Studies of genetic diversity in a homogeneous population of *Withania somnifera* (L.) Dunal *using* DNA based markers

A thesis submitted in partial fulfillment of the degree of

MASTER OF TECHNOLOGY

IN

BIOTECHNOLOGY

By

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June 2023

CERTIFICATE

This is to certify that dissertation entitled "**Studies of genetic diversity in a homogeneous population of *Withania somnifera* (L.) Dunal using DNA based markers**" submitted by **Miss. Ruchika Singh** (Roll no. 602104012) in the partial fulfilment of the requirements for the award of the degree of Master of Technology in Biotechnology, Thapar Institute of Engineering and Technology, Patiala is a record of student's own work carried out under my guidance and supervision.

It is also certified that the matter embodied in this thesis has not been submitted in part or full to any other institute or university for the award of any degree or diploma.

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DECLARATION

I hereby declare that the work presented in the thesis entitled "**Studies of genetic diversity in a homogeneous population of *Withania somnifera* (L.) Dunal *using* DNA based markers**" is a bonafide work under the supervision and guidance of **Dr. Anil Kumar**, Professor, Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala.

I also declare that this thesis or any other part of this thesis has never been submitted for any degree in this or any other university.

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ACKNOWLEDGMENT

First of all, I would like to express my deepest gratitude to **Dr. Anil Kumar**, Professor in the Department of Biotechnology, **Thapar Institute of Engineering and Technology Patiala,** for giving me the golden opportunity to work under his invaluable guidance and suggestions throughout the course of dissertation. I would also thank him for his constant encouragement, oversight of my work, and advice which enabled me to finish my research work.

I am grateful to the head of Department of Biotechnology and TIFAC-CORE for providing me with the equipment, infrastructure and resources I needed for the completion of my work.

I would also like to express my sincere thanks to **Ms. Shivani Guleria**, (PhD student) and **Ms. Nisha Chauhan** (PhD student) Department of Biotechnology, for their ongoing help, encouragement, and direction helped me finish my research project.

I would like to express my sincere appreciation to my parents **Mr. Anil Singh** and **Mrs. Chhaya Singh** for their emotional and financial support throughout my journey.

I would also like to thank my sibling **Dr. Disha Singh** and friends **Ms. Warsha Koul**, **Ms. Navgeet Kaur**, and **Mr. Sunny Yadav** for their constant support and encouragement throughout my journey.

Ruchika Singh

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List of Abbreviations

µL	Microliter
AFLP	Amplified fragment length polymorphism
Cm	Centimetre
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate
EtBr	Ethidium bromide
EMR	Effective multiplex ratio
G	gravitational force
HPTLC	High Performance Thin Layer Chromatography
Gm	Grams
ISSR	Inter simple sequence repeat
Kbp	Kilogram base pair
M	Meter
M	Molar
MI	Marker index
mL	Milliliter
Mm	Millimeter
mM	Millimolar
ºC	degree Celsius
PCA	Principal component analysis
PCR	Polymerase chain reaction
PIC	Polymorphic information content
RAPD	Random amplified polymorphic DNA
Rf	Resolution power
SCoT	start codon targeted
SSR	simple sequence repeats
Taq	Thermus aquaticus
TE	Tris-EDTA
w/v	weight per volume

Abstract

Withania somnifera (L.) Dunal (2n=48) family Solanaceae is an important medicinal plant that contains many active ingredients used to treat various ailments. Most of the wild population are seed raised and show a great deal of variations in active ingredients which could be due to (i) genetic variations and (ii) environmental effects. The aim of the present study is to investigate the genetic diversity in these seed raised plants of *W. somnifera* using morphological and molecular markers (Random Amplified Polymorphic DNA: RAPD, Inter Simple Sequence Repeats: ISSR and start codon targeted polymorphism: SCoT). Morphological studies revealed that the leaves were oval or oblong in shape having pinnate venation. The average leaf length and width recorded were 6.06 cm and 4.15 cm respectively. The average internodal distance recorded was 3.39 cm and in an average 5-6 berries were recorded in a cluster. This data provided limited morphological variations among plants. Therefore, PCR based amplification of RAPD, ISSR and SCoT makers was caried out using isolated DNA of selected plants. RAPD primers amplified 95 band out of which 94 were polymorphic (98%), followed by ISSR which amplified 245 band and 229 were polymorphic (93.6%). The SCoT primers produced 200 bands out of which 118 were polymorphic (56.6%) thereby detecting least polymorphism among the three markers. The dendrogram constructed grouped the 12 plants in 2 clusters, in case of ISSR plant WS 12 was out grouped whereas dendrogram constructed based on the combined data of all three molecular markers out grouped WS 06 plant. The PCA analysis for RAPD and ISSR recorded 27.2%, 12.8% and 36.5%, 9.8% component variance respectively. This study suggests that RAPD marker were more efficient in the detection of polymorphism in *W. somnifera* population. The information gathered here might be used to develop conservation plans and routine maintenance strategies for this population.

Chapter 1: Introduction

Withania Somnifera (L.) Dunal (2n=48) is a valuable medicinal plant belonging to family Solanaceae. It is also known as Ashwagandha, Winter Chery, Indian ginseng, and poison gooseberry (Chauhan et al. 2017). The name "ashwagandha" is derived from the smell of the roots, which resembles horse (ashwa) urine (Gaurav et al. 2015). It is one of the most important plants with immense therapeutic value in the ayurvedic and indigenous medicinal system. Since ancient times it has been used in Ayurveda as "*rasayana'* and '*medha rasayana'* (Saroya and Singh 2013). The medicinal value of the plant has been depicted in Ayurveda as "scared text," including *Charak Samhita* (Chauhan et al. 2017). It is regarded as a valuable species due to its "*Vata*" appeasing properties (Sangwan et al. 2004; Tripathi et al. 2012).

Withania somnifera	
	
Scientific classification	
Kingdom	Plantae
Clade	Tracheophytes
Order	Solanales
Family	Solanaceae
Genus	Withania
Species	*W. Somnifera*
Binomial name	
Withania somnifera (L.) Dunal	

Fig:1 Classification of *Withania somnifera*

The plant of *W. somnifera* is about 90 to 140 cm tall, erect, and covered in tiny hairs all over the surface. The leaves are ovate or oblong in shape, with wavy edges arranged in a simple or alternating manner. The flowers are generally tiny, greenish, axillary, monoecious, or bisexual enclosed inside a corolla. The corolla is pale yellow to yellow-green measuring about 5-8 mm in length, distinctively long, and bell-shaped with five globes. The fruits are generally smooth berries with a diameter of 6 mm. The berries are green when immature and change their colour from green to orange-red upon ripening. These berries are enclosed in a globular calyx of about more than 25 mm in diameter. The seeds are white and yellow, disc-shaped, complotropus. The roots are generally stout, long, tuberous, fleshy, and whitish brown in colour.

Fig 2: Morphological features of *Withania somnifera*

Nearly all parts of *W. somnifera* are used for a variety of therapeutic purposes in Ayurvedic medicines (Gurav et al. 2023). Its leaves are bitter and used for the treatment of fever, as an anthelmintic, and for releasing painful swelling. The flowers have astringent, aphrodisiac, depurative, and diuretic properties, while the fruits have been used to treat tumours and tubercular glands, carbuncles, and skin ulcers (Kaur et al. 2004; Singh et al. 2011). Furthermore, the fruits are used as a coagulant agent in the curdling of plant milk to make vegetarian cheese (Hussain et al. 2015). *W. somnifera* seeds are used to remove white spots from the cornea, boost sperm count, testicular growth and has anti-helminthic property (Azgomi et al. 2018). The roots are the most popularly used for wide range of health-promoting

effects. Their powder and preparations are widely used as functional foods to promote vitality and virility (Polumackanycz et al. 2023). They are used to make a tonic that revitalises the body, promotes longevity, strengthens the immune system, and slows down ageing process (Singh et al. 2008; Bhat et al. 2022). According to ethnobotanical research, the roots and leaves are also used as a hypnotic in alcoholism and emphysematous dyspnea (Maccioni et al. 2021; Murthy et al. 2022). *W. somnifera* also has anti-diabetic (Kulkarni and Dhir 2008), immunomodulatory (Ziauddin et al. 1996), anti-inflammatory (Pawar et al. 2011), neuroprotective (Kulkarni and Dhir 2008), and anticancer (Singh et al. 2011) activities. It is also a diuretic, deobstruent, antibiotic, antioxidant, aphrodisiac, and sedative (Kulkarni and Dhir 2008; Mishra et al. 2000).

The plant contains various classes of chemical compounds and vast array of nutrients and phytochemicals, which lead to increased interest of many researchers in this plant (Ahmad and Dar 2017). The primarily recognised bioactive compounds are withanolides A-Y, withaferin A, withasomnierose A–C, withasomniferin A, withasomnidienone, withanone, etc (Srivastava et al. 2019). Withanolides are a class of naturally occurring polyoxygenated steroidal lactones that are arranged on a C28 ergostane skeleton (Mirjalili et al. 2009). The number and nature of oxygenated substituents, as well as the degree of unsaturation of the rings, cause structural variation in withanolides (Chen et al. 2011). The presence of certain withanolides, such as withaferin A, withanoside V, withanolide B, withanone, and 1,2-deoxywithastramonolide, is thought to be responsible for the plant's antioxidant and free-radical scavenging activities (Polumackanycz et al. 2023). Withanolides also stimulate immune system cell activation (Teixeira et al. 2017). Furthermore, these compounds are reported to have antiviral activity, with distinct effects on the viral receptor, and may also be effective against corona virus (Kumar et al. 2021; Singh et al. 2021).

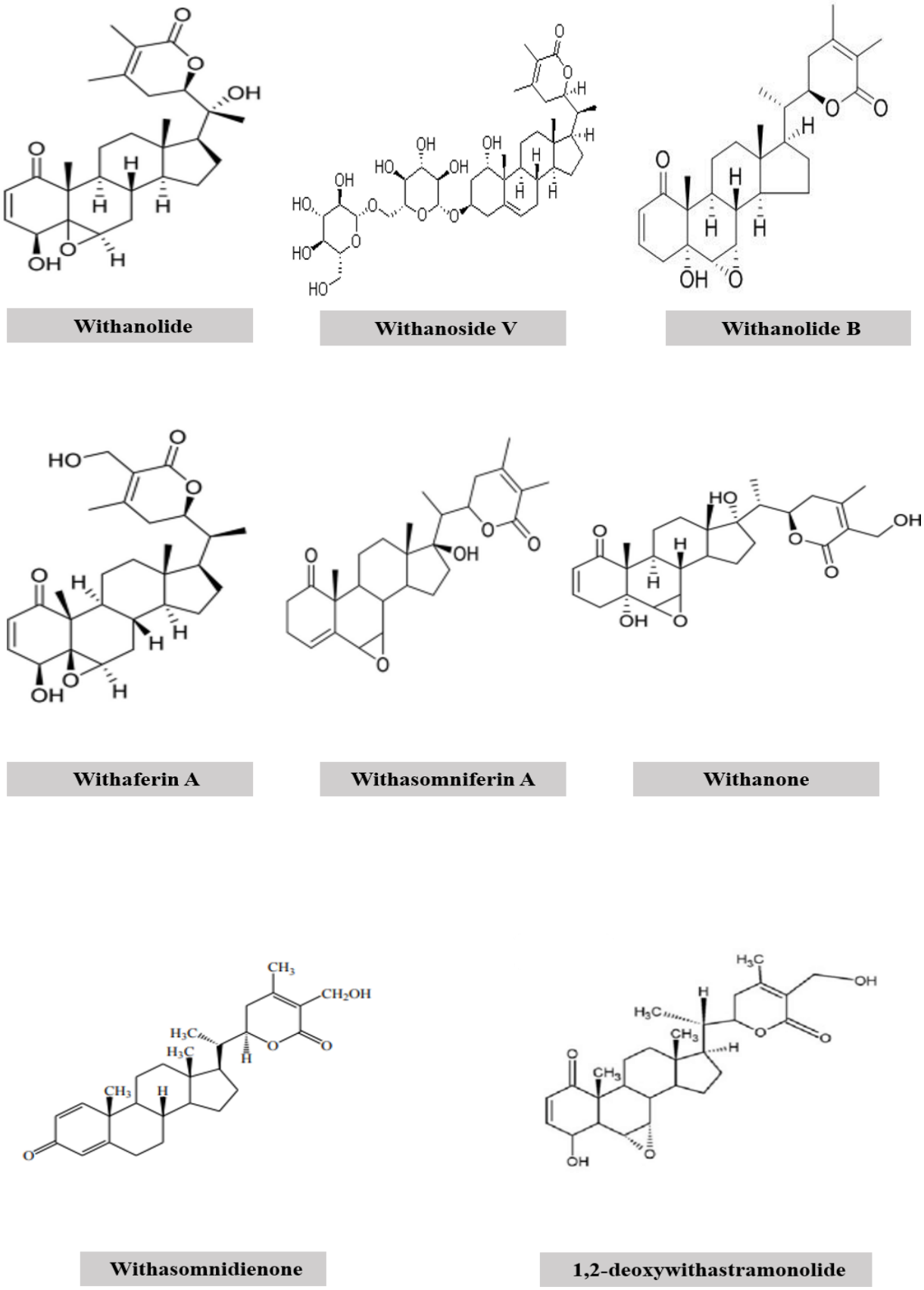

Fig 3: Chemical structure of some important compounds present in *W. somnifera.*

The plant extract also contains alkaloids such as isopelletierine, anaferine, cuseohygrine and anahygrine in addition to these lactones. Furthermore, it has been shown that the plant's extract contains withanamides, peroxidases, sitoindosides, reducing sugars, glycosides, withanicil, benzoic acid, phenyl acetic acid, benzyl alcohol, starch, dilcitol, 2-phenyl ethanol and 3, 4, 5-trihydroxy cinnamic acid. More than 40 withanolides 12 alkaloids and several sitoindosides have been reported from *W. somnifera* (Saleem et al. 2020). Withanolides and phenolic compounds are primarily responsible for *W. somnifera*'s widely acclaimed medicinal properties (Kumar et al. 2015).

W. somnifera is found in India, Baluchistan, Pakistan, Afghanistan, Sri Lanka, Congo, South Africa, Egypt, Morocco, and Jordan. India is the leading supplier of the *W. somnifera*. The total dry root production in India is 8,429 tonnes with an approximate area of 10,780 ha. From January 2014 to November 2016, India exported roots of about 8.17 crores accumulating to a total quantity of about 132.72 tons. The United States is the India's largest buyer of *W. somnifera*, accounting for 5.2 crores (USD 779,111 in exports), followed by Australia and China which imported *Withania* worth 57.7 lakhs (USD 84,946) and 54.6 lakhs (USD 80,407), respectively (Singh and Sharma et al. 2018; Hiremath et al. 2021).

It is widely grown in the Indian provinces of Madhya Pradesh, Maharashtra, Uttar Pradesh, Haryana, the plains of Punjab, and the north western states of Gujarat and Rajasthan. Furthermore, it grows wild in Himachal Pradesh and Jammu and Kashmir (Mir et al. 2009). Plants grown in various agroclimatic conditions frequently exhibit qualitative and quantitative variations in their phytoconstituents. *W. somnifera* has five primary chemotypes that originate in different areas and are classified as Form I to Form V. Form I is grown in Madhya Pradesh and is main source of production in India. Form II grows in Rajasthan's sandy deserts, Form III in Chandigarh and other hilly areas of Punjab, Form IV in Delhi and its surrounding areas, and Form V near Delhi and Ahmedabad (Khan and Shah 2016).

