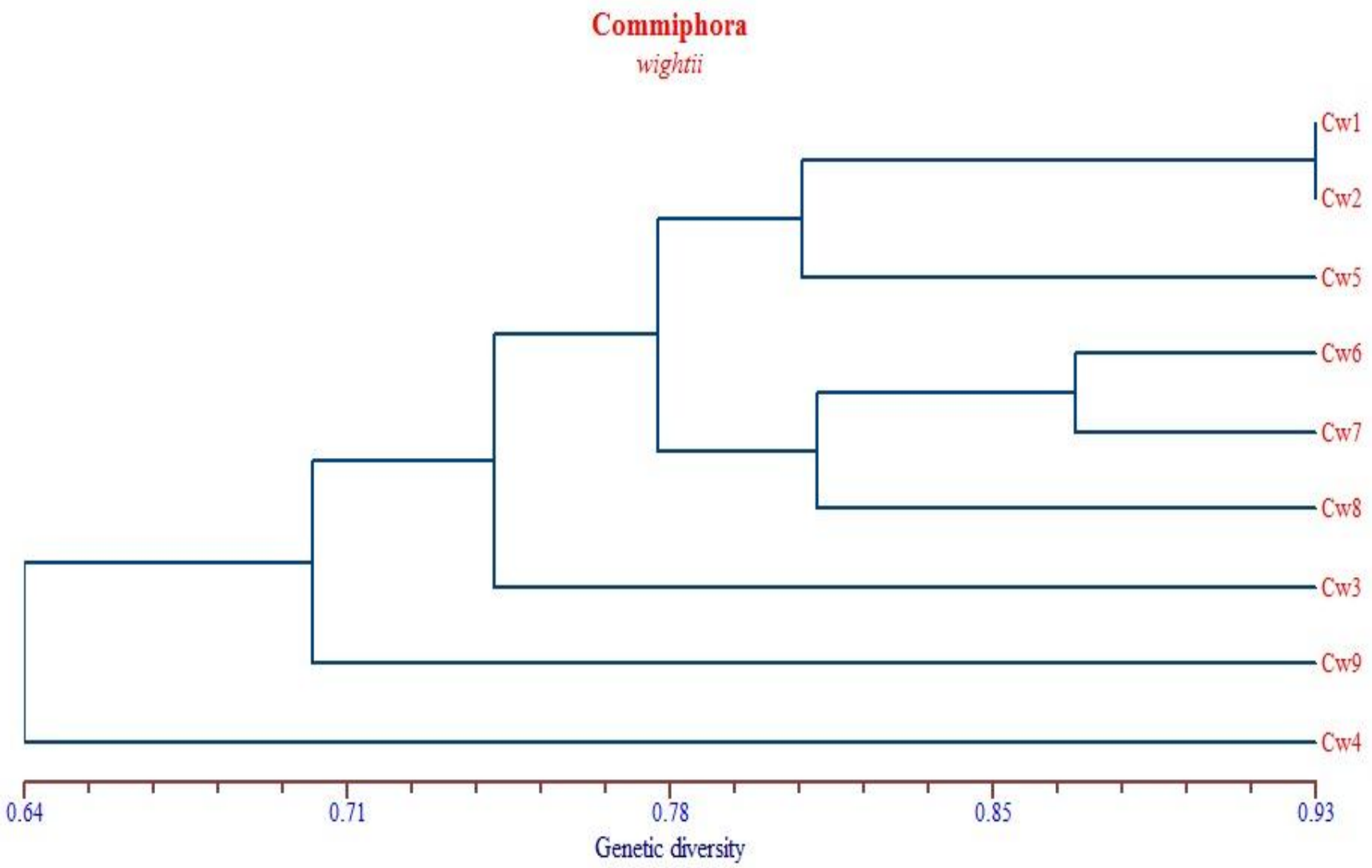
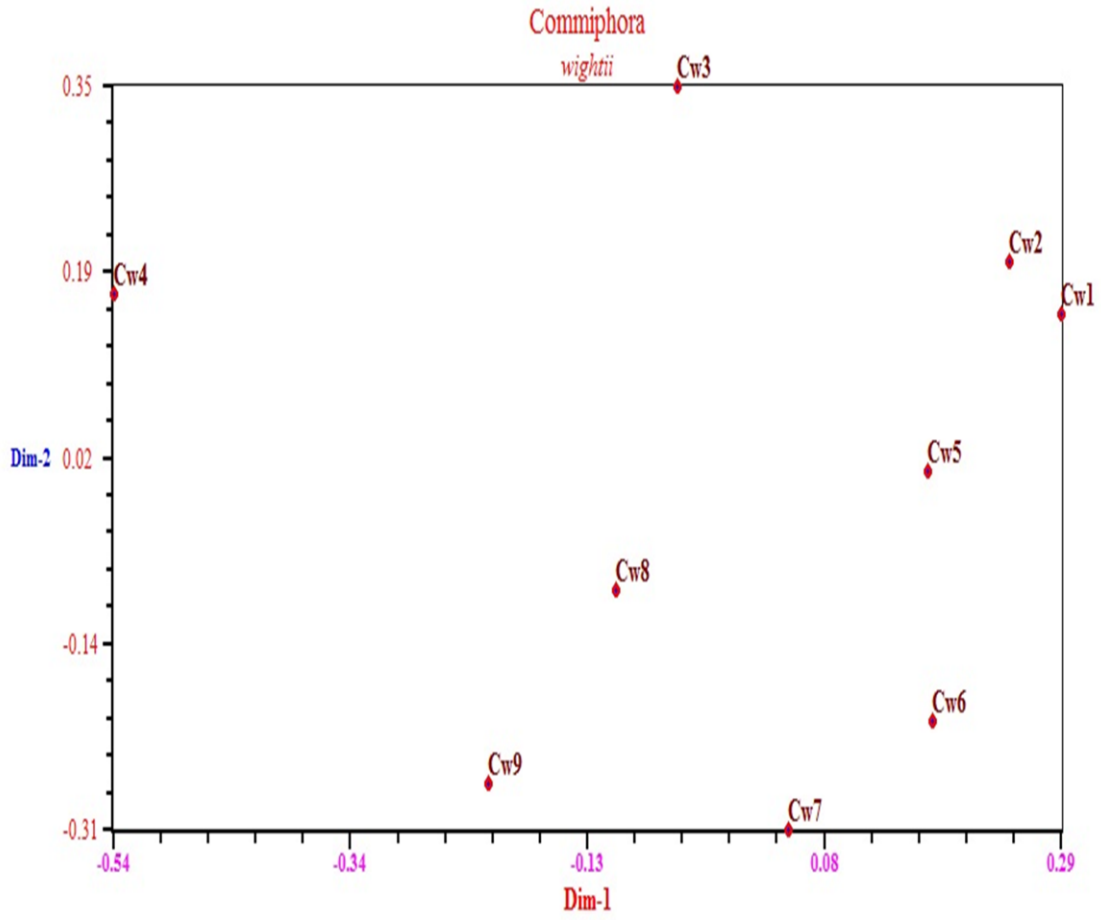
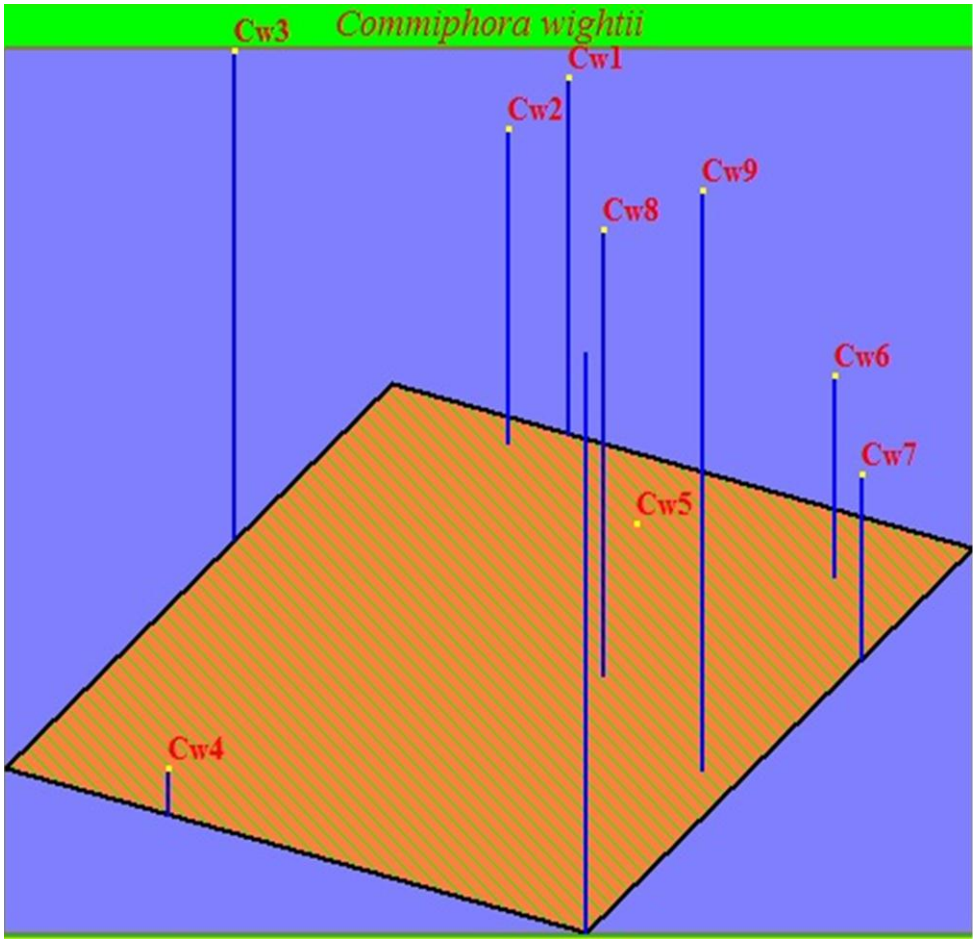


Fig-4- 2D Dendrogram



2 Dimensions graph

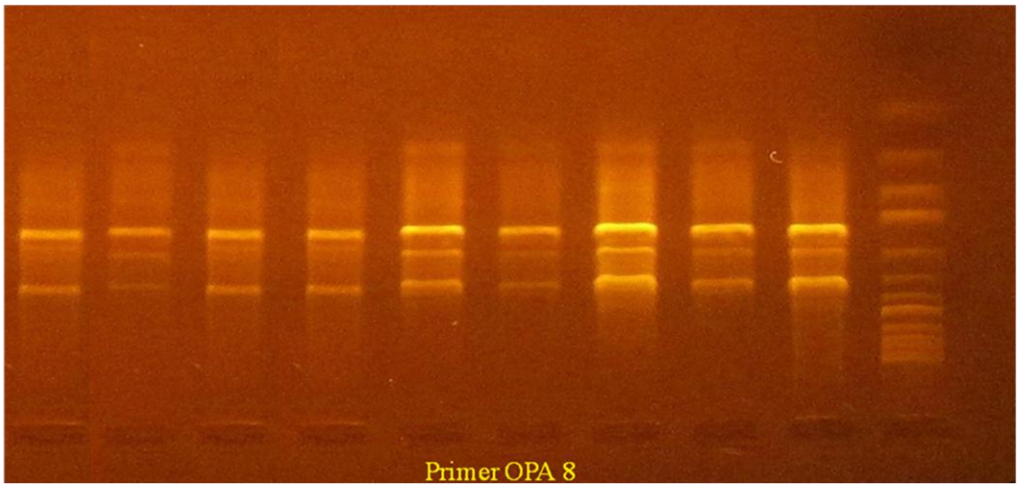
Fig- 5- 3D Dendrogram



