Studies of genetic diversity in the seed raised population of *Withania somnifera* (L.) Dunal using RAPD markers

A thesis submitted in partial fulfillment of the degree of

MASTER OF SCIENCE

IN

BIOTECHNOLOGY

THAPAR INSTITUTE
OF ENGINEERING & TECHNOLOGY
(Deemed to be University)

by

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June, 2022

CERTIFICATE

I hereby certify that the work entitled **"Studies of genetic diversity in the seed raised population of *Withania somnifera* (L.) Dunal using RAPD markers"** submitted by **Navneet Kaur**, Reg. no. **302001009** for the partial fulfillment of the requirement for the award of the degree of Masters of Science in Biotechnology. The work has been carried out under my supervision.

It is also certified that the work of present thesis or any part of this has not been submitted, to this university or any other university for the award of any other degree or diploma.

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DECLARATION

I, hereby declare that the work presented in this thesis entitled **"Studies of genetic diversity in the seed raised population of *Withania somnifera* (L.) Dunal using RAPD markers"** for the award of degree Masters of Science in Biotechnology is an authentic record of my work. The work has been performed under the supervision of **Dr. Anil Kumar,** Department of Biotechnology at Thapar Institute of Engineering and Technology (Patiala).

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Place: Patiala, Punjab

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Date: 20 Sept, 2022

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ACKNOWLEDGEMENT

First and foremost, I would like to show my gratitude to my supervisor **Dr. Anil Kumar**, Professor, Department of Biotechnology at **Thapar Institute of Engineering and Technology, Patiala** for giving me the opportunity to work under his guidance. His continuous efforts and monitoring of my work, guidance helped me in completing my research work.

I am also thankful to Department of Biotechnology and TIFAC-CORE for providing me necessary infrastructure and resources for my work

I am also grateful to **Ms. Shivani Guleria** (PhD scholar) Department of Biotechnology. Her continuous help, support and guidance in completion of my research work.

At last, I owe my special gratitude to my parents and family. Without their blessings and financial support, it would not have been possible to accomplish what I am today.

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List of Abbreviations

µl	Microliter
AFLP	Amplified fragment length polymorphism
ASAPs	Amplification, sequencing & annotation of plastomes
CAPs	Cleaved amplify ed polymorphic
cm	Centimeter
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate
EST	Expressed sequence tag
EtBr	Ethidium bromide
g	gravitational force
gm	Grams
ha	Hectare
ISSR	Inter simple sequence repeat
kbp	Kilogram base pair
m	Meter
M	Molar
ml	Milliliter
mm	Millimeter
mM	Millimolar
ºC	degree Celsius
PCR	Polymerase chain reaction
PIC	Polymorphic information content
RAPD	Random amplified polymorphic DNA
SCAR	Sequence characterized amplified region
SSCP	Single-strand conformation polymorphism
STMS	Sequence tagged microsatellite site
STS	Sequence-tagged site
Taq	*Thermus aquaticus*
TE	Tris-EDTA
w/v	weight per volume

Abstract

Withania somnifera (L.) Dunal is one of the important medicinal plants having immense therapeutic value. It is mainly known for its bioactive compounds withanolides present in the roots. The aim of the present study is to study the genetic diversity among the wild seed raised population of *W. somnifera* from Thapar Institute of Engineering and Technology (Patiala) using RAPD molecular markers and morphological markers. Morphological data showed good variations among the plants studied. The RAPD markers revealed much more information. A total of 15 RAPD primers were selected on the basis of those produced clear and good amplification. In total, 89 markers were scored. In which 77 markers were observed as polymorphic (86.52%) and 12 markers were monomorphic (13.48%). The size of amplified fragments was ranged between 250 to 6000 bp. The similarity indices were calculated using Jaccard’s similarity coefficient which ranged from 0.383 to 0.695. The cluster analysis by UPGMA (unweighted pair group method with average) formed two clusters. In one cluster, only two accessions were grouped (WS 9 and WS 12) whereas, in another cluster two sub-groups were generated consisted of 10 accessions. The highest percentage of polymorphism (100%) was seen with RAPD 3, RAPD 10, RAPD 11, RAPD 16, RAPD 1 and RAPD 32 and, lowest percentage of polymorphism (60%) was noticed with RAPD 7. The highest PIC value was generated by RAPD 7 which was 0.400 and the least value was observed with RAPD 1 i.e., 0.159. High genetic diversity was revealed by RAPD primers among all the accessions of *W. somnifera*.