In view of the great demand from phyto-pharmaceutical industries, commercial cultivation is carried out in different parts of the country. *W. somnifera* is mainly cultivated through seeds. This cultivation method is associated with issues such as poor seed viability, seed germination (due to ineffective storage conditions and the presence of inhibitory substances in seeds and fruit walls) and variation among the seed raised population resulting in decreased yield and production. To address such issues, new varieties of *W. somnifera* with improved traits are urgently needed. Traditionally the breeding programs were initiated but soon it was observed that these efforts were not sufficient to meet the growing demands. Moreover, the process is lengthy and time-consuming and it fails to provide satisfactory crop yield response. Despite its commercial value, there is a paucity of improved varieties of *W. somnifera* and improvement studies need to be taken vigorously. Therefore, it is important to study the genetic variations among the wild population of species in order to develop improved varieties in short span of time. Once recorded, diversity can be successfully used for crop improvement programmes to develop new and improved varieties with increased dry root weight (Singh and Sharma 2018), high germination rate, and higher withanolide content (Hiremath et al. 2021).

The term "genetic diversity" refers to the traits that parents pass on to their offspring's, whereas "diversity" refers to having a variety of different traits. Genetic diversity refers to the various inherited characteristics within a species. Pollination (genetic) and different geographical conditions cause genetic diversity (epigenetic). Crop variety development and improvement rely on genetic diversity, so it is necessary to precisely study and catalogue the species' genetic diversity at biochemical, morphological and molecular level (Negi et al. 2006). The biochemical and morphological markers provide a limited knowledge as they are also influenced by environmental factors (Bekele and Bekele 2014). As a result, the molecular marker serves as robust tool for studying genetic diversity. Molecular markers work by detecting variations (polymorphism) at DNA level, which can occur in the form of insertion,

deletion, translocation, duplication, or point mutation (Mondini et al. 2009). Molecular markers have many advantages over other markers, such as the ability to be applied at any point of the genome, the lack of pleiotropic or epistatic effects, the ability to co-dominate, and so on (Mondini et al. 2009). As a result, in this study, RAPD (Random Amplified Polymorphic DNA), ISSR (Inter Simple Sequence Repeats), and SCoT (Start Codon Targeted Polymorphism) molecular markers were used to identify genetic diversity in the members of a population of *W. somnifera.*

OBJECTIVES

- To study the morphological variations of *Withania somnifera* (L.) Dunal plants growing at TIET campus.
- To study the genetic diversity in a homogeneous population of *Withania somnifera* (L.) Dunal growing at TIET campus DNA based markers.

Chapter 2: Review of Literature

The assessment of genetic diversity is critical for crop improvement programmes and breeding practises. For proper crop development and maintenance, it is critical to have a thorough understanding of the various quality characteristics of new and improved varieties or genotypes. Furthermore, the reproduction process and environmental conditions are two important factors that influence how characters are expressed and are considered a critical component for both genotypic and phenotypic diversity. It was reported that to obtain the maximum amount of information regarding such genetic diversity, the use of morphological, biochemical, and DNA-based molecular markers is recommended (Sikdar et al. 2010). Molecular markers that are primarily DNA-based produce better results and are more informative (Dekkers and Hospital 2002). Various molecular markers, such as RAPD (Random amplified polymorphic DNA), ISSR (Inter simple sequence repeats) and SCoT (Start codon target polymorphism), have proven to be beneficial by detecting a significant amount of polymorphism (Gogoi et al. 2020)

Mirjalili et al. (2009) examined the genetic diversity between two Iranian natural populations of *W. somnifera* and *W. coagulans* with respect to their withaferin A concentration. Among 16 RAPD markers used, the highest polymorphism was recorded with OPAD -15 primer while lowest polymorphism was recorded with OPC-06 primer. The RAPD results also revealed a significant amount of genetic diversity, which was attributed to differences in geographical conditions and reproduction processes. Withaferin A concentration was also detected by HPTLC analysis. Furthermore, the withaferin A was present in all of the samples tested. The concentration was much higher in the aerial regions than in the root regions. The study concluded that high level of genetic diversity was observed among the Iranian natural

population of *W. somnifera* and *W. coagulans,* and there is a need for conservation of such resources which can also be used for the development of better varieties.

Mir et al. (2011) studied the genetic diversity among the 23 accessions of *W. somnifera*. These accessions were obtained from different locations in India. This study was performed with 8 RAPD primers that detected 37.80% polymorphism. UPGMA analysis was carried out using Jaccard's similarity coefficient matrix which reveals two main clusters one for wild accessions and the other for the cultivated ones. A low similarity value of 0.3 showed that the wild and cultivated accessions were highly distinct. Moreover, cultivated and wild accessions also showed differences in their morphological characteristics. Overall, this revealed a strong correlation between morphological and molecular marker systems.

Tripathi et al. (2012) studied 16 accession of *W somnifera* collected from central India using RAPD and ISSR molecular markers. In this approach 25 RAPD primers were selected for analysis and showed 81.1% polymorphism while 28 ISSR primers showed 74.5% polymorphism. RAPD marker were seen to be more informative as they amplified genotype specific allele which helped in authentication and identification of adulteration in plant species. Similarity matrix for RAPD and ISSR was generated using Jaccard's similarity coefficient. UPGMA clustering was done using dendrogram. Based on the dendrogram it was observed that the given genotypes were clustered into two groups. Moreover, the grouping pattern was observed to be similar in case of RAPD and ISSR markers. This study indicates that RAPD and ISSR polymorphism can be used as a powerful tool for detection of genetic similarity and phylogenetic relationships in *W. somnifera* which can further help in conservation of genetic resources.

Khatak et al. (2013) conducted a study to identify the genetic diversity in 30 variants of *W. somnifera* that were collected from various states of India. In this study a considerable level of variation, across 30 variants of *W. somnifera* were found. These findings were beneficial because they established a distinct DNA fingerprint and demonstrated excellent polymorphism. The findings of this study demonstrated that RAPD patterns can be used to supplement existing techniques for varietal identification and long-term gene bank maintenance, which can be exploited to maximise the efficacy of new cultivar.

Khanna et al. (2013) conducted a study to identify genetic diversity in 14 *W. somnifera* accessions by using chemical, morphological and RAPD molecular markers. In this study, wild accessions were found to be superior to cultivated accessions in terms of morphological characteristics and maximum accumulation of chemical constituents. 5 RAPD primers were used to amplify a total of 12 DNA fragments, out of which 75.00 % were polymorphic. The dendrogram was constructed by using dice index coefficient, which ranged between 0.55 to 0.94. All the accessions were divided into two groups, with the first group consisting of 9 accessions and the second group consisting of the remaining 5 accessions. The dendrogram generated assisted in identifying the most distinct populations (AGB-053 and AGB-055) as well as accessions with the greatest similarity among themselves. The study concluded that when all three chemical, morphological, and RAPD markers are combined, to yield excellent and promising results for assessing the genetic diversity of *W. somnifera*. This can be useful for the conservation and maintenance of species with elite bioactive metabolites.

Sahu et al. (2015) investigated the molecular diversity of 20 ashwagandha genotypes. In this study, 9 RAPD primers were used to produced 46 bands, 41 of which were polymorphic. The UPGMA method was used to generate a dendrogram. The similarity coefficient was calculated to be between 0.37-0.86. The study concluded that there was a high variation among the 20 different genotypes of *W. somnifera*. In the future, these findings may help in improvement of

W. somnifera through genetic modifications and marker assisted selection of lines with desired traits.

Khan and Shah (2016) studied the genetic diversity of 16 genotypes of *W. somnifera* using RAPD and ISSR molecular markers. The samples were collected from four different locations namely Lucknow, Nimuch, Kalasapur (Karnataka) and Mumbai. RAPD and ISSR combinedly produced 89 bands, 39 of which were polymorphic, revealing 47.9% polymorphism and 90.00% population uniformity. The collected samples were also grouped according to their origin as a result of the experiment. The dendrogram revealed that the samples of *W. somnifera* from Kalasapur (Karnataka) and Nimuch were closely related, whereas the samples from Mumbai and Lucknow were evolutionary related. In population analysis, genotypes from Lucknow and Nimuch were divided into two sub-groups with slight diversity, whereas genotypes from Mumbai and Kalasapur (Karnataka) had less diversity within the population.

Tiwari and Shrivastava (2016) investigated the genetic diversity of 11 *W. somnifera* samples. In this study 4 RAPD primers were used which produced clear banding profile. The PIC value ranged from 0.44 to 0.49. The similarity coefficient was in the range of 0.46–0.95 indicating that the genetic distance between the *W. somnifera* samples was high. Jaccard's similarity matrix was used to construct dendrogram. The high genetic diversity among the *W. somnifera* samples was recorded by this dendrogram generated by UPGMA.

Chauhan et al. (2017) investigated the genetic diversity in 25 genotypes of *W. somnifera*. In this study, 15 RAPD primers were used, yielding 150 bands, 141 of which were polymorphic. UPGMA dendrogram was generated using Jaccard's similarity coefficient matrix. The dendrogram classified the 25 genotypes into two cluster: major and minor. 20 genotypes were grouped in major cluster, while 5 genotypes were grouped in the minor cluster. The overall

results showed that genetic diversity was high among these genotypes. It also revealed that genotypes in each cluster have a higher degree of similarity to those in the other cluster.

Hiremath et al. (2021) studied genetic diversity of 9 ashwagandha accessions using ISSR markers. Out of 9 accessions, 4 were collected from CSIR, Bangalore and 5 were local cultivars. In this study 20 ISSR primers were used to amplify 224 bands, 86.10% of which were polymorphic and 13.82% were monomorphic. The PIC and RP value for the accessions ranged from 0.160-0.360, 2.220-7.990 respectively. UPGMA dendrogram classified the accessions into two clusters: major and minor. Major cluster contains 7 accessions while the minor cluster contains only 2 accessions. Furthermore, this study revealed that ISSR-based genetic diversity assessment is an efficient tool for determining similarity and phylogenetic relationships among *W. somnifera* samples collected from different locations.

Chapter 3: Materials and Methods

3.1. Selection of plant materials

Twelve different plants of *W. somnifera* growing at the Thapar Institute of Engineering and Technology campus were randomly selected to identify genetic diversity among them. The selected plants were tagged and labelled as WS-01 to WS-12 (Table 2).

Table 1: Places of collection of *W. somnifera* plant's from TIET campus.

S.no	Plants name	GPS coordinates	
		Latitude	**Longitude**
1	WS-01	30°21'22.5"N	76°22'02.9"E
2	WS-02	30°21'22.5"N	76°22'02.9"E
3	WS-03	30°21'22.5"N	76°22'02.9"E
4	WS-04	30°21'22.5"N	76°22'02.9"E
5	WS-05	30°21'21.4"N	76°22'03.1"E
6	WS-06	30°21'24.7"N	76°22'03.0"E
7	WS-07	30°21'23.8"N	76°22'02.8"E
8	WS-08	30°21'23.8"N	76°21'59.9"E
9	WS-09	30°21'23.8"N	76°21'59.0"E
10	WS-10	30°21'25.2"N	76°22'07.2"E
11	WS-11	30°21'23.4"N	76°21'56.9"E
12	WS-12	30°21'23.7"N	76°21'54.7"E

The selected plants were tagged and used for the study of morphological parameters and genetic diversity.

3.2. Morphological Studies:

Morphological studies were performed by considering various parameters that were measured in three replicates. For statistical data collection, the various parameters were calculated for their mean values and standard error (SE). The following morphological parameters of the selected plants are studied:

1. Shape of the leaves
2. Arrangement of the leaves
3. Leaf venation
4. Average leaf length
5. Average leaf width
6. Average internodal distance
7. Average number of berries in each cluster

Leaf arrangement, shape, and venation in leaves were scored by closely observing their pattern.

3.3. Molecular Analysis

3.3.1 Genomic DNA extraction

The genomic DNA was extracted from the leaf samples of all the 12 selected plants following Cetyl trimethyl ammonium bromide (CTAB) method (Annexure) as reported by Doyle and Doyle (1990). A sample of 3 to 5 g of fresh (young) leaves were collected. The leaf surfaces were cleaned by using ethanol. The sample was grinded in liquid nitrogen with the help of mortar pestle. The sample is transferred to 50 mL Oakridge tube, followed by addition of 10 mL prewarmed extraction buffer and 0.2% (w/v) β-mercaptoethanol. Incubation was done at 60 ℃ for 1 hr. After incubation, equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed for 5 min. Centrifugation was performed at 10,000 g for 15 min at 4℃. To the aqueous phase 0.66 volume of cold isopropanol was added followed by incubation at -20 ℃ for 1 hr. samples were again centrifuged (10000 x g; 15min) and pellets were dissolved in 1

mL of TE buffer (Annexure). To the isolated DNA, 2 µL (10 µg mL^{-1}) RNAase enzyme was added followed by incubation at 37 ℃ for 1 hr. The samples were again extracted with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and centrifuged (10,000 x g;10 min at 4℃). After this, aqueous layer was collected into new vials and 0.3 volume of 3M sodium acetate and 0.6 volume of chilled isopropanol was added followed by incubation at -20 ℃ for 1 hr. The samples were again centrifuged (10000 x g;10min at 4℃) and finally pellets were retained and dissolved in 30 µL TE buffer (Annexure). In all the samples the concentration of DNA was adjusted to 20 ng μL^{-1}.

3.3.2 Quantitative and Qualitative estimation of genomic DNA

Qualitative estimation of isolated DNA was performed through agarose gel electrophoresis. A 0.24 gm of agarose (0.8 % w/v) was weighed and added to 30 mL (0.5X) Tris-acetate EDTA (TAE) buffer (Annexure). The mixture was heated in microwave for 1 min till it was completely dissolved followed by cooling at room temperature (RT). To this molten agarose solution 1µL (0.5µg $mL^{-1)}$ ethidium bromide (EtBr) was added. The molten agarose was then poured in gel electrophoresis casting tray, already inserted with the comb. After complete gelling of agarose, comb was removed and DNA sample (2 µL each) mixed with 6X bromophenol dye (0.5 µL) were loaded in the wells. The electrophoresis was carried out at 50 V for 1 hr and gel was visualized and photographed with the help of UV-transilluminator (Gel Doc Mega: Biosystematics USA). The number of bands produced by each primer, as well as their sizes were determined with the help of a 1kbp (kilobase pair) ladder.

The quantity of DNA sample was estimated with the help of Nanodrop 1000^{TM} UV/VIS spectrophotometer using 1µL of TE buffer as a blank and diluted DNA (20 ng μL^{-1}) as sample.

3.3.3 Testing of Primers

A total of 15 RAPD, 25 ISSR and 30 SCoT primers were tested. Amplification of each primer is performed with DNA of *W. somnifera* plant 1 (WS 1). A total of 10 RAPD, 20 ISSR and 25 SCoT primers were selected based on primers producing clear and scorable bands.

3.3.4 PCR Amplification

The12 DNA samples of *W. somnifera* (WS 1-WS 12) were amplified using each of the selected (RAPD, ISSR, SCoT) primers in the PCR-Veriti 96 Well thermocycler (Applied Biosystem). The reaction mixture in PCR tubes consisted of 2 µL Taq buffer (10X), dNTPs (200 µM), template DNA (20 ng μL^{-1}), 10.0 nmol primer and 1.0 U Taq polymerase. The final volume was made up to 20 µL with sterile MilliQ water. The following amplification conditions were used: initial denaturation at 94 °C for 4 minutes, subsequent denaturation at 94 °C for 45 seconds, annealing at 32 °C for 1.5-minute, extension at 72 °C for 2 minutes, and final extension at 72 °C for 5 minutes, followed by 35 cycles. The amplified products were then separated on 1.2 % (w/v) agarose gel containing 1µL (0.5µg $mL^{-1)}$ ethidium bromide (EtBr) and were visualised and photographed using UV transilluminator (Gel Doc Mega: Biosystematics USA).