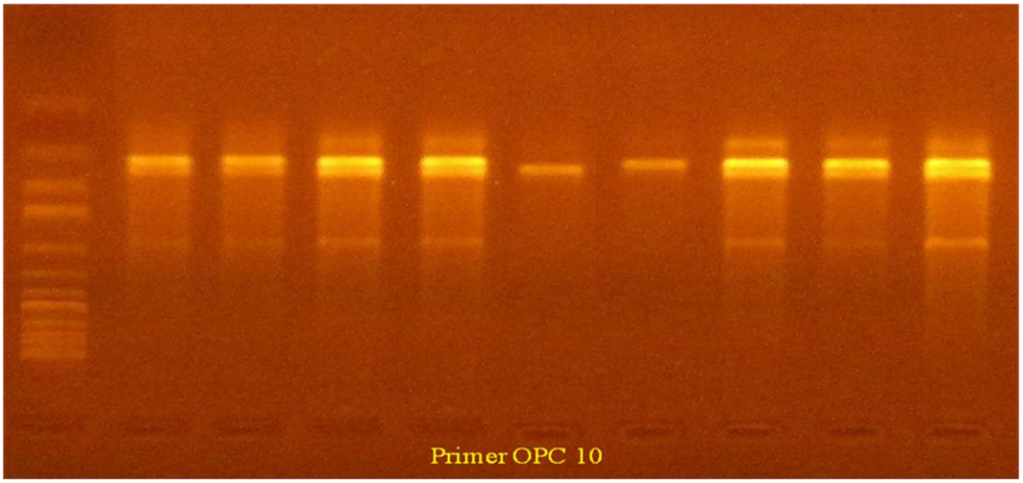
3 Dimensions plot graph

CHAPTER- 5

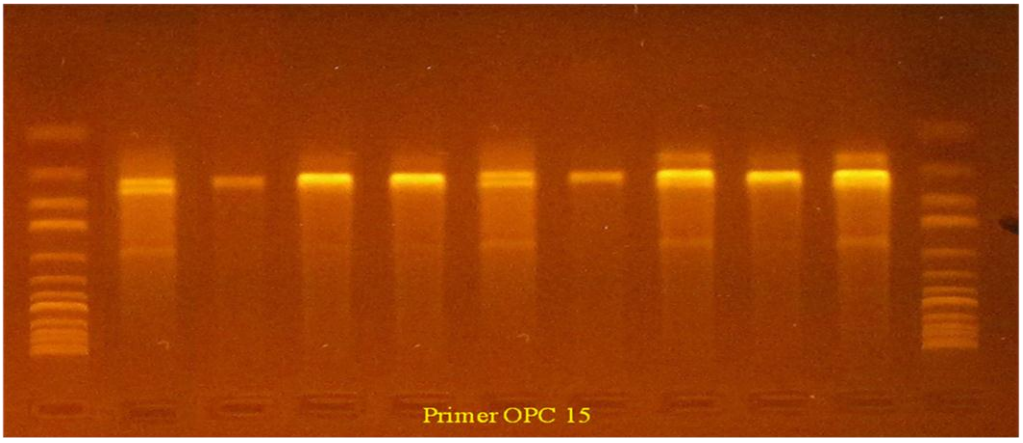
PHOTO PLATES OF DNA BANDS OBTAINED FROM GEL DOCUMENTATION SYSTEM



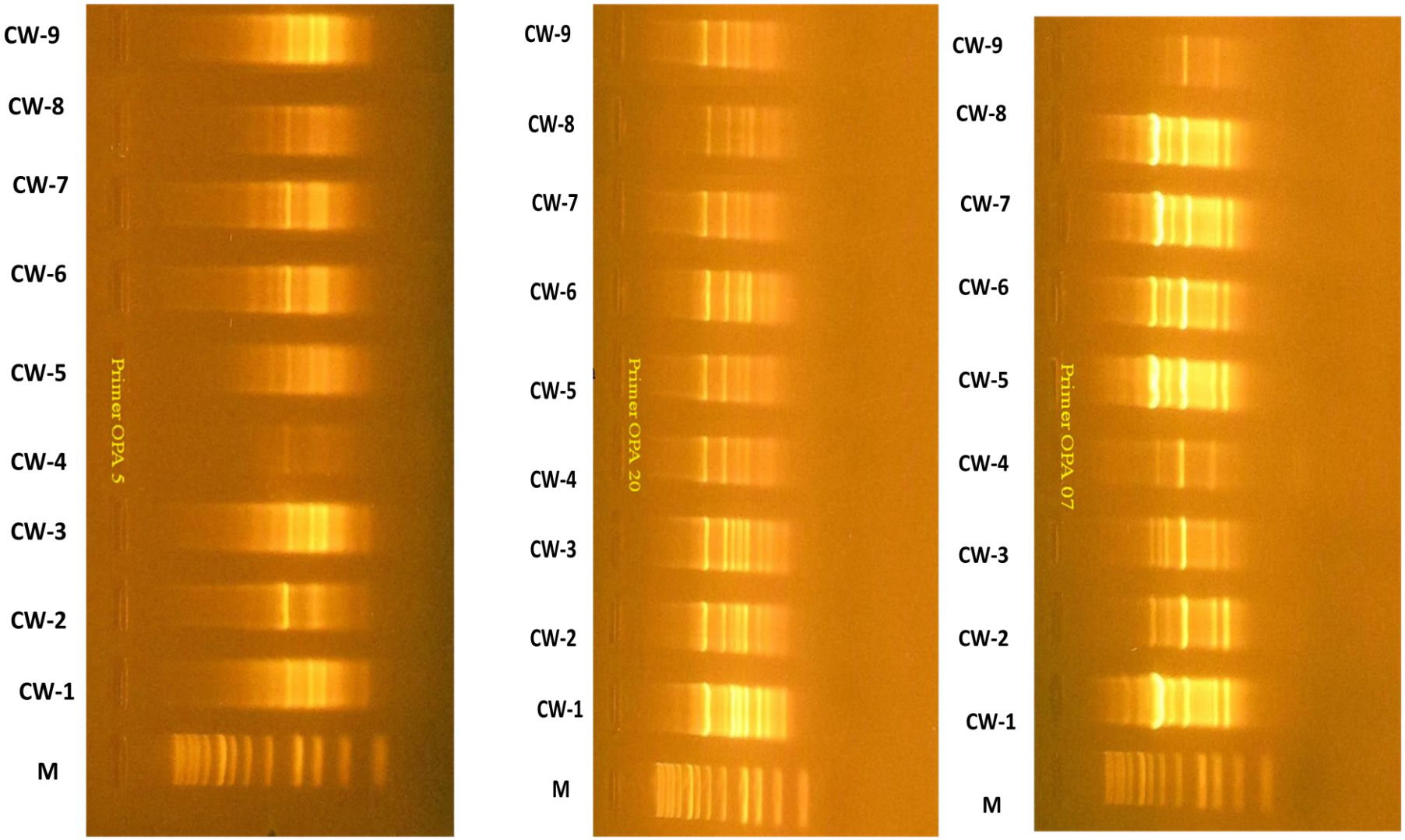
CW-9 CW-8 CW-7 CW-6 CW-5 CW-4 CW-3 CW-2 CW-1 M