Withania somnifera (L.) Dunal is an important medicinal plant of family Solanaceae. It is commonly known as ashwagandha, winter cherry and Indian ginseng. In Ayurveda, it had been used since ancient times as an indigenous medicine (Srivastava et al. 2018). It is rich source of many pharmaceutical active compounds but is specially known for "withanolides", credited with wide range of remedying properties (Dhar et al. 2015).

Scientific Classification

Kingdom: Plantae
    Clade: Tracheophytes
        Order: Solanales
            Family: Solanaceae
                Genus: *Withania*
                    Species: *W. somnifera*

W. somnifera is an evergreen shrub of about 1 to 2 meter in height (Fig 1a). It is all covered with trichomes or small fine hairs which are white or grey in color. Leaves are usually elliptical or ovate, and arranged in spiral manner. The length and width of leaves is about 3 to 8 cm and 2 to 6 cm respectively. Flowers are generally tiny (1 cm long), light yellowish to green in color, monoecious (Fig 1b) and which later develop into fruits. The corolla having 5 lobed structures. Fruit is usually a small hairless berry of about 5 to 8mm, orange or red in colour and enclosed within the papery, brownish calyx (Fig 1c). Roots are generally whitish-brown, long tuberous, stout and fleshy in nature. The roots have taproot system which means grows directly downwards as straight and cylindrical (Fig 1d). The flowering season of W. *somnifera* is from October to June, whereas the fruiting season is usually from October to July (Gaurav et al. 2015).

W. somnifera thrives on sandy silt or lighter red sand with a pH of 7.5-8.0 and adequate irrigation. It could be grown at elevation range from 600 to 1200 meters. Most suitable temperature is required for its growth, which usually ranges between 20° to 35° C. (Verma and Singh 2014). *W. somnifera* favors dry subtropical conditions, which it finds in the northwestern locations, North Africa & central India (Afewerky et al. 2021 and Tripathi and Awasthi 2018).

In India, it is mainly cultivated on 10,780 acres with a productivity of 8429 tons (Verma and Singh 2014). It is widely cultivated in Madhya Pradesh, Gujarat, Uttar Pradesh, Andhra Pradesh, Rajasthan, Punjab and Maharashtra, as well as in Himachal Pradesh & Jammu's mountain areas up to an altitude of 1500 metres (Tripathi and Awasthi 2018).