3.4. Data scoring and statistical analysis

Binary data was created for each band making present as (1) and band absent for (0). Only clear and unambiguous bands were considered for scoring. This data were then used in NTSYS-pc-v 2.10 (Numerical Taxonomy and Multivariate Analysis System) to create a Jaccard's similarity matrix**.** This Jaccard's similarity matrix helps to build a UPGMA (Unweighted Pair Group Method with Arithmetic Mean) dendrogram. Using the previously mentioned binary data, a scatter plot was also created using Principal Coordinate Analysis (PCA) option in SPSS (Statistical Package for Social Sciences) software for these plants. Other factors like polymorphic information content (PIC), marker index (MI), resolution power (RP), effective

multiplex ratio (EMR) was calculated using formulas:

Table 2: Formulas used to calculate these important factors applied in this study.

S.No.	Factors	Formula
1.	Polymorphic Information Content	PIC→ $2f_i(1-f_i)$
2.	Marker Index	MI→ PIC* EMR
3	Effective Multiplex Ratio	EMR→ n_p (n_p/n)
4.	Resolution Power	RP → lb= 1-(2*(0.5-P) (*Ib is band informativeness)

Chapter 4: Results

1. Morphological analysis

Twelve different plants of *W. somnifera* were randomly selected from the TIET campus. At first mrphological study was conducted using different morphological characters like leaf shape, venation of leaf, arrangement of leaves, average number of berries in a cluster, inter nodal distance, average leaf length and average leaf width (Table 3). It was observed that leaves showed a spiral arrangement while having pinnate venation. The shape of the leaves was noted as oval or ovate. The average leaf length of plant was recorded in a range of 4.25- 5.77 cm, the average leaf width was between 2.66- 4.54 cm, the average internodal distance was recorded in a range between 2.46-3.50 cm and lastly the average number of berries in cluster were in a range between 4.44-5.22.

The data that were collected, implies that there was no significant amount of morphological difference present in leaf shape, width, length, internodal distance and number of barriers in a cluster. It was also noted that the data obtained appeared to be somewhat similar in all 12 plants of *W. somnifera*, which means that the morphological studies alone are insufficient for this study of diversity in selected plants. Hence, in order to obtain insight of genetic diversity, DNA based molecular markers were used to study the selected *W. somnifera* plants.

Table 3: Morphological studies of 12 plants of *W. somnifera* with respect to selected parameters.

Plants No.	Leaf shape	Leaf arrangement	Leaf venation	Average leaf length	Average leaf width	Average internodal distance	Average number of berries in a cluster
WS1	Ovate	Spiral	Pinnate	4.34±0.38	2.66±0.38	2.50±0.31	5.11±0.41
WS2	Ovate	Spiral	Pinnate	4.98±0.56	2.82±0.33	2.50±0.20	5.11±0.11
WS3	Ovate	Spiral	Pinnate	4.60±0.50	2.66±0.17	2.74±0.15	4.44±0.51
WS4	Ovate	Spiral	Pinnate	5.52±0.33	3.67±0.34	2.46±0.27	4.66±0.57
WS5	Ovate	Spiral	Pinnate	4.96±0.30	3.28±0.52	2.74±0.33	4.55±0.41
WS6	Ovate Oval	Spiral	Pinnate	5.10±0.37	3.62±0.56	2.85±0.27	4.88±0.48
WS7	Ovate, Oval	Spiral	Pinnate	5.62±0.54	3.02±0.41	3.50±0.47	4.66±0.91
WS8	Ovate	Spiral	Pinnate	5.77±0.74	3.87±0.42	2.92±0.28	5.22±0.44
WS9	Ovate	Spiral	Pinnate	5.03±0.72	3.66±0.43	2.70±0.40	4.88±0.52
WS10	Ovate, Oval	Spiral	Pinnate	4.25±0.54	4.54±0.37	3.15±0.35	4.77±0.59
WS11	Ovate	Spiral	Pinnate	5.23±0.49	3.55±0.17	3.03±0.24	5.11±0.33
WS12	Oval	Spiral	Pinnate	5.27±0.56	4.18±0.47	2.88±0.25	5.22±0.22

*WS 1- 12 refers to as *W. somnifera* plants 1 to 12.

2. Genomic DNA Isolation

After morphological analysis, the DNA was isolated from the selected plants using CTAB method (Cetyl trimethyl ammonium bromide). The isolated DNA was diluted to the concentration of 40 ng μL^{-1} and quality of isolated DNA was analysed using gel electrophoresis as shown in (Fig 4).

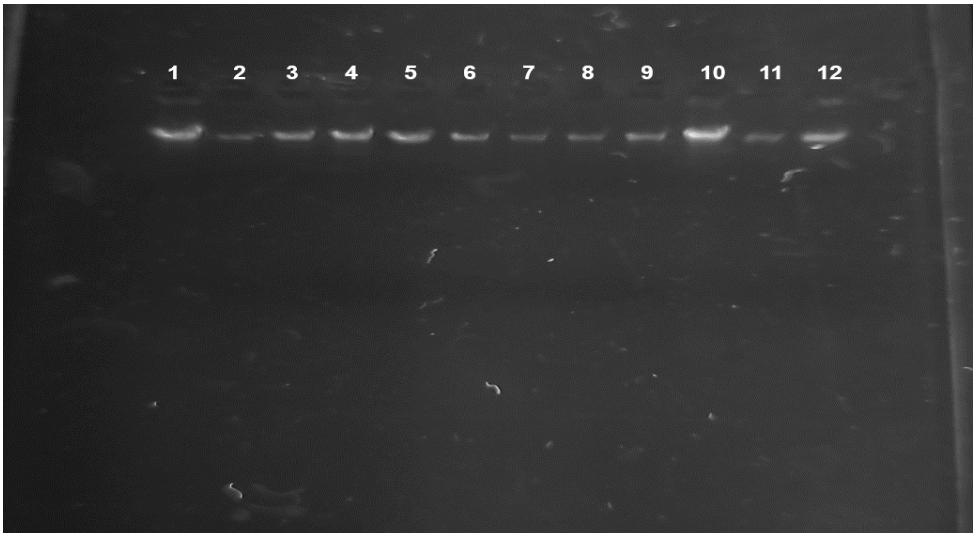

Fig 4: Estimation of isolated DNA for 12 plants of *W. somnifera* on 0.8% agarose gel.

*1- 12 refers to as *W. somnifera* plants 1 to 12.

3. ISSR Analysis

In total 25 ISSR primers were tested out of which 20 ISSR primers were selected. The primers with clear and scorable bands were selected. The DNA isolated from 12 different *W. somnifera* plants were amplified using the ISSR primers (table 4) followed by agarose gel electrophoresis. The amplified bands were recorded in a size range between 250-3000 bp. The highest number of amplified bands were recorded in ISSR 27, with total of 19 bands which were in a size range of 250-2000 bp, whereas the ISSR 1 amplified the minimum number of bands (6). A total of 245 bands were recorded out of which 229 were polymorphic. The total polymorphism calculated was 93.6%. The highest PIC (Polymorphic Information Content) was calculated in ISSR 15 (0.415), whereas lowest PIC value was calculated in ISSR 3 (0.208). The marker index (MI) ranges from 0.52-6.78. ISSR 27 recorded the highest MI value whereas ISSR 3 gave the lowest MI value. The ISSR 24 have highest resolution power (RP) of 26.2 and lowest resolution power was observed in ISSR 15 (6.2). The effective multiplex ratio (EMR) is basically the sum of multiple polymorphic bands and polymorphic band fraction. The average EMR value for ISSR was 10.84.

Table 4: DNA amplification profile, list of ISSR primers and polymorphism generated in *W. somnifera* using 20 ISSR primers.

Markers	Sequence (5'-3')	Size range (bp)	TB	NB	MB	PM (%)	PB	PP (%)	PIC	EMR	MI	RP
ISSR 1	CACACACACACACACG	250-500	38	6	0	0.00	6	100	0.294	6	1.76	6.3
ISSR 2	GAGAGAGAGAGAGACG	250-2000	76	15	0	0.00	15	100	0.309	15	4.63	12.7
ISSR 3	GAGAGAGAGAGAGATC	500-1500	86	10	5	50.00	5	50.00	0.208	2.5	0.52	14.3
ISSR 4	ACACACACACACACGCGC	250-750	46	9	0	0.00	9	100	0.299	9	2.69	7.7
ISSR 6	CACACACACACACATG	250-750	56	10	0	0.00	10	100	0.325	10	3.25	9.3
ISSR 7	CACACACACACACAGC	250-1500	55	12	1	08.33	11	91.66	0.277	10.08	2.79	9.2
ISSR 8	GAGAGAGAGAGAGATA	250-1000	65	11	1	09.09	10	90.90	0.319	9.09	2.9	11.5
ISSR 13	CTCTCTCTCTCTCTA	500-2000	42	9	0	0.00	9	100	0.355	9	3.19	7.0
ISSR 14	CTCTCTCTCTCTCTAG	250-3000	68	13	0	0.00	13	100	0.348	13	4.52	11.3
ISSR 23	GAGAGAGAGAGAGACT	250-2000	102	18	2	11.11	16	88.88	0.306	14.22	4.35	17.0
ISSR 15	GTGTGTGTGTGTGTA	250-2000	37	8	0	0.00	8	100	0.415	8	3.32	6.2
ISSR 16	GTGTGTGTGTGTGTC	250-1000	43	11	0	0.00	11	100	0.372	11	4.09	7.2
ISSR 19	ATATATATATATATGC	250-750	46	10	0	0.00	10	100	0.311	10	3.11	7.7
ISSR 21	GAGAGAGAGAGAGATG	250-1500	82	15	3	20.00	12	80.00	0.291	9.6	2.79	13.7
ISSR 22	GAGAGAGAGAGAGAC	250-750	48	12	0	0.00	12	100	0.326	12	3.91	8.0
ISSR 24	GAGAGAGAGAGAGACA	250-1500	111	18	2	11.11	16	88.88	0.288	14.22	4.09	26.2
ISSR 25	GAGAGAGAGAGAGACC	250-1500	53	12	1	08.33	11	91.66	0.267	10.08	2.69	8.8
ISSR 26	GAGAGAGAGAGAGAT	250-1500	64	12	1	08.33	11	91.66	0.324	10.08	3.26	10.7
ISSR 27	CTCTCTCTCTCTCTTA	250-2000	75	19	0	0.00	19	100	0.357	19	6.78	12.5
ISSR 12	CTCTCTCTCTCTCTGA	250-1500	46	15	0	0.00	15	100	0.307	15	4.60	7.7
		Total	1239	245	16	126.3	229	1873.6	6.298	216.87	69.2	215
		Average	61.9	12.2	0.8	6.315	11.4	93.68	0.3149	10.84	3.46	10.7

(* TB: Total Bands * NB: Number of Bands *MB: Monomorphic Bands *PM: Percent Monomorphism *PB: Polymorphic Bands *PP Percent polymorphism*PIC: Polymorphic information content, *EMR: Effective multiplex ratio, *MI: Marker index and *RP: Resolution power).

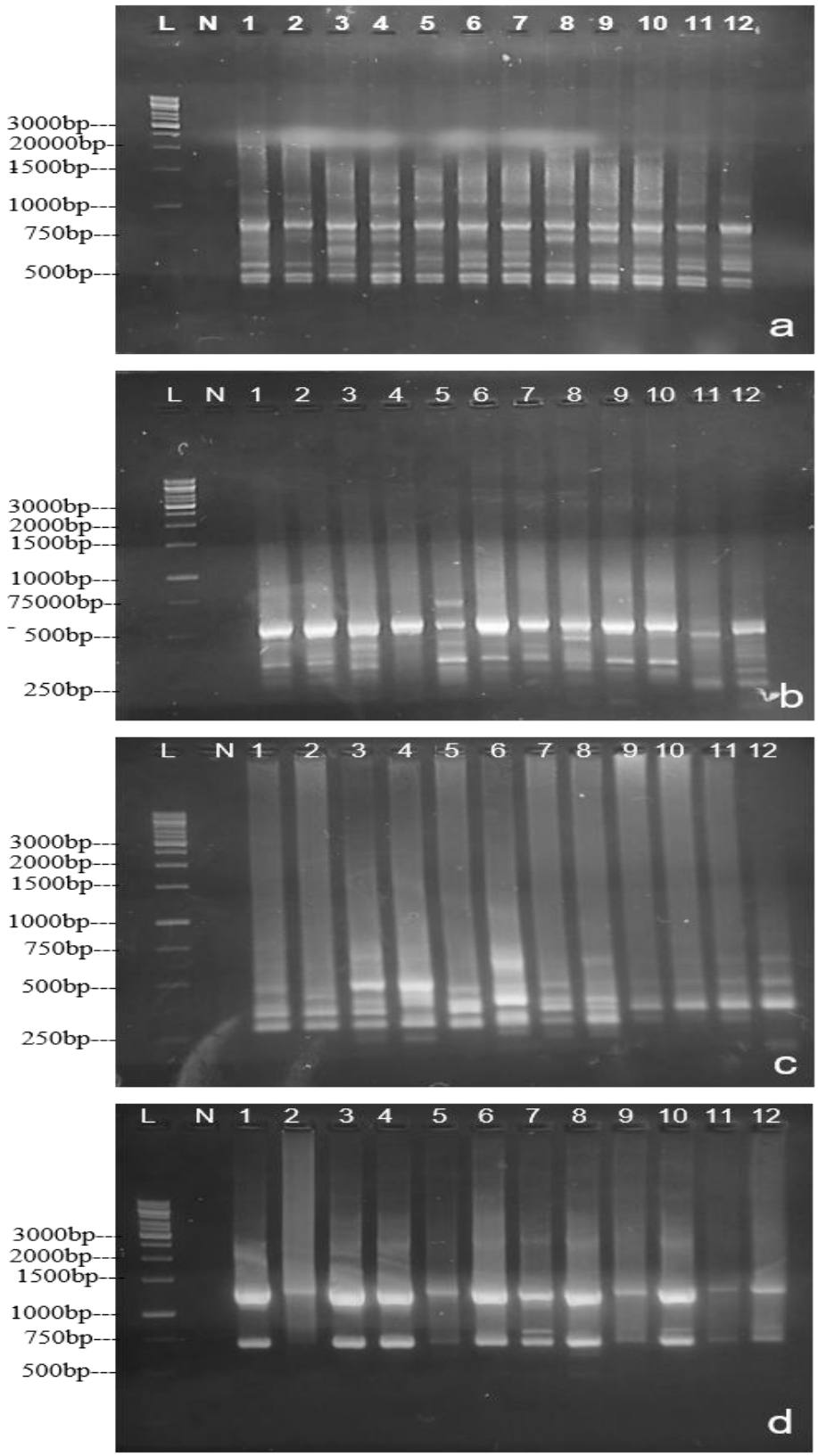

Fig 5: PCR amplification products of genomic DNA of 12 plants of *W. somnifera* using ISSR primers (a) ISSR 3, (b) ISSR 4, (c) ISSR 6 (d) ISSR 13. Lane L:1kbp ladder; Lane N: negative control; Lane 1-12: 12 plants of *W. somnifera*