M CW-1 CW-2 CW-3 CW-4 CW-5 CW-6 CW-7 CW-8 CW9



M CW-9 CW-8 CW-7 CW-6 CW-5 CW-4 CW-3 CW-2 CW-1 M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CW-7
CW-6
CW-5
CW-4
CW-3
CW-2
CW-1
M
Primer OPA 5
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CW-8
CW-7
CW-6
CW-5
CW-4
CW-3
CW-2
CW-1
M
Primer OPA 20
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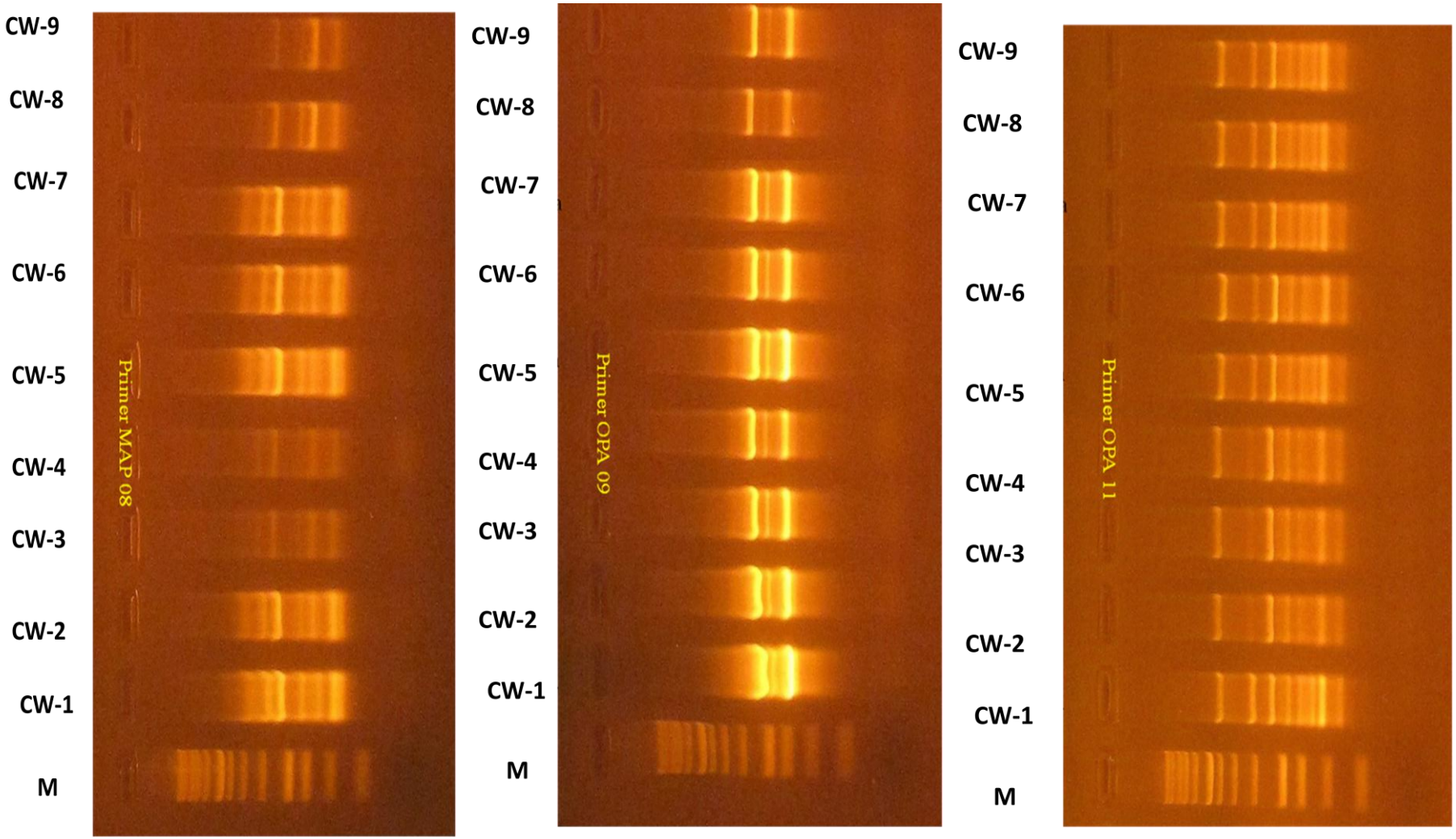
CW-6
CW-5
CW-4
CW-3
CW-2
CW-1
M
Primer OPA 07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CW-6