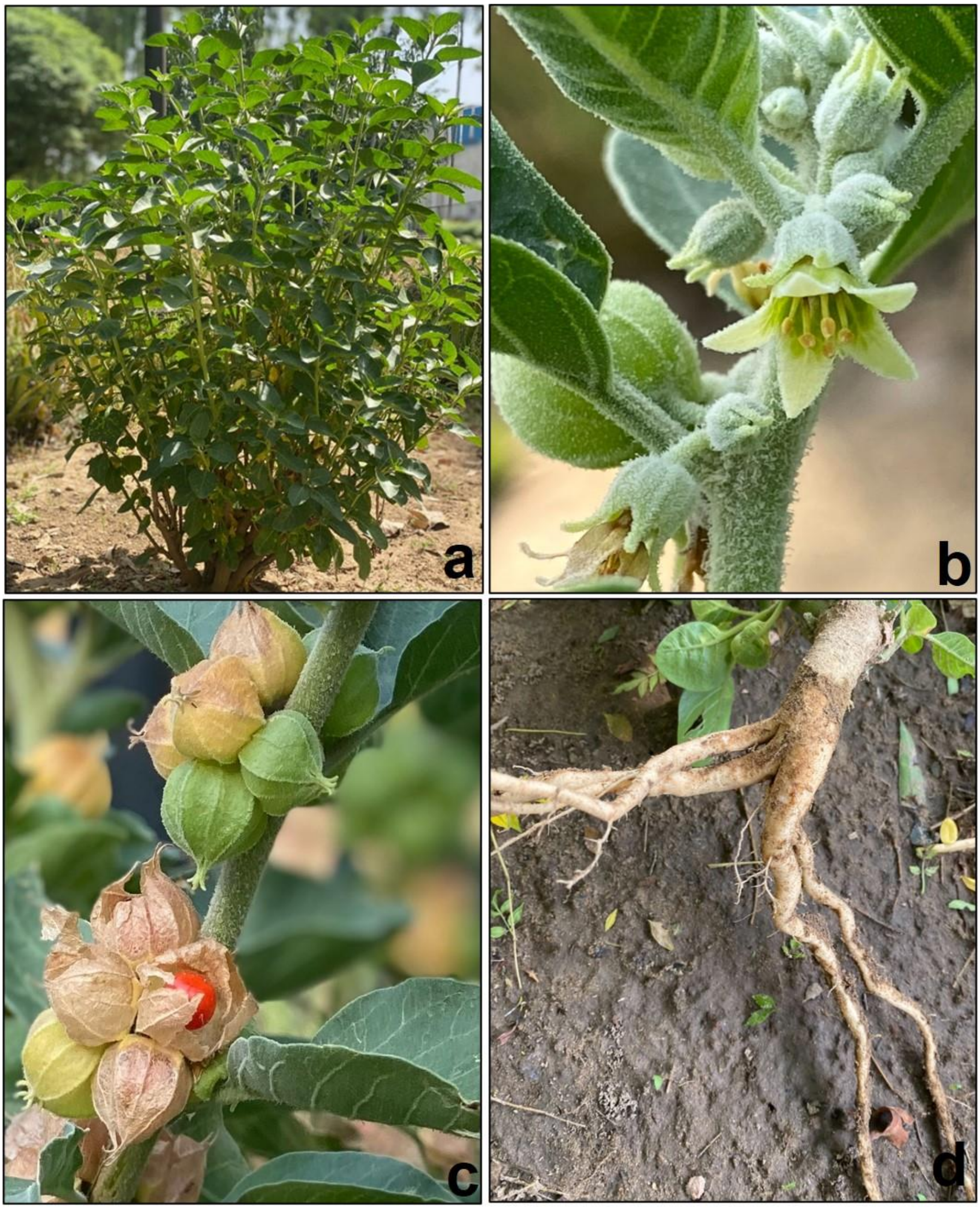


Figure 1: Morphological characters of *W. somnifera* (a) fully grown plant (b) flowers (c) berries and seed (d) root part.

W. somnifera is a rich source of many phytochemicals such as alkaloids, starch, steroidal lactones, phenolic content, sitoindosides, β-sitosterol, tannin, chlorogenic acid, somniferinine, carbohydrates, pseudotropine, anaferine, saponin, anahygrine, cysteine, 14-α-hydroxywithanone, withanine, cuscohygrine, scopoletin, withananine, somniferiene, tropanol, and 6,7 β-Epoxywithanon, Viscosalactone-B (Saleem et al. 2020; Naz and Choudhary 2003; Arya and Chauhan 2019; Uddin et al. 2012), but it is mainly known for withanolide A, withanolide D, withaferin A and withanone (Mirjalili et al. 2009). These are the compounds that mainly attribute to medicinal and pharmacological properties of *W.somnifera.*