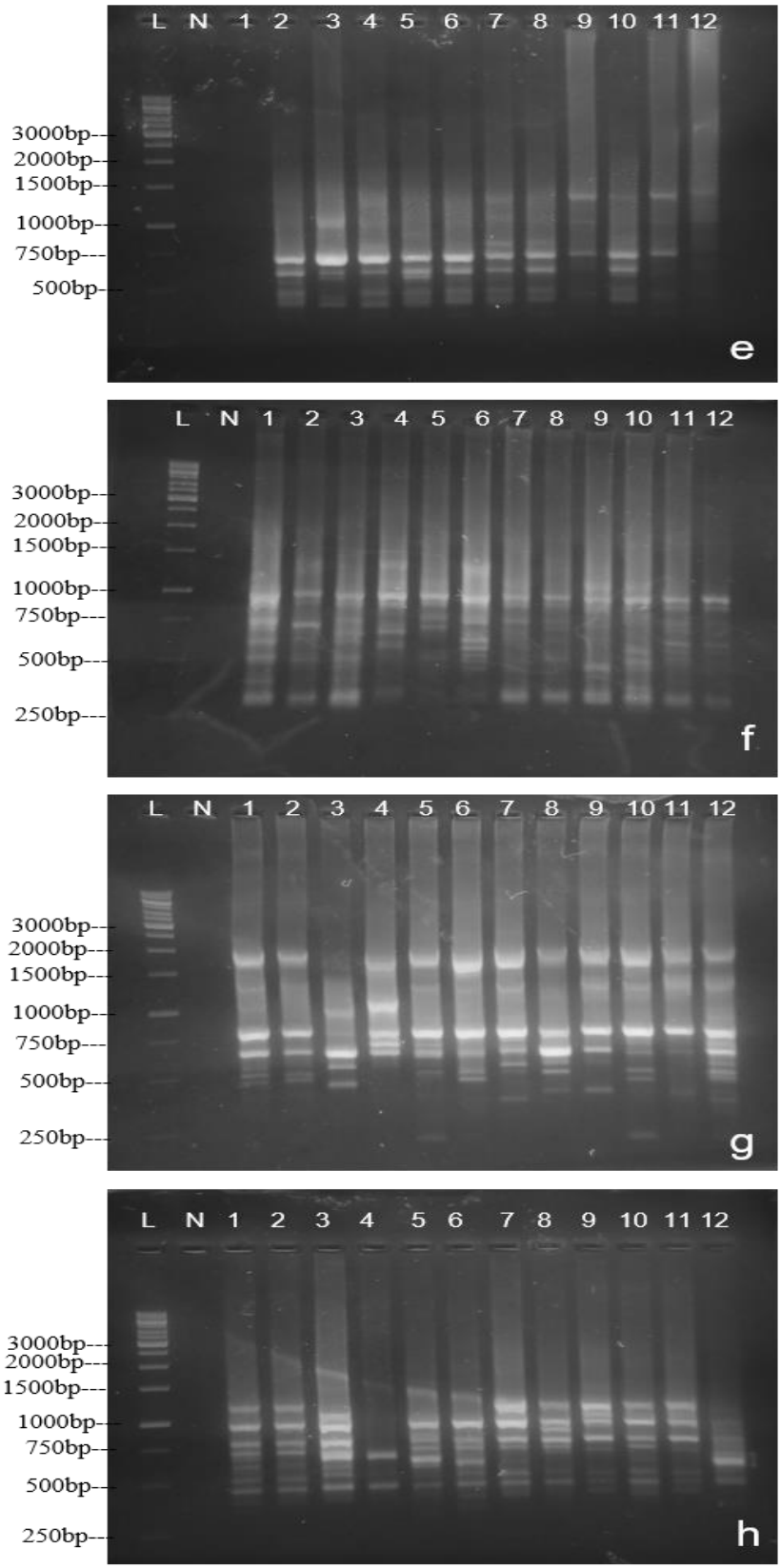

Fig 7: PCR amplification products of genomic DNA of 12 plants of *W. somnifera* using ISSR primers (e) ISSR 24, (f) ISSR 21, (g) ISSR 23 (h) ISSR 24. Lane L:1kbp ladder; Lane N: negative control; Lane 1-12: 12 plants of *W. somnifera*

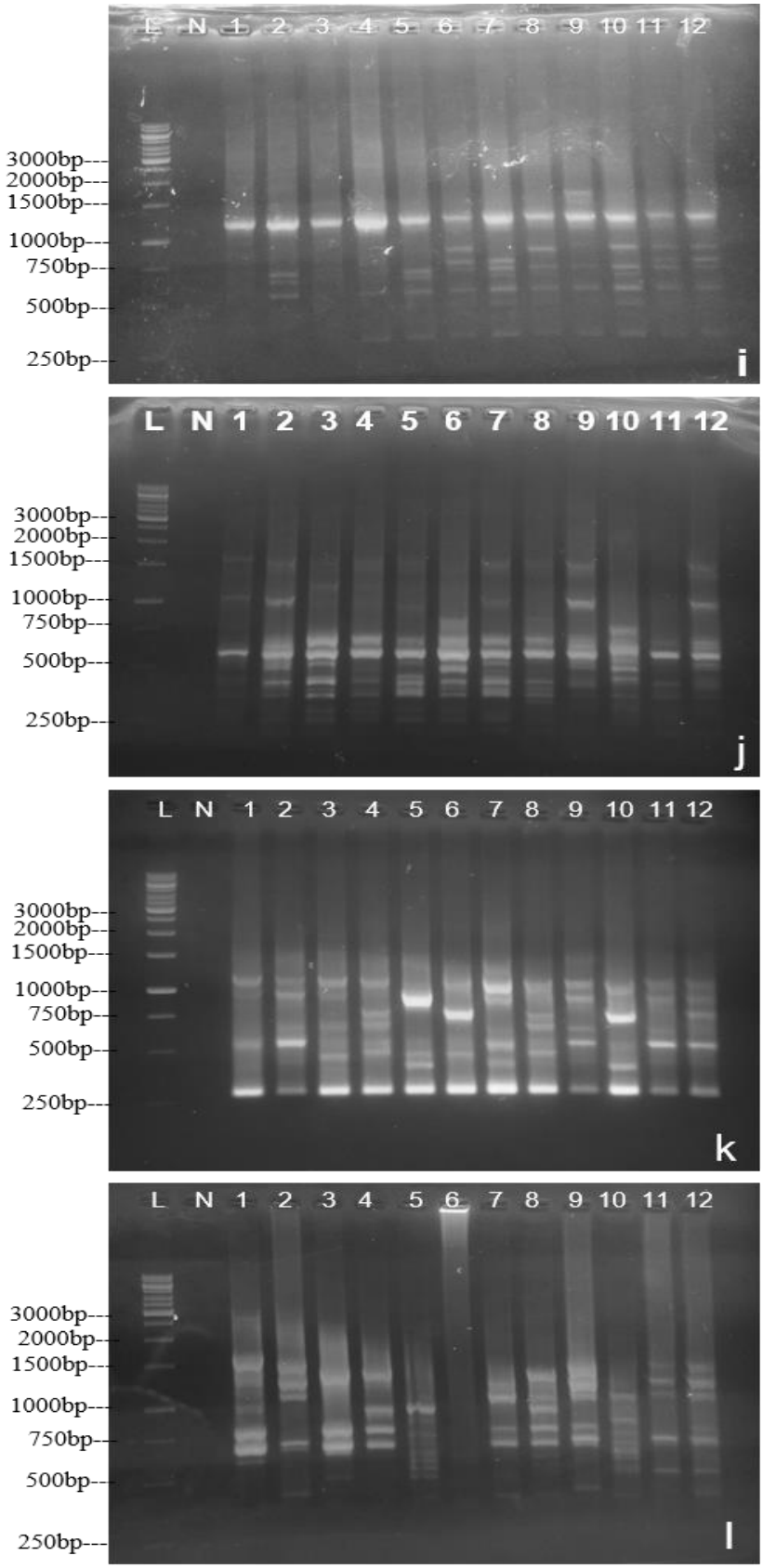

Fig 6: PCR amplification products of genomic DNA of 12 plants of *W. somnifera* using ISSR primers (i) ISSR 8, (j) ISSR 25, (k) ISSR 26 (l) ISSR 27. Lane L:1kbp ladder; Lane N: negative control; Lane 1-12: 12 plants of *W. somnifera*

3.1 Jaccard's Similarity Matrix

The Jaccard's similarity matrix was produced using binary data. This Jaccard's similarity coefficient value ranges from 0.321 to 0.592 (Table 5). These results demonstrate that the plants of *W. somnifera* exhibited substantial genetic variation. The plants WS 8 and WS 10 showed the most genetic similarity, with a similarity coefficient of 0.592, followed by WS 1 and WS 2, with a similarity coefficient of 0.557. Additionally, according to the similarity matrix, plants WS 1 and WS 11 showed the least genetic similarity with a similarity coefficient of 0.321.

Table 5: Jaccard's similarity coefficient matrix among 12 *W. somnifera* plants using ISSR markers.

	WS1	**WS2**	**WS3**	**WS4**	**WS5**	**WS6**	**WS7**	**WS8**	**WS9**	**WS10**	**WS11**	**WS12**
WS1	1.000											
WS2	0.557	1.000										
WS3	0.437	0.420	1.000									
WS4	0.403	0.368	0.519	1.000								
WS5	0.391	0.387	0.401	0.449	1.000							
WS6	0.383	0.411	0.467	0.424	0.462	1.000						
WS7	0.389	0.395	0.444	0.503	0.478	0.476	1.000					
WS8	0.396	0.432	0.443	0.433	0.439	0.507	0.519	1.000				
WS9	0.401	0.417	0.401	0.383	0.391	0.403	0.506	0.527	1.000			
WS10	0.381	0.438	0.374	0.374	0.524	0.462	0.506	0.592	0.493	1.000		
WS11	0.321	0.422	0.347	0.347	0.366	0.459	0.428	0.465	0.493	0.462	1.000	
WS12	0.324	0.350	0.357	0.331	0.350	0.358	0.407	0.354	0.366	0.384	0.414	1.000

*WS 1- 12 refers to as *W. somnifera* plants 1 to 12.

3.2 UPGMA dendrogram:

The ISSR cluster tree analysis was carried out on Jaccard's similarity matrix by using UPGMA method. It was observed that the 12 *W. somnifera* plants were grouped into two clusters, one major and one minor cluster. The major cluster contains 11 plants. It is further divided into, the subcluster I having two plants WS 1 and WS 2 with 30% bootstrap value. The subcluster II has two plants WS 3 and WS 4 with 70% bootstrap value. The subcluster III has four plants WS 7, WS 8, WS 9 and WS 10 with 81% bootstrap value. The subgroup IV comprises of rest remaining 5 plants that are WS 5, WS 6, and WS 11. It is speculated that high genetic diversity is observed among the plants which have low similarity coefficient value of 0.32. The second minor cluster contain WS 12 plants which have a 31% of genetic similarity.

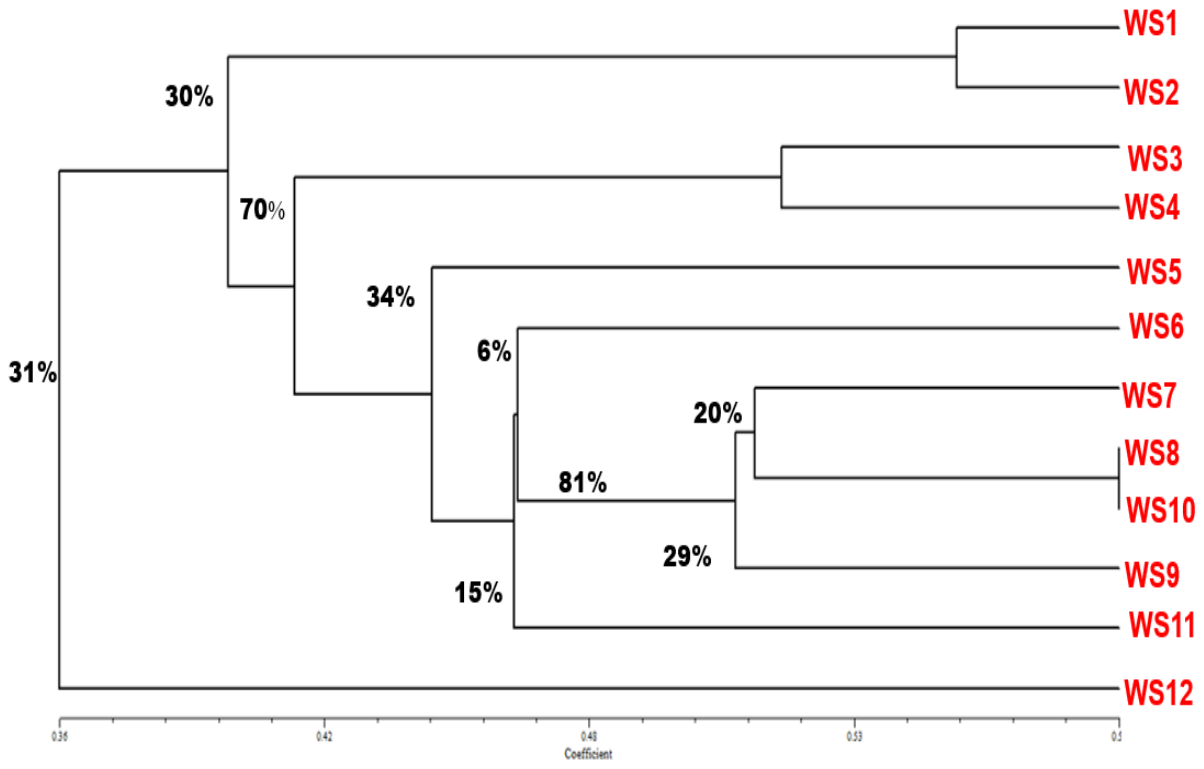

Fig 8: A dendrogram constructed from ISSR data collected for homogeneous population *of W. somnifera* using UPGMA method.

* WS 1- 12 refers to as *W. somnifera* plants 1 to 12

3.3 Principal Component analysis (PCA)

Using PCA, a scatter plot of all 12 *W. somnifera* plants was generated. In figure 9, component 1 and component 2 had total variances of 36.53% and 9.83% respectively. There were two groupings for the plants. The group I has plants WS 1, WS 3, and WS 4 and the remaining plants i.e., WS 5, WS 6, WS 7, WS 8, WS 9, WS 10 and WS 11, were all present in group II. It was observed that the WS 12 and WS 2 plants were out grouped.

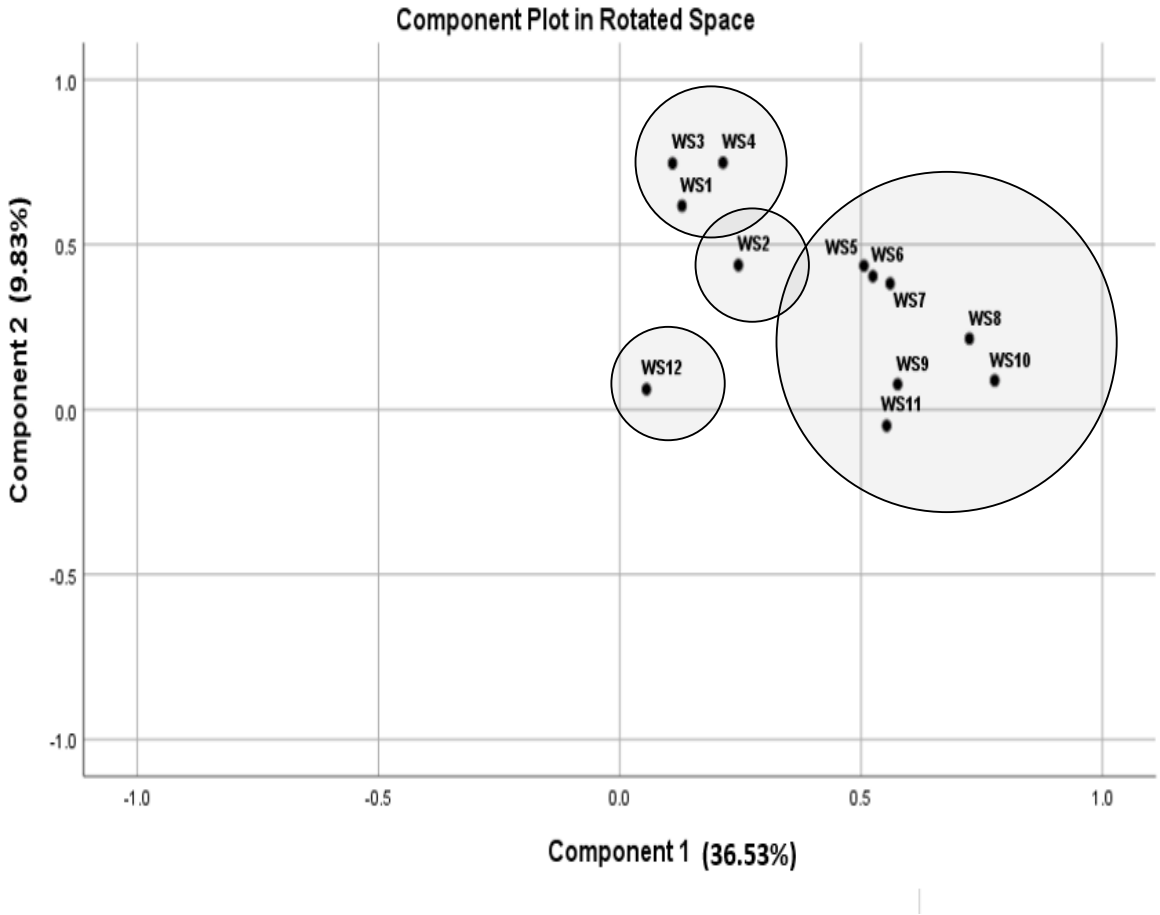

Fig 9: Two dimensional PCA scaling of 12 plants of *W. somnifera* using ISSR data

* WS 1- 12 refers to as *W. somnifera* plants 1 to 12.