CW-5
CW-4
CW-3
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CW-1
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Primer MAP 08
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CW-8
CW-7
CW-6
CW-5
CW-4
CW-3
CW-2
CW-1
M
Primer OPA 09
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CW-6
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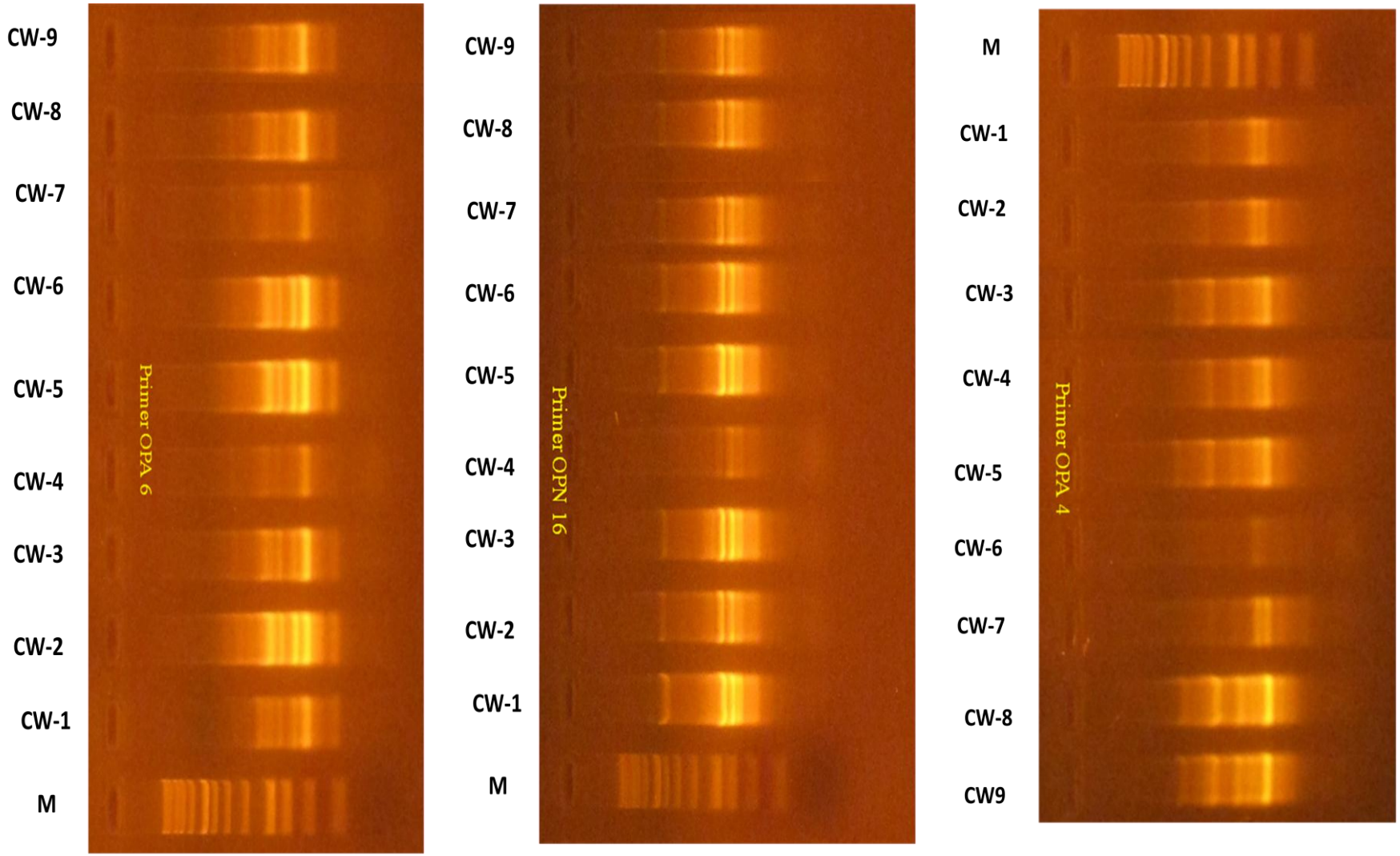
CW-3
CW-2
CW-1
M
Primer OPA 11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CW-1