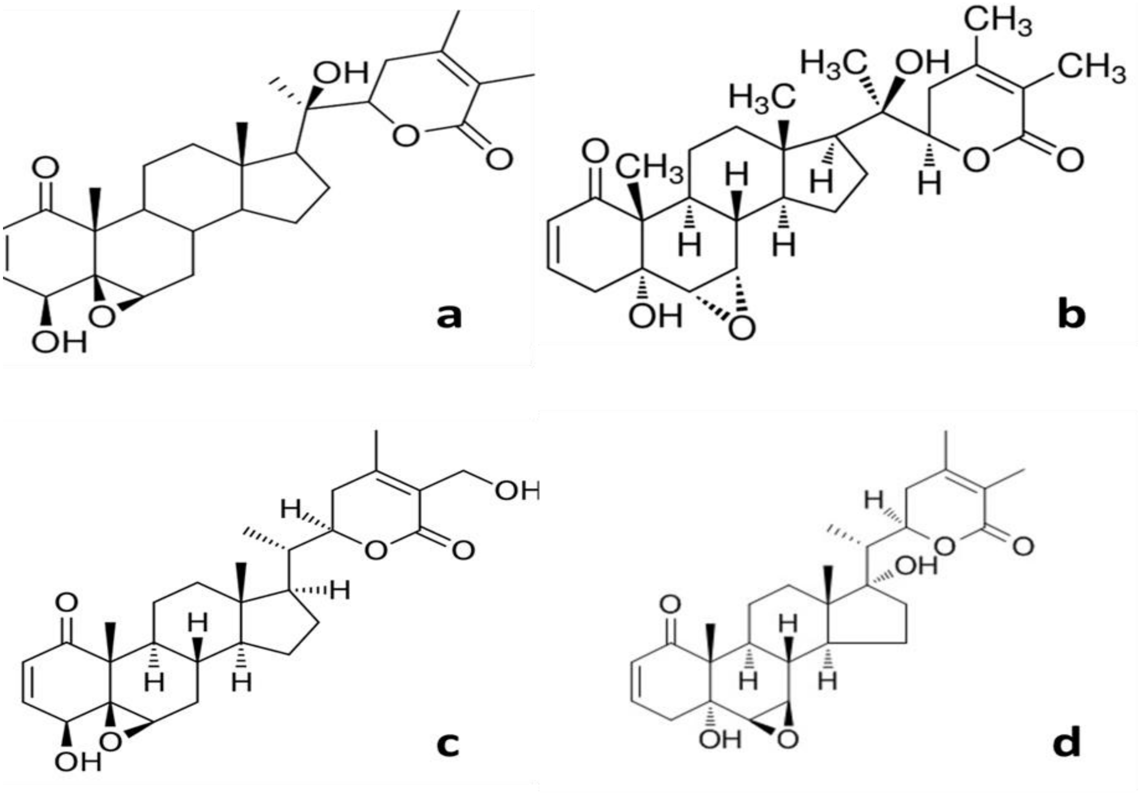


Figure 2: Molecular structure of bioactive compounds present in *W. somnifera* **(a)** Withanolide D **(b)** Withanolide A **(c)** Withaferin A **(d)** Withanone.

These phytochemicals possess anti-inflammatory, antibiotic, immunomodulatory, antitumour, anti-stress, anticonvulsant, neuropharmacological, hypoglycemic effect, musculotropic, anti-oxidant and hepatoprotective activities (Umadevi et al. 2012; Kaur et al. 2013; Dar et al. 2015; Nazir and Chauhan 2018; Sharma et al. 2011; Uddin et al. 2012). Gynecological problems, rheumatism, pneumonia, rheumatism and even drug addiction have all been linked to these components. The phytochemical of this plant is used to treat these disorders (Afewerky et al. 2021; Umadevi et al. 2012; Azgomi et al. 2018). They are also used to treat Parkinson's disease, neurological diseases, and anxiousness problems (Kuboyama et al. 2005; Bhattacharya et al. 2000; Ahmed et al. 2005; Srivastava et al. 2019).

Every part of the plant is enriched with different phytochemicals that are helpful in treating various diseases

Table 1: The use of different organs of *Withania somnifera* for the treatment of various ailments.

Plant part	Treatment
Whole plant	Antidote of snake venom, cardioprotective, immunomodulatory, larvicidal, neurotic regenerator, antioxidant, adaptogenic, hepatoprotective, and insecticidal (Saleem et al. 2020; Kuboyama et al. 2005)
Roots	Arthritis, tuberculosis, bronchitis, heart disorders, leucoderma, liver problems, and Asthma (Jayaprakasam et al. 2003; Bhattacharya and Muruganandam 2003; Singh et al. 2010; Rayees and Malik 2017; Saleem et al. 2020)
Leaves	Hemorrhoids, swellings, syphilis, Ulcers, external pains, edema, boils, and eyesores, (Singh et al. 2011, Saleem et al. 2020, Singh and Singh 2019)
Fruits	Tuberculosis and ulcer (Afewerky et al. 2021; Saleem et al. 2020)
Seeds	Hypnotic, diuretic and narcotic (Saleem et al. 2020; Singh and Singh 2019)
Stem	Antibacterial, antitumor and herbicidal (Singh et al. 2010; Saleem at al. 2020)

There are two types of variations that can be seen in plants such as genetic variations and epigenetic variations. Genetic variations are heritable and it may pass from one generation to another. These variations in plants occur due to sexual reproduction and is most likely to occur through pollination (Lele et al. 2018). The plants of *W. somnifera* are generally propagated through seeds (Khanna et al. 2013). Therefore, there might be possible chances of occurrence of genetic variations among seed raised plants of *Withania somnifera*. Furthermore, epigenetic variations can arise in plants due to certain environmental factors. The environment conditions in which plants are growing generally contribute to these kinds of variations (John 2014). It has been reported that both genetic and epigenetic variations also affect the production of phytochemicals within the plant (Borges et al. 2017). Moreover, the variations can have both positive or negative effect on genetic, morphology, physiology and therapeutic value of plants. Therefore, it is necessary to evaluate genetic variability of plants.