4. SCoT Analysis

25 SCoT primers were used, for the amplification of genomic DNA of 12 selected plants of *W. somnifera*. The results obtained are shown in Table 6. The amplified bands recorded were observed to be in a size range from 250 bp to 2000 bp. The highest number of amplified bands were recorded in SCoT 3 and SCoT 18 with total number of 12 bands, which were in a size range from 250 bp to 750 bp. Whereas, the SCoT 1, SCoT 11, SCoT 24 were recorded to amplify lowest number of bands (5), giving an average of about 8 bands per primer. Twenty-five SCoT primers amplified a total of 200 bands, out of which 118 were polymorphic (56.6%) and remaining were monomorphic (45.1%). The highest polymorphism was detected in SCoT 9, SCOT 15, SCoT 23, SCoT 31 and SCoT 34 (100%), while the highest monomorphism was detected in 2 primers SCoT 2 and SCoT 22 (100%). The average percent polymorphism was noted to be 56.65%.

The highest polymorphic information content (PIC) calculated was in SCoT 15 (0.391), whereas lowest PIC value was calculated in SCoT 2, SCoT 22 (0). Marker index (MI) was calculated by product of PIC and EMR. It ranges from 0 to 0.47, with SCoT 16 giving the highest marker index value. Amplification with primer SCoT 8 recorded the highest resolution power of 17.7 and lowest resolution power was observed in SCoT 1 (6.3). The effective multiplex ration (EMR) is basically the sum of multiple polymorphic bands and polymorphic band fraction. The highest EMR value was recorded in two primers; Scot 2 and Scot 22 (6) whereas, lowest EMR was recorded in four primers that were SCoT 9, SCoT 15, SCoT 21, SCoT 31 and SCoT 34. The average EMR value for SCoT primer was calculated to be 1.94.

Table 6: DNA amplification profile, list of SCoT primers and polymorphism generated in *W. somnifera* using 25 SCoT primers.

Markers	Sequence (5'-3')	Size range (bp)	TB	NB	MB	PMB (%)	PB	PPB (%)	PIC	EMR	MI	RP
SCoT 1	CAACAATGGCTACCACCA	1500-2000	38	5	3	60.00	2	40.00	0.061	1.8	0.109	6.3
SCoT 2	CAACAATGGCTACCACCC	250-750	72	6	6	100	0	0.00	0	6	0	12.0
SCoT 3	CAACAATGGCTACCACCG	250-750	101	12	2	16.66	10	83.33	0.212	0.33	0.07	16.8
SCoT 4	CAACAATGGCTACCACCT	250-1500	81	9	5	55.55	4	44.44	0.162	2.77	0.45	13.5
SCoT 5	CAACAATGGCTACCACGA	250-1000	70	6	5	88.33	1	16.66	0.046	4.16	0.19	11.7
SCoT 6	CAACAATGGCTACCACGC	250-1000	65	8	4	50.00	4	50.00	0.214	2.00	0.428	10.8
SCoT 8	CAACAATGGCTACCACGT	250-1000	106	11	4	36.36	7	63.63	0.184	1.45	0.267	17.7
SCoT 9	CAACAATGGCTACCAGCA	250-1000	82	8	0	0.00	8	100.0	0.243	0.0	0	13.7
SCoT 10	CAACAATGGCTACCAGCC	250-750	74	9	4	44.44	5	55.55	0.201	1.77	0.35	12.3
SCoT 11	CAACAATGGCTACCACCA	250-1500	39	5	1	20.00	4	80.00	0.303	0.2	0.060	6.5
SCoT 12	ACGACATGGCGACCAACG	250-750	63	7	5	71.42	2	28.57	0.062	3.57	0.221	10.5
SCoT 13	ACGACATGGCGACCATCG	250-1000	84	10	2	20.00	8	80.00	0.169	0.4	0.067	14.0
SCoT 14	ACGACATGGCGACCACGC	250-750	59	8	3	37.50	5	62.50	0.189	1.12	0.212	9.8
SCoT 15	ACGACATGGCGACCGCGA	250-1500	57	8	0	0.00	8	100	0.391	0.0	0	9.5
SCoT 16	ACCATGGCTACCACCGAC	250-1000	81	9	5	55.55	4	44.44	0.171	2.77	0.475	13.5
SCoT 17	ACCATGGCTACCACCGAG	250-1000	111	10	6	60.00	4	40.00	0.113	3.6	0.406	17.2
SCoT 18	ACCATGGCTACCACCGCC	250-750	86	12	2	16.66	10	83.33	0.222	0.33	0.074	14.3
SCoT 21	ACGACATGGCGACCCACA	250-500	43	6	3	50.00	3	50.00	0.248	0.0	0	7.2
SCoT 22	AACCATGGCTACCACCAC	250-750	43	6	6	100	0	00	0	6.0	0	12.0
SCoT 23	CACCATGGCTACCACCAG	250-1000	42	10	4	40.00	6	100.0	0.218	1.6	0.348	16.2
SCoT 24	CACCATGGCTACCACCAT	250-750	55	5	4	80.00	1	20.00	0.097	3.2	0.310	9.2
SCoT 26	ACCATGGCTACCACCGTC	250-500	69	6	5	83.33	1	16.66	0.062	4.16	0.258	11.5
SCoT 27	ACCATGGCTACCACCGTG	250-500	62	7	3	42.85	4	57.14	0.155	1.28	0.199	10.3
SCoT 31	CCATGGCTACCACCGCCT	250-1500	56	8	0	0.00	8	100.0	0.264	0.0	0	9.3
SCoT 34	ACCATGGCTACCACCGCA	250-1500	41	9	0	0.00	9	100.0	0.363	0.0	0	6.8
		Total	1680	200	82	1128.6	118	1416.25	4.35	48.5	4.49	292.6
		Average	67.2	8	3.28	45.14	4.96	56.65	0.174	1.94	0.17	11.7

(* TB: Total Bands * NB: Number of Bands *MB: Monomorphic Bands *PM: Percent Monomorphism *PB: Polymorphic Bands *PP Percent polymorphism*PIC: Polymorphic information content, *EMR: Effective multiplex ratio, *MI: Marker index and *RP: Resolution power).

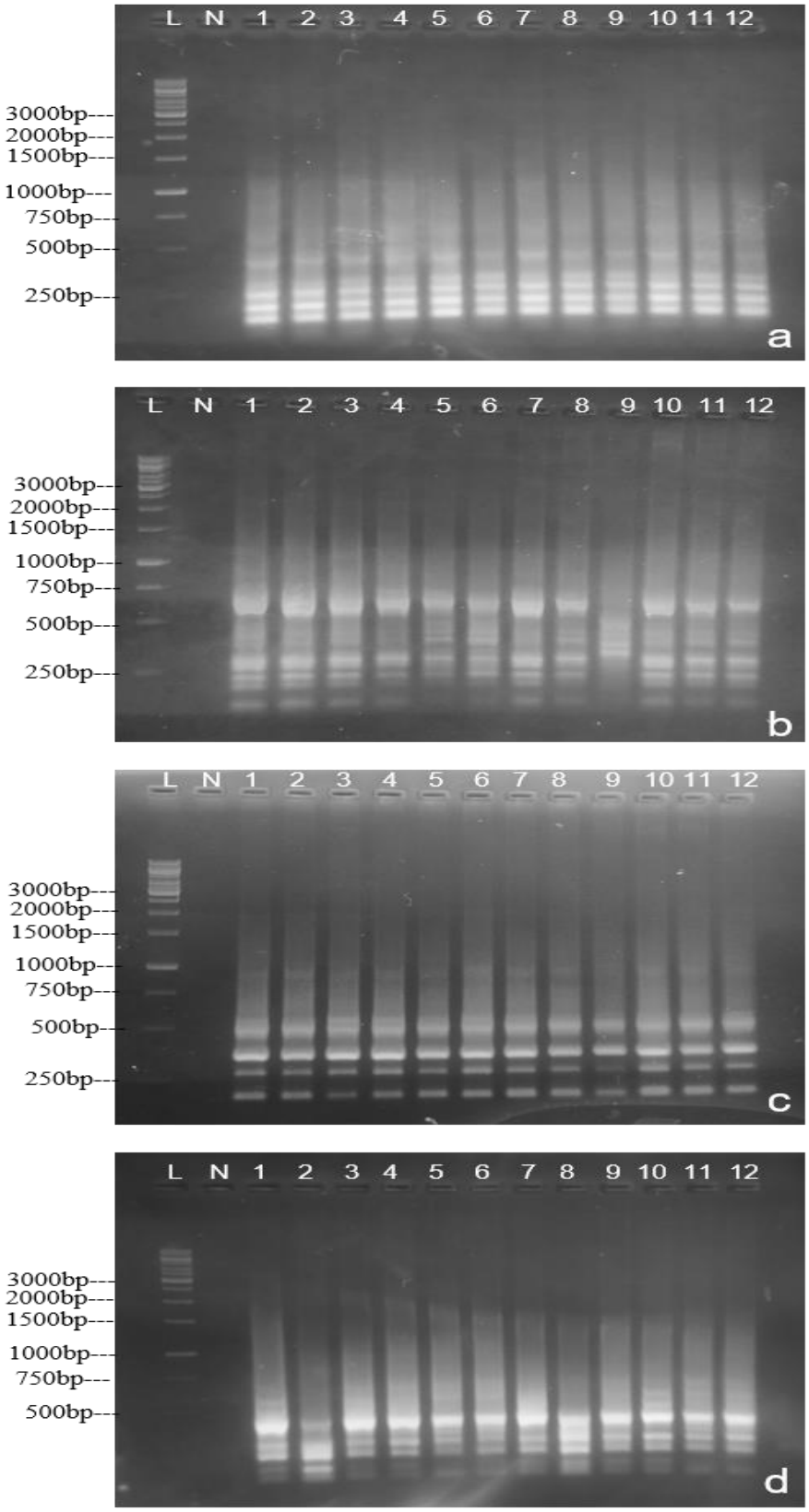

Fig 10: PCR amplification products of genomic DNA of 12 plants of *W. somnifera* using SCoT primers (a) SCoT 2, (b) SCoT 3, (c) SCoT 5 (d) SCoT 10. Lane L:1kbp ladder; Lane N: negative control; Lane 1-12: 12 plants of *W. somnifera*

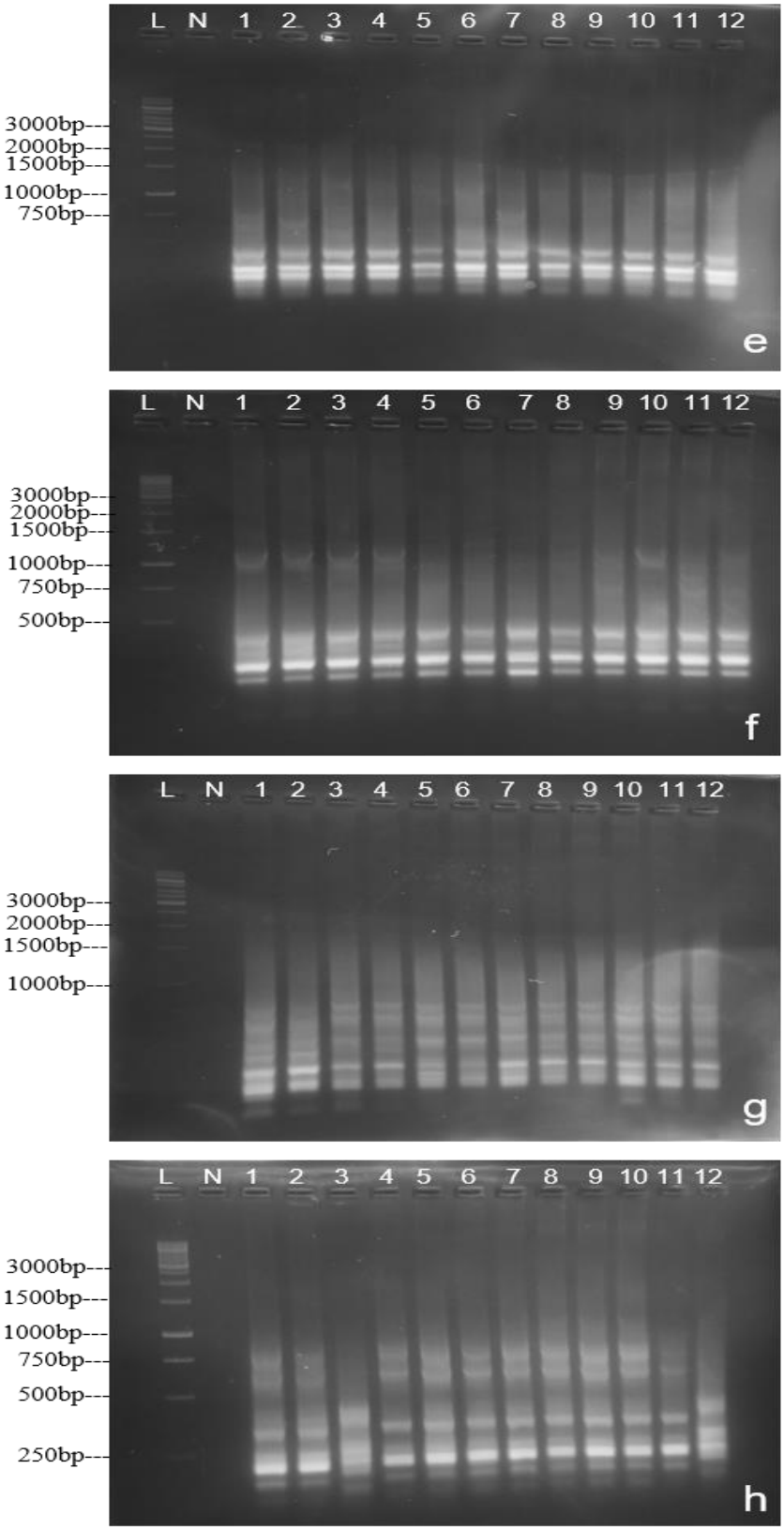

Fig 11: PCR amplification products of genomic DNA of 12 plants of *W. somnifera* using SCoT primers (e) SCoT 13, (f) SCoT 16, (g) SCoT 17 (h) SCoT 18. Lane L:1kbp ladder; Lane N: negative control; Lane 1-12: 12 plants of *W. somnifera*