M
Primer OPA 6
CW-9
CW-8
CW-7
CW-6
CW-5
CW-4
CW-3
CW-2
CW-1
M
Primer OPN 16
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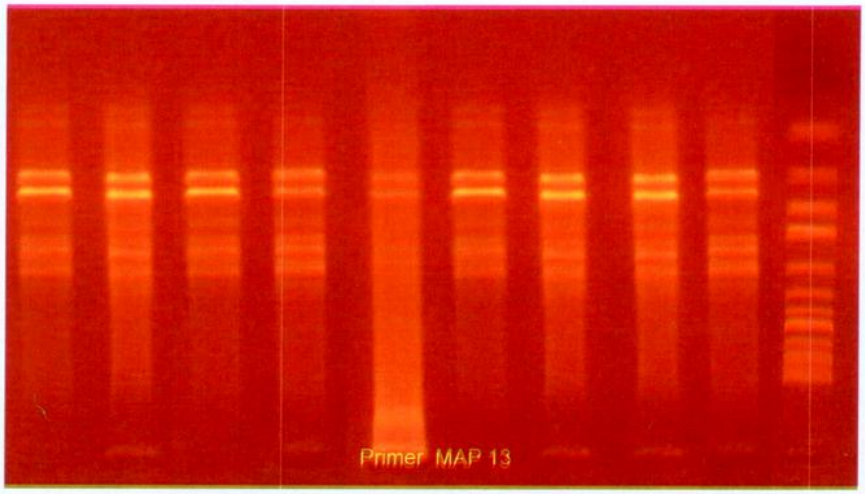
Primer OPA 4



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