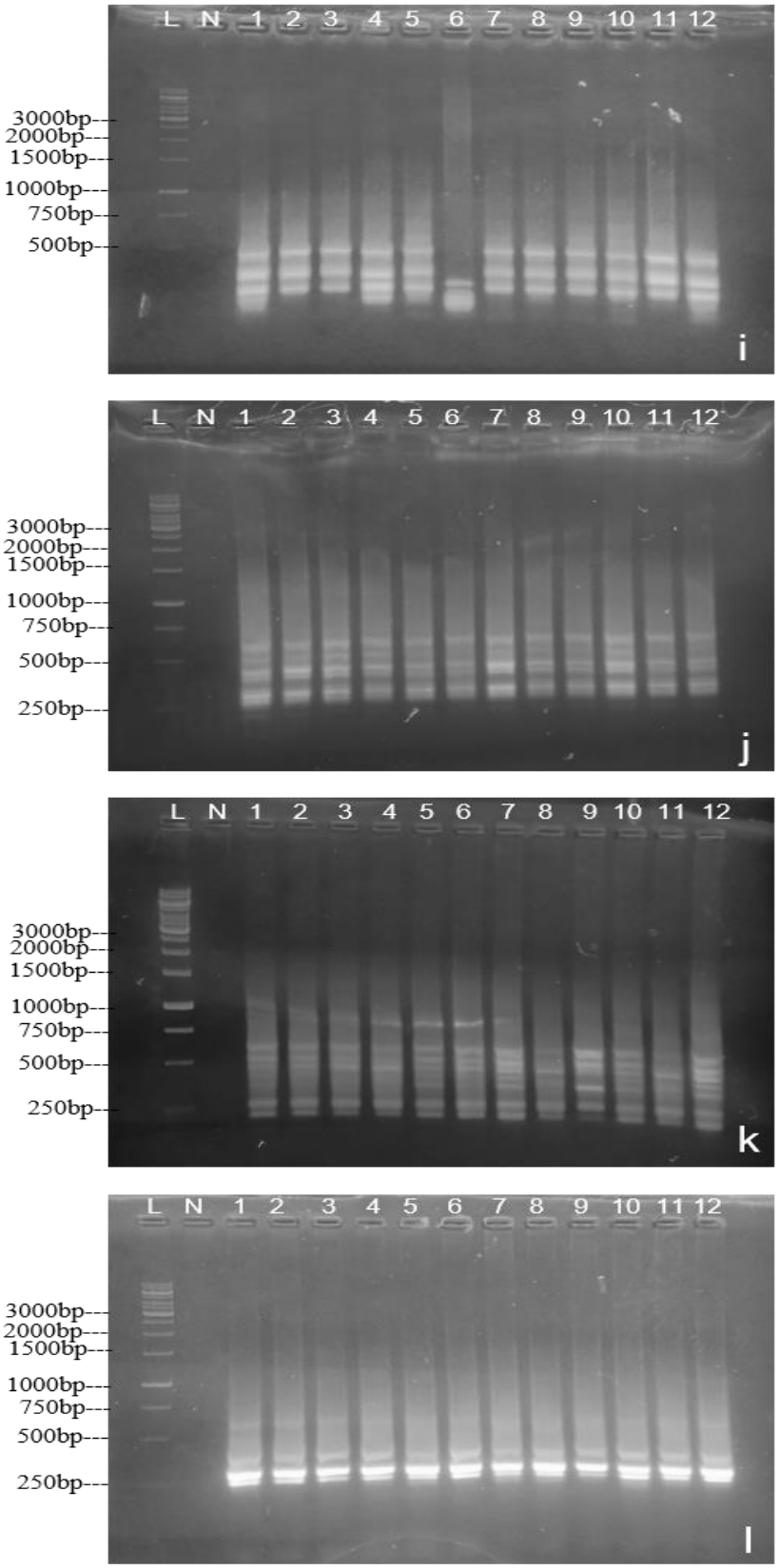

Fig 12: PCR amplification products of genomic DNA of 12 plants of *W. somnifera* using SCoT primers (i) SCoT 21, (j) SCoT 22, (k) SCoT 23 (l) SCoT 24. Lane L:1kbp ladder; Lane N: negative control; Lane 1-12: 12 plants of *W. somnifera*

4.1 Jaccard's Similarity Matrix

The Jaccard's similarity matrix was produced using binary data. According to Table 7, Jaccard's similarity coefficient value ranges from 0.625 to 0.859. These results demonstrate that the plants of *W. somnifera* exhibited substantial genetic variation. The plants WS 1 and WS 4 showed the most genetic similarity, with a similarity coefficient of 0.859, followed by WS 1 and WS 2, with a similarity coefficient of 0.840. Additionally, according to the similarity matrix plants WS 9 and WS 11 showed the least genetic similarity with a similarity coefficient of 0.625.

Table 7: Jaccard's similarity coefficient matrix among 12 *W. somnifera* plants using SCoT markers.

	WS1	**WS2**	**WS3**	**WS4**	**WS5**	**WS6**	**WS7**	**WS8**	**WS9**	**WS10**	**WS11**	**WS12**
WS1	1.000											
WS2	0.840	1.000										
WS3	0.822	0.823	1.000									
WS4	0.859	0.802	0.807	1.000								
WS5	0.804	0.783	0.799	0.834	1.000							
WS6	0.729	0.720	0.714	0.745	0.804	1.000						
WS7	0.792	0.835	0.798	0.778	0.822	0.781	1.000					
WS8	0.752	0.764	0.768	0.780	0.794	0.728	0.782	1.000				
WS9	0.665	0.716	0.690	0.720	0.744	0.681	0.756	0.813	1.000			
WS10	0.753	0.785	0.789	0.801	0.793	0.721	0.813	0.784	0.768	1.000		
WS11	0.724	0.675	0.720	0.730	0.755	0.700	0.715	0.723	0.625	0.726	1.000	
WS12	0.753	0.725	0.789	0.728	0.804	0.701	0.782	0.753	0.697	0.774	0.768	1.000

*WS 1- 12 refers to as *W. somnifera* plants 1 to 12

4.2 UPGMA dendrogram

Using the Jaccard's similarity coefficient values for 12 plants of *W. somnifera*, a dendrogram was created. Two clusters—Cluster I and Cluster II—were established in this dendrogram and subsequently subdivided into two sub-cluster.

- WS 11 and WS 12 were the only plants present in Cluster I, which is supported by 36% bootstrap value. Representing more similarity between them.
- Cluster II consist of 10 plants which were further subdivided into 2 subgroup the first subgroup has three plants WS 6, WS 8 and WS 9 having a bootstrap value of 17%. The second subgroup consists of 8 plants WS 1, WS 2, WS 3, WS 4, WS 5, WS 7 and WS 10. These plants show 62% of genetic similarity among themselves.

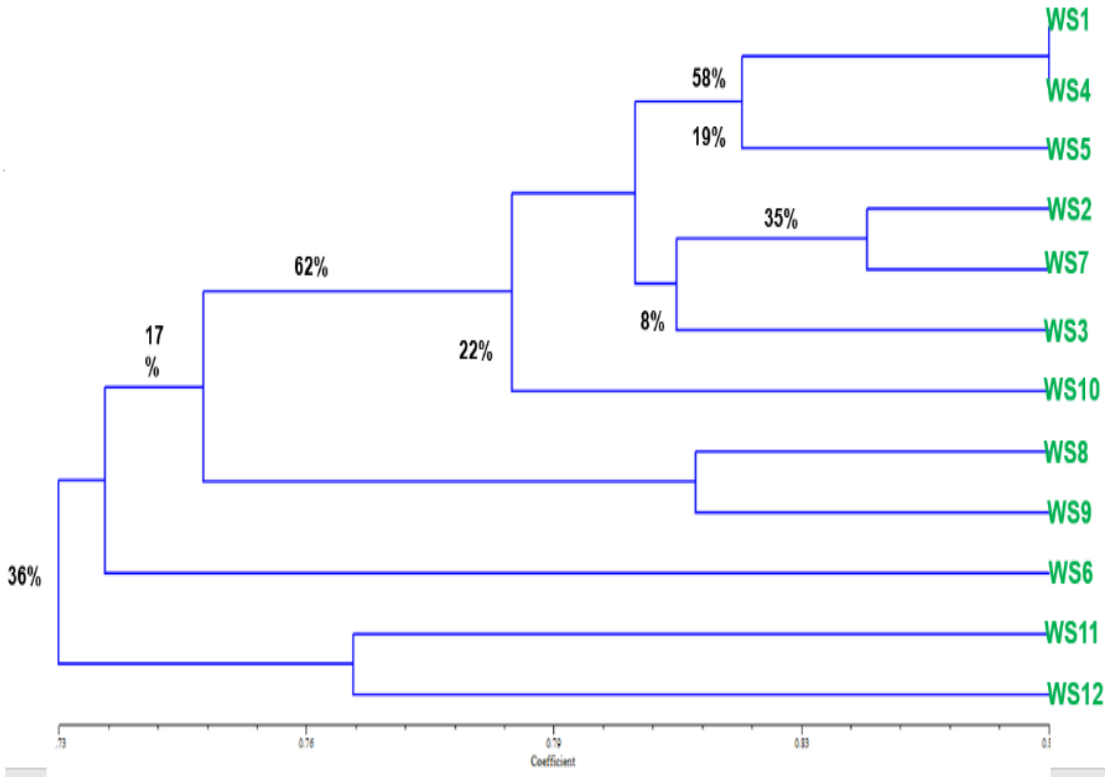

fig 13: A dendrogram constructed from SCoT data collected for homogeneous population *of W. somnifera* using UPGMA method.

* WS 1- 12 refers to as *W. somnifera* plants 1 to 12

5. RAPD Analysis

A total of 15 RAPD primers were tested, out of which 10 RAPD primers were selected. Only the primers showing clear and scorable bands were selected. The DNA isolated from 12 different *W. somnifera* plants was amplified by using the RAPD primer and agarose gel electrophoresis was carried out for further analysis. The results recorded are shown in table 8. After amplification by 10 RAPD primers, the bands produced were in a size range from the 250bp to 6000bp. The highest number of bands were obtained in RAPD 24 with total of 16 amplified bands under size range of 250bp to 6000bp, whereas the lowest number of bands were produced in RAPD 34 and RAPD 38 (5), giving an average of about 9.5 bands per primer. Ten RAPD primers amplified a total of 95 bands, out of which 94 were polymorphic (98%) And 1 were monomorphic (1%). The highest polymorphism was detected in RAPD (4,13,21,22,24,36,37,38,40) primer (100%), while the lowest polymorphism was detected in primer RAPD 34 (80%). The average percent polymorphism was noted to be 98%.

The highest PIC value was obtained in RAPD 38 (0.403) and lowest PIC value was observed in RAPD 36 (0.282). Marker index ranged from 0.979 to 4.65, with highest marker index value of RAPD 24 and lowest of RAPD 34. Amplification with primer RAPD 22 resulted in highest resolution power 9.5 and lowest resolution power was observed in RAPD 38 (4.5). The highest EMR value was recorded in primers RAPD 24 (14.06) whereas, the lowest EMR was recorded in RAPD 34 (3.2). The average EMR value for RAPD primer was calculated as 9.12.

Table 8: DNA amplification profile, list of RAPD primers and polymorphism generated in *W. somnifera* using 10 RAPD primers

Markers	Sequence (5'-3')	Size range (bp)	TB	NB	MB	PMB (%)	PB	PPB (%)	PIC	EMR	MI	RP
RAPD 4	CTGGGGGACT	500-3000	34	10	0	0.00	10	100	0.358	10	3.58	4.8
RAPD 13	CTCTGGAGAC	1000-3000	43	9	0	0.00	9	100	0.344	9	3.096	7.2
RAPD 21	CAGGCCCTTC	500-1500	39	9	0	0.00	9	100	0.387	9	3.483	6.5
RAPD 22	TGCCGAGCTG	1000-3000	57	10	0	0.00	10	100	0.399	10	3.99	9.5
RAPD 24	AATCGGGCTG	250-6000	55	16	0	0.00	16	100	0.331	14.062	4.65	9.2
RAPD 34	TCTGTCCTGG	250-1000	32	5	1	20.00	4	80.0	0.306	3.2	0.979	5.3
RAPD 36	AGCCAGCGAA	250-1500	46	12	0	0.00	12	100	0.282	12	3.384	7.7
RAPD 37	GACCGCTTGT	750-6000	45	8	0	0.00	8	100	0.387	8	3.096	7.5
RAPD 38	AGGTGACCGT	250-1500	27	5	0	0.00	5	100	0.403	5	2.015	4.5
RAPD 40	GTTGCGATCC	500-3000	41	11	0	0.00	11	100	0.324	11	3.564	6.8
		Total	419	95	1	20	94	980	3.521	91.26	31.83	69
		Average	41.9	9.5	0.1	2	9.3	98	0.3521	9.126	3.18	6.9

(* TB: Total Bands * NB: Number of Bands *MB: Monomorphic Bands *PM: Percent Monomorphism *PB: Polymorphic Bands *PP Percent polymorphism*PIC: Polymorphic information content, *EMR: Effective multiplex ratio, *MI: Marker index and *RP: Resolution power).

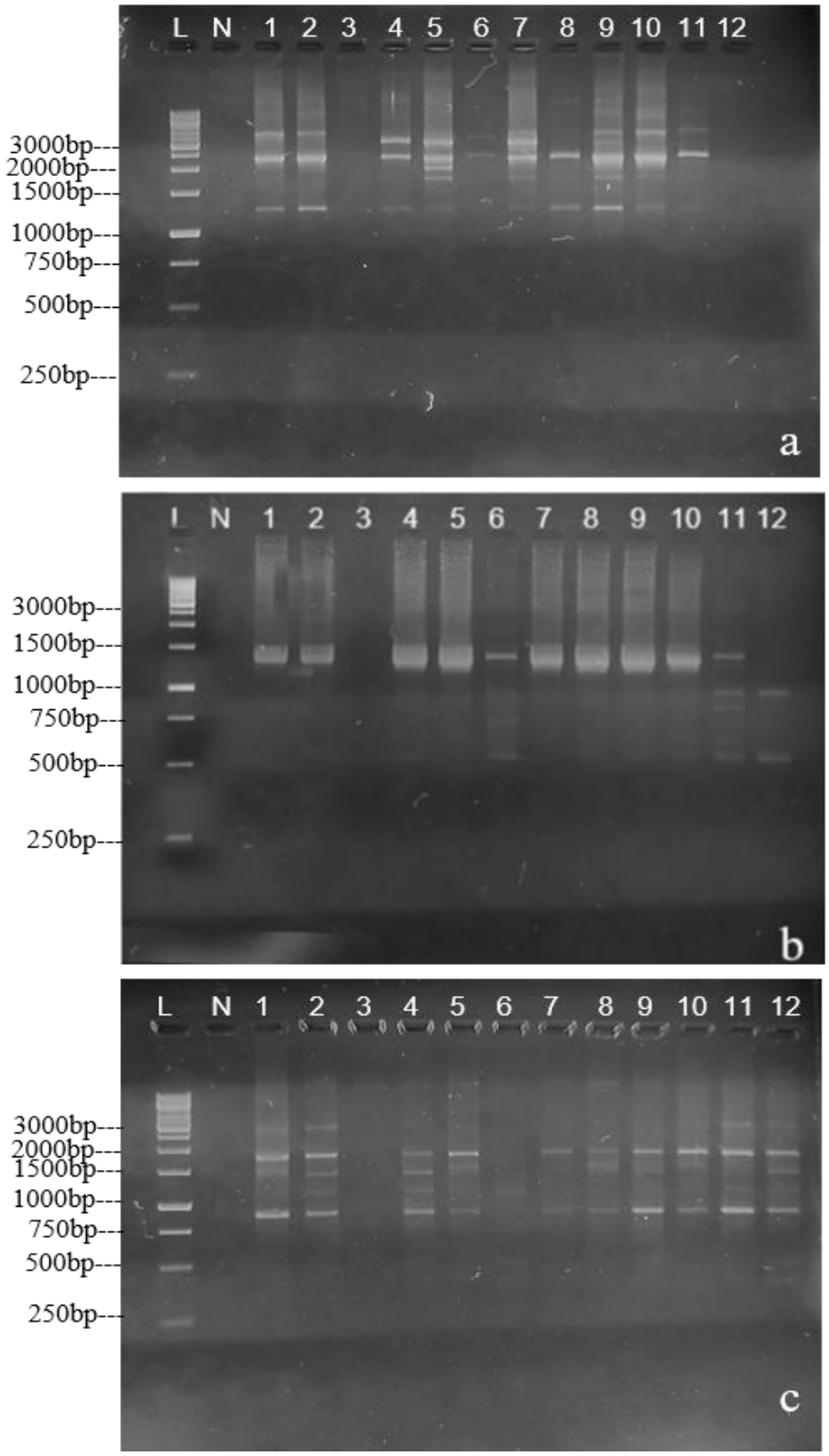

Fig 14: PCR amplification products of genomic DNA of 12 plants of *W. somnifera* using RAPD primers (a) RAPD 13, (b) RAPD 21, (c) RAPD 22. Lane L:1kbp ladder; Lane N: negative control; Lane 1-12: 12 plants of *W. somnifera*

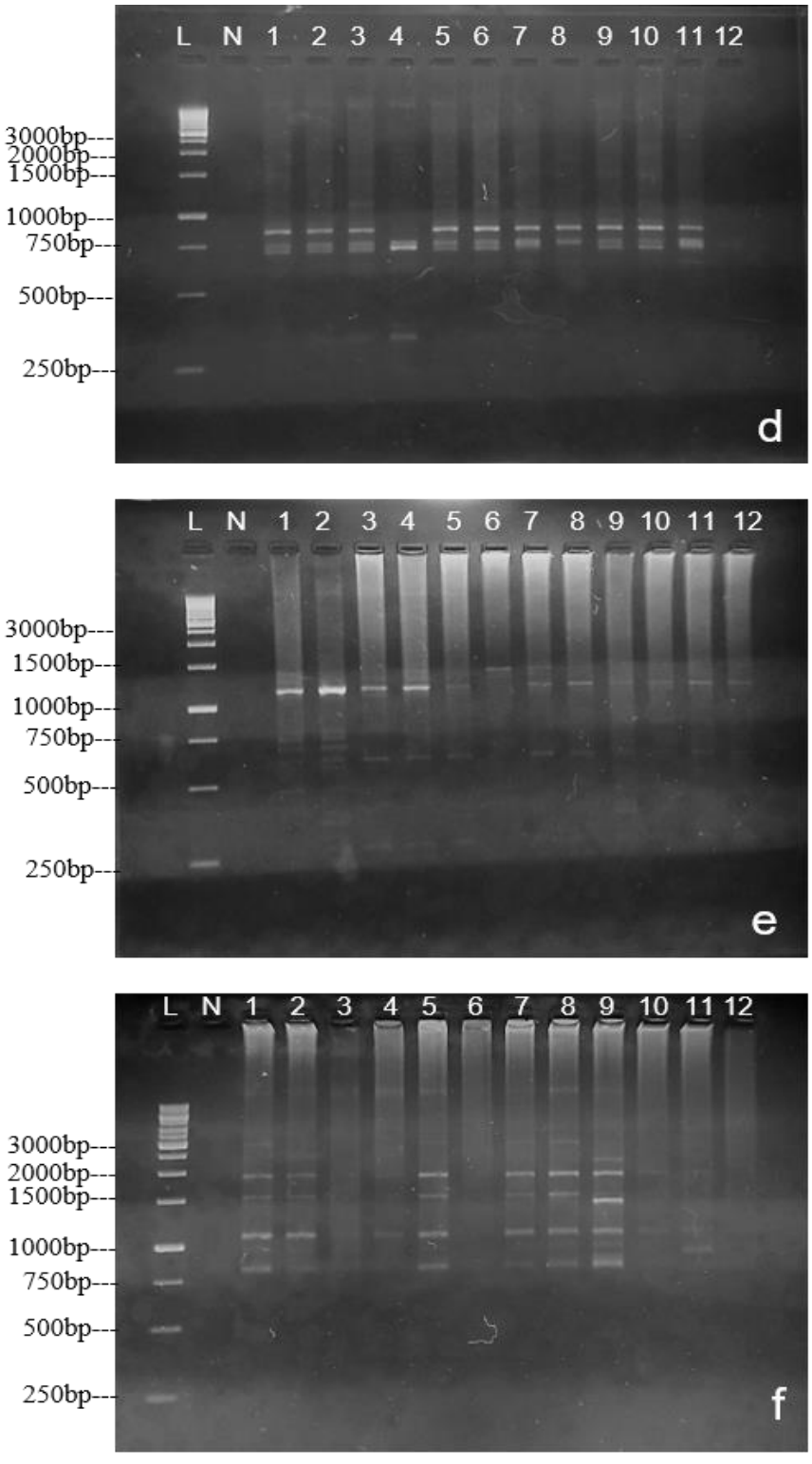

Fig 15: PCR amplification products of genomic DNA of 12 plants of *W. somnifera* using RAPD primers (d) RAPD 34, (e) RAPD 36, (f) RAPD 37. Lane L:1kbp ladder; Lane N: negative control; Lane 1-12: 12 plants of *W. somnifera*

5.1 Jaccard's Similarity Matrix

The Jaccard's similarity matrix was created using binary data. According to Table 9, Jaccard's similarity coefficient values range from 0.086 to 0.540. These results demonstrate that the plants of *W. somnifera* exhibited substantial genetic variation. The plants WS 9 and WS 10 showed the most genetic similarity, with a similarity coefficient of 0.540, followed by WS 1 and WS 2, with a similarity coefficient of 0.536. According to the similarity matrix plants WS 3 and WS 12 showed the least genetic similarity with a similarity coefficient of 0.086.

Table 9: Jaccard's similarity coefficient matrix among 12 *W. somnifera* plants using RAPD markers.

	WS1	**WS2**	**WS3**	**WS4**	**WS5**	**WS6**	**WS7**	**WS8**	**WS9**	**WS10**	**WS11**	**WS12**
WS1	1.000											
WS2	0.536	1.000										
WS3	0.294	0.259	1.000									
WS4	0.362	0.397	0.488	1.000								
WS5	0.407	0.393	0.250	0.418	1.000							
WS6	0.231	0.200	0.179	0.204	0.235	1.000						
WS7	0.333	0.258	0.208	0.392	0.500	0.217	1.000					
WS8	0.350	0.361	0.260	0.382	0.509	0.220	0.521	1.000				
WS9	0.394	0.423	0.286	0.403	0.485	0.254	0.400	0.500	1.000			
WS10	0.323	0.292	0.306	0.375	0.373	0.240	0.321	0.386	0.540	1.000		
WS11	0.314	0.302	0.195	0.292	0.269	0.270	0.255	0.306	0.262	0.327	1.000	
WS12	0.191	0.234	0.086	0.214	0.222	0.161	0.116	0.152	0.164	0.125	0.393	1.000

*WS 1- 12 refers to as *W. somnifera* plants 1 to 12.

5.2 UPGMA dendrogram:

The cluster analysis for RAPD was done using UPGMA method on Jaccard's similarity matrix where the selected plants were grouped into two major cluster one major and one minor cluster. The major cluster comprises of 9 plants which were further divided. The first subcluster has a 55% genetic similarity among WS 1 and WS 2 plants. The second subcluster have 5 plants, where WS 5, WS 7 and WS 8 have a bootstrap value of 14%. WS 9 and WS 10 have 18% bootstrap value. The third subcluster has a genetic similarity of 2%, showing a similarity value of 0.488 among WS 3 and WS 4 plants. The minor cluster has WS 11 and WS 12 which have a bootstrap value of 47 %. The WS 6 plant which has a bootstrap value of 24% was out groped. It is observed that WS 3 and WS 12 shows the highest genetic diversity as they have the lowest similarity value of 0.086.

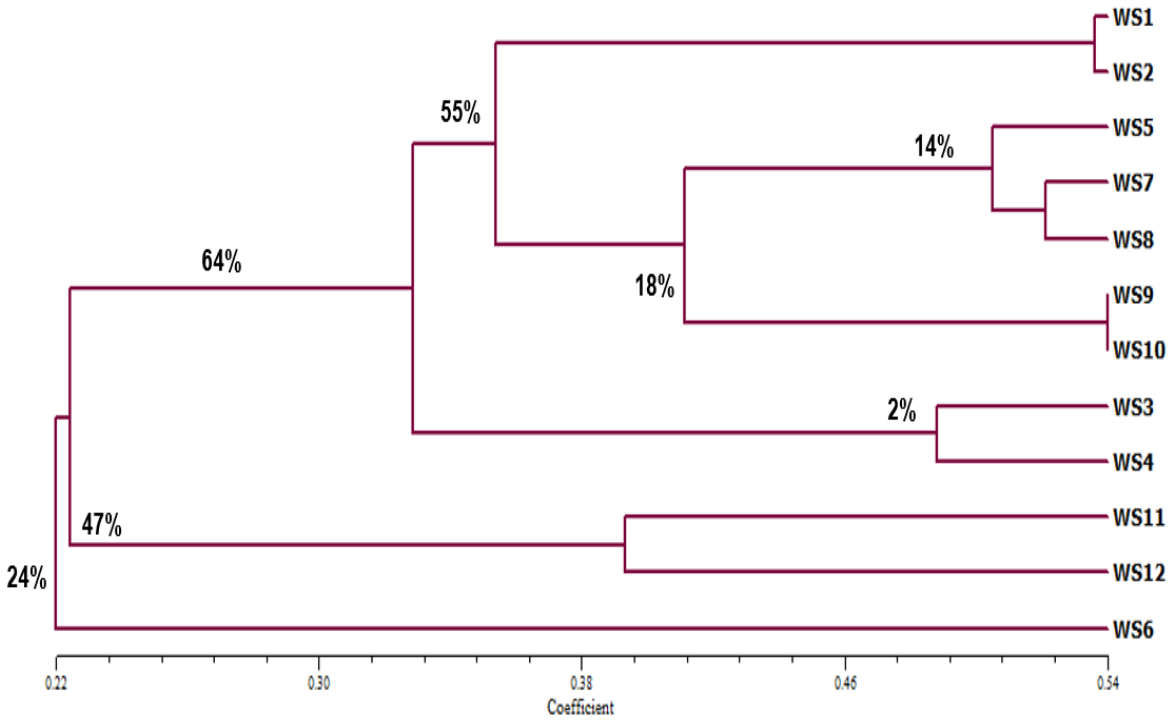

Fig 16: A dendrogram constructed from RAPD data collected for homogeneous population *of W. somnifera* using UPGMA method.

* WS 1- 12 refers to as *W. somnifera* plants 1 to 12

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5.3 Principal Component analysis (PCA):

Using PCA, a scatter plot for 12 *W. somnifera* plants was generated. In the figure 17, component 1 and component 2 had a total variance of 27.27 percent and 12.83 percent respectively. There were three groupings for the selected plants. Only WS 5, WS 7 and WS 8 were discovered in first group. Another group II countians plants WS 1, WS 2, WS 3, WS 4, WS 11, WS 12. The three remaining plants WS 6, WS 9 and WS 10, were present in group III.

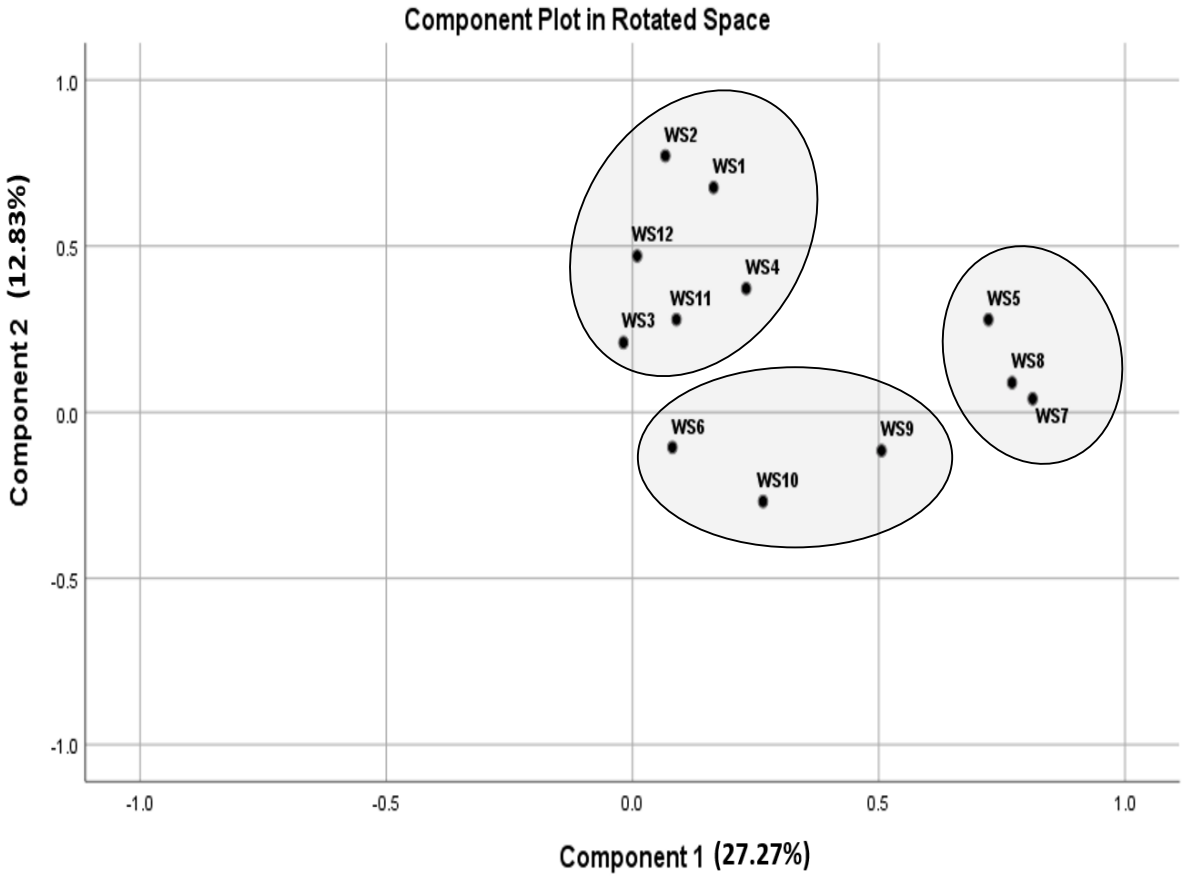

Fig 17: Two dimensional PCA scaling of 12 plants of *W. somnifera* using RAPD data

* WS 1- 12 refers to as *W. somnifera* plants 1 to 12.

6. RAPD, ISSR and SCoT Combined Data Analysis

6.1 Jaccard's similarity matrix

The Jaccard's similarity matrix was produced using binary data. According to Table 10, Jaccard's similarity coefficient values range from 0.481 to 0.694. These results demonstrate that the plants of *W. somnifera* exhibited substantial genetic variation. The plants WS 1 and WS 2 showed the most genetic similarity, with a similarity coefficient of 0.694, followed by WS 3 and WS 4, with a similarity coefficient of 0.654. According to the similarity matrix plants WS 9 and WS 12 showed the least genetic similarity with a similarity coefficient of 0.481.

Table 10: Jaccard's similarity coefficient matrix among 12 *W. somnifera* plants using combined data of RAPD, ISSR and SCoT markers.

	WS1	**WS2**	**WS3**	**WS4**	**WS5**	**WS6**	**WS7**	**WS8**	**WS9**	**WS10**	**WS11**	**WS12**
WS1	1.000											
WS2	0.694	1.000										
WS3	0.600	0.585	1.000									
WS4	0.603	0.570	0.654	1.000								
WS5	0.581	0.566	0.557	0.613	1.000							
WS6	0.526	0.526	0.556	0.542	0.582	1.000						
WS7	0.567	0.570	0.578	0.615	0.636	0.586	1.000					
WS8	0.549	0.570	0.565	0.577	0.603	0.568	0.640	1.000				
WS9	0.520	0.551	0.510	0.530	0.555	0.500	0.598	0.643	1.000			
WS10	0.544	0.572	0.555	0.562	0.621	0.553	0.619	0.648	0.622	1.000		
WS11	0.509	0.526	0.503	0.511	0.522	0.555	0.539	0.560	0.512	0.567	1.000	
WS12	0.512	0.516	0.536	0.503	0.537	0.508	0.549	0.508	0.481	0.531	0.585	1.000

*WS 1- 12 refers to as *W somnifera* plants 1 to 12.

6.2 UPGMA dendrogram:

Using the Jaccard's similarity coefficient for 12 plants of *W. somnifera*, a dendrogram was created. Two clusters—Cluster I and Cluster II—were established in this dendrogram and subsequently subdivided into two sub-cluster.

- ❖ WS 11 and WS 12 were the only plants present in Cluster I, which is supported by an 86 % bootstrap value, representing more similarity between them.
- ❖ Cluster II contain 10 plants which were further divided into two subgroups. Subgroup I contain plant WS 6 that has a bootstrap value of 12%. The subgroup II has 9 plants WS 1 WS 2, WS 3, WS 4, WS 5, WS 7, WS 8, WS 9, and WS 10 with 75% genetic similarity among each other.

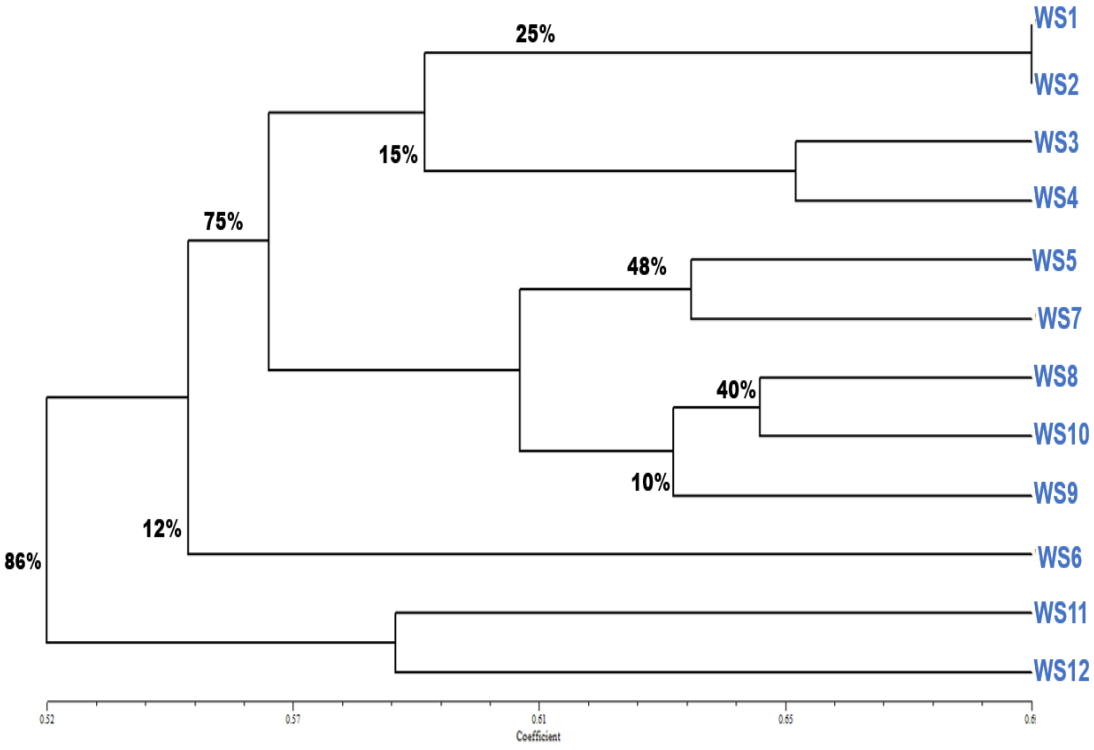

Fig 18: A dendrogram constructed from combined data of RAPD, ISSR and SCoT collected for homogeneous population *of W. somnifera* using UPGMA method.

* WS 1- 12 refers to as *W. somnifera* plants 1 to 12

Chapter 5: Discussion and Conclusion

Withania somnifera (Solanaceae), is a medicinal plant which is widely used in ancient systems of medicine as a rasayana and medhya rasayana. It has been used in Ayurvedic and Unani medicine for 3,000-4,000 years (Bamhania et al. 2013). A large number of medicinally important phytochemicals called withanolides are present in *W. somnifera* that contribute to its medicinal activity. Plant to plant variations in the quantity and quality of bioactive constituents seems to exist and geographical conditions also seem to have the greatest influence on bioactive constituents and activity profiles of medicinal plants (Oleszek et al. 2002). Despite being one of the world's most important medicinal plants, it has received little attention from geneticists, cytogeneticists and molecular biologists. For future quality improvement programmes, systematic morphochemical and molecular characterization of *W. somnifera* germplasm is required. Thus, the genetic diversity analysis of *W. somnifera* is important for future use (Gupta and Rana 2007).

Assessment of genetic diversity, prevailing for each trait in the germplasm of any crop species is not only important for crop improvement programmes but also for effective management and conservation of genetic resources (Ramachandran et al. 2013). According to researchers' higher levels of genetic diversity is generally regarded as a beneficial trait, whereas low genetic diversity is regarded as an immediate threat to survival of the species (Tiwari and Shrivastava 2016). Genetic diversity certainly benefits plant breeding programmes for a variety of reasons, including crop product improvement. Therefore, genetic diversity research and quantification are essential for the wise and sustainable use of plant resources. Furthermore, plants that thrive under harsh conditions likely to have useful alleles that can be beneficial for the adaptations to such environmental traits. Thus, Genetic diversity evaluation is critical for the improvement of

desirable qualities in crop plants, selection, genetic advancement, conservation and management of the species (Kumari et al. 2018).

The objective of the current study is to assess genetic diversity within the homogeneous population of *W. somnifera* growing at Thapar Institute of Engineering and Technology, Patiala, Punjab. Twelve randomly selected plants were identified from different location for study of genetic diversity among them using morphological characteristics such as leaf venation, leaf shape, leaf arrangement, average leaf length, width, internodal distance and number of berries in a cluster. In this study, information gathered through morphological marker data revealed no consistent variations within the plants. Morphological markers are highly influenced by environmental factors (Bruschi et al. 2003). Therefore, lower level of morphological variation among the plants could be due to the uniform environment. On the contrary many researchers have also reported morphological variations in plants growing in the same environment conditions (Kandalkar et al. 1993; Misra et al. 1998; Sahu et al. 2015).

Since the morphological marker did not produce consistent results, more precise DNA-based molecular markers were used to study the genetic variations in the selected plants. Three types of molecular markers were used in this study namely, Random Amplified Polymorphic DNA (RAPD), Inter Simple Specific Repeats (ISSR), and Start Codon Targeted polymorphism (SCoT) (Negi et al. 2006; Gogoi et al. 2020; Tabasi et al. 2020). The effectiveness of these markers have been evaluated using several parameters and some of which includes, % polymorphism, PIC, and RP values. The level of polymorphism provides an indication as to how much genetic diversity is present in plant species (Pfeifer et al. 2011). In our study RAPD, ISSR and SCoT markers revealed 98%, 93.6% and 56.6% polymorphism respectively, which is much higher than the previously reported values in *W. somnifera*.(Mirjalili et al. 2009; Khatak et al. 2013; Khanna et al. 2014; Khan and shah 2016; Ramachandran et al. 2017) Currently the average percent polymorphism for RAPD marker was 98% which was higher

than the average percent polymorphism for ISSR marker (93.6%).This pattern of observation was similar to the findings by Tripathi et al. (2012) in which RAPD markers were found to be more polymorphic as compared to ISSR.

It was observed that in case of RAPD a total of 95 bands were scored out of which 94 were polymorphic (98%), in SCoT 200 bands were amplified out of which 118 were polymorphic (56.6%) and similarly in case of ISSR 245 bands were amplified out of which 229 were polymorphic (93.6%). In this study RAPD primers amplified higher number of polymorphic bands as compared to ISSR and SCoT primer and also reported more discriminating ability among *W. somnifera* plants. RAPD marker amplified more complex pattern of marker in comparison to SCoT and ISSR (Parsons et al. 1997; Chowdhury et al. 2002), which can be beneficial during differentiating closely related plants. The result of this study is in line with this observed however, this study identifies that RAPD, ISSR, and SCoT polymorphisms can be used to determine the evolutionary connections and similarities among *W. somnifera* plants.

PIC value is another factor that is used to quickly assess genetic diversity in plants. In general, PIC>0.50 is considered a very informative marker, the range between 0.25-0.50 is considered a fairly informative marker value (Bostein et al. 1980). In our study, the average PIC values for RAPD, ISSR, and SCoT were 0.35, 0.31, and 0.17, respectively. These values are an indication of their effectiveness (Mandal and Reddy 2017). Our findings show that RAPD and ISSR markers have a higher PIC value, which is consistent with the findings of Tiwari et al. (2009), who recorded an average PIC value of 0.327. However, Tripathi et al. (2012) in their analysis recorded a slightly lower average PIC value (0.29) of these markers. Similarly, the average RP value for RAPD, ISSR, and SCoT are 6.9, 10.75 and 11.70 which are little higher than previous studies conducted by Khan and Shah (2016), Barvaliya et al. (2018), and Gilberto et al. (2020) who observed slightly lower RP values in *W. somnifera* and *Stevia rebaudiana*.

The cluster tree analysis done using UPGMA method were based on RAPD, ISSR and SCoT data. The plants were distributed into two clusters. It was observed that in case of RAPD and SCOT plant WS 6 was out grouped where as in case of ISSR the plant WS 12 was reported to be out grouped from the two-cluster formation. Similarly previous studies were in line with our current outcomes where the plants were observed to be divided into two clusters, with a reasonable variation that could be used to select parents for the breeding of high-yielding plants varieties (Khanna et al. 2014; Chauhan et al. 2017; Hiremath et al. 2021). In addition, a two-dimensional PCA scatter plot created using RAPD and ISSR data differed from UPGMA clustering. The PCA scatter plot constructed from RAPD and ISSR data grouped these plants into three major categories. In the case of ISSR, plant WS 12 was out grouped, whereas WS 06 plant was out grouped in the case of RAPD.

It is worth noting that RAPD and ISSR markers showed more promising results in identifying genetic diversity in the selected plants, resulting in a higher degree of polymorphism. Therefore, molecular markers allow the rapid detection of DNA polymorphism in different plant samples. The advantages of these dominant molecular marker lies in its rapidity, simplicity, usage of small quantity of DNA and ability to generate higher polymorphism (Mondini et al. 2009). It has been emphasised that molecular marker approach can be effectively used for genotype assessment, genomic modelling, and genetic variability evaluation (Gurudeeban et al. 2011).

It can be summarised that genetic diversity exhibited in the homogeneous population of *W. somnifera* collected from TIET campus (Patiala, Punjab). It was observed that the morphology of the plants was very similar and it was very challenging to record any phenotypic differences. In our analysis 12 plants of *W. somnifera* from same geographical locations showed the presence of genetic variations. It is feasible to speculate that the genetic diversity in this group of *W. somnifera* plants was caused due to sexual reproduction within populations and selection

pressure over time. *W. somnifera* is thought to have originated in one of these locations, travelled a considerable distance, and then adapted to its new habitat by interacting with local factors. This research shows, how polymorphisms observed with the help of molecular markers (RAPD, ISSR and SCoT) could be exploited to demonstrate similarities and evolutionary relationships among *W. somnifera* plants. The information gathered here might be useful in developing conservation and maintenance strategies for *W. somnifera* population.

Salient points

- It's crucial to note that population of *W. somnifera* collected from the same area exhibit considerable genetic diversity.
- This study reported that RAPD and ISSR marker revealed higher degree of polymorphisms in plants of *W. somnifera* as compared to SCoT markers.
- The diversity seen in naturally existing plants at TIET campus might be due to the cross pollination of plants.
- The data generated in this study could be utilized for planning future breeding and conservation strategies by characterizing variable genotypes.
- The study also validates that the DNA based molecular marker approach is a rapid, straightforward, inexpensive and easy-to-use technique.

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Annexture

1. Preparation for extraction buffer

Components	Quantity (100 mL)
2% CTAB	2 gm
20 mM EDTA	0.585 gm
100mM Tris HCL	1.576 gm
1.4 M NaCL	8.181 gm

2. Preparation for TAE buffer

Components	Quantity (100 mL)
Tris base	24.2 gm
Glacial acetic acid	5.71 mL
NaEDTA	3.72 gm
pH	8-8.5

3. Preparation for TE buffer

Components	Quantity (100 mL)
100mM Tris buffer	0.121 gm
1mM EDTA	0.029 gm

4. Preparation for 3M Sodium acetate

Components	Quantity (100 mL)
3M Sodium acetate	40.824 gm

5. Preparation for ethidium bromide strain

Components	Quantity (2 mL)
Ethidium Bromide	10 mg
Sterile Milli-Q water	2 mL

6. Preparation for Phenol: Chloroform: Isoamyl alcohol

Components	Quantity (100 mL) (25:24:1)
phenol	50 mL
Chloroform	48 mL
Isoamyl alcohol	2mL

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Studies of genetic diversity in a homogeneous population of Withania somnifera (L.) Dunal using DNA based markers
A thesis submitted in partial fulfillment of the degree of MASTER OF TECHNOLOGY IN BIOTECHNOLOGY
By Ruchika Singh (Roll no. 602104012)
Under the Guidance of Dr. Anil Kumar Professor
Department of Biotechnology Thapar Institute of Engineering & Technology (Deemed to be University) Patiala-147004, Punjab, India June 2023
CERTIFICATE
This is to certify that dissertation entitled "Studies of genetic diversity in a homogeneous population of Withania somnifera (L.) Dunal using DNA based markers" submitted by Miss. Ruchika Singh (Roll no. 602104012) in the partial fulfilment of the requirements for the award of the degree of Master of Technology in Biotechnology, Thapar Institute of Engineering and Technology, Patiala is a record of student's own work

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carried out under my guidance and supervision. It is also certified that the matter embodied in this thesis has not been submitted in part or full to any other institute or university for the award of any degree or diploma. Dr. Anil Kumar Professor Department of Biotechnology Thapar Institute of Engineering & Technology Patiala Punjab DECLARATION I hereby declare that the work presented in the thesis entitled "Studies of genetic diversity in a homogeneous population of Withania somnifera (L.) Dunal using

DNA based markers" is a bonafide work under the supervision and guidance of

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Dr. Anil Kumar, Professor, Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala.

I also declare that this thesis or any other part of this thesis has never been submitted for any degree in this or any other university.
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ACKNOWLEDGMENT
First of all, I

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would like to express my deepest gratitude to Dr. Anil Kumar, Professor in the Department of Biotechnology, Thapar Institute of Engineering and Technology Patiala, for giving me the golden opportunity to work under his invaluable guidance

and suggestions throughout the course of dissertation. I would also thank him for his constant encouragement, oversight of my work, and advice which enabled me to finish my research work. I am grateful to the head of Department of Biotechnology and TIFAC-CORE for providing me with the equipment, infrastructure and resources I needed for the completion of my work. I would also like to express my sincere thanks to Ms. Shivani Guleria, (PhD student) and Ms. Nisha Chauhan (PhD student) Department of Biotechnology, for their ongoing help, encouragement, and direction helped me finish my research project. I would like to express my sincere appreciation to my parents Mr. Anil Singh and Mrs. Chhaya Singh for their emotional and financial support throughout my journey. I would also like to thank my sibling Dr. Disha Singh and friends Ms. Warsha Koul, Ms. Navgeet Kaur, and Mr. Sunny Yadav for their constant support and encouragement throughout my journey.
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