The identified alleles can be helpful for crop improvement and breeding programs. These studies require great efforts to interpret the pattern of variations in plants (James and Ashburner

1997) To identify the alleles contributing to variations various markers such as biochemical markers, morphological markers and molecular markers are being used. Among these, molecular markers are mainly used which include Random amplified polymorphic DNA (RAPD), Sequence-tagged site (STS), amplification, sequencing & annotation of plastomes (ASAPs), expressed sequence tag (EST), single-strand conformation polymorphism (SSCP), sequence tagged microsatellite site (STMS), inter simple sequence repeat (ISSR), sequence characterized amplified region (SCAR), cleaved amplified polymorphic (CAPs) and amplified fragment length polymorphism (AFLP). Among these, RAPD markers has been frequently used to study the genetic diversity among the wild population of many plants (Tripathi et al. 2012; Bansal et al. 2014). Therefore, in present study, PCR-based RAPD molecular markers are used. This technique is quite fast, simple, efficient and cost effective and require very small amount of DNA. Moreover, this do not require any prior information of template sequence (Chauhan et al. 2018). It is mainly used to determine genetic relationship among species, taxonomic identity and gene mapping.

Therefore, in present study, RAPD markers were used to analyse genetic diversity in *Withania somnifera*

Objective

- To study the morphological diversity of *Withania somnifera* (L.) Dunal wild population.
- To study the genetic diversity among the wild population of *W. somnifera* (L.) Dunal using RAPD markers.

For any crop improvement programme or breeding, evaluation of genetic diversity is important. The improved genotypes or varieties are required for any crop improvement programmes which involves a comprehensive understanding of various quality characteristics. Furthermore, reproductive function and environment are two significant elements that play a key role in character expression and taken into consideration as an essential aspect of each genotypic and phenotypic variability. Specific alleles of relevance can be identified based on these variations. To extract the most information from the matrix data, morphological and molecular markers were suggested (Sikdar et al. 2010). Genetic diversity can be analysed by various morphological, molecular and biochemical markers. Among these three markers, DNA based molecular markers are very effective tools for analyses of genetic diversity within a crop (Dekkers and Hospital 2002). The RAPD molecular markers have already been widely employed as genetic markers for genetic diversity assessment and pedigree evaluation (Dawson et al. 1993). The majority of RAPD markers are dominant, and they have been used to fingerprint diverse plant genotypes (Connolly et al. 1994). RAPD markers have been used successfully in a variety of phylogenetic and taxonomic research because they are a quick and inexpensive technique to collect molecular data (Tripathi et al. 2012). Several studies were carried out to analyse genetic diversity in *Withania* species using RAPD marker. Those are summarized in the following paragraphs.

The study was carried out by Mirjalili et al. (2009) to check genetic variations among *W. somnifera* and *W. coagulans* in Iranian natural populations by using RAPD markers in relation with their withaferin A content. The RAPD results of Iranian natural *Withania* populations revealed high genetic diversity, confirming that geographic distribution and reproduction are the two major factors that influences genetic diversity. HPTLC examination also revealed the presence of withaferin A in *W. coagulans* and *W. somnifera*. Also, in all used samples, the content of withaferin A was higher in the aerial region than in the roots, which is essential for conservation, also breeding practices to enhance withaferin A production. It was mentioned that assessing genetic diversity is critical for successful medicinal species conservation and breeding, as well as determining the quantity of phytochemical consitutents.

Mir et al. (2009) studied genetic diversity of 23 genotypes of *Withania somnifera* which were collected from different geographical regions of India. The cultivated genotypes were very well isolated from the wild genotypes and had a lower similarity value, showing that the wild and cultivated genotypes were very distinctive. The cultivated accessions were likewise morphologically dissimilar from the wild ones, and RAPD cluster analysis clearly distinguished the five cultivated *W. somnifera* genotypes. The morphological and molecular marker were shown to have a high association. This genetic diversity aids in the generation of a large number of new variants through hybridization and gene transfer, enhancing the usage of existing germplasms as genetic resource materials for breeders.

Punetha et al. (2011) carried out a study on *W. somnifera*. In total, 36 genotypes were evaluated, including 35 selection lines and one released *W. somnifera* variety. At the intra-specific level, the high level of genetic diversity found in selection lines compared to released variants of *Withania somnifera* indicates that selection line variants have a larger gene pool than released varieties, which is useful in developing improved crop varieties of *Withania somnifera*.

Tripathi et al. (2012) analyzed 16 *W. somnifera* genotypes taken from various parts of India. The study revealed that the genetic basis is limited, and that the insertion of genes from previously unexploited sources requires more consideration. Traditional breeding efforts to develop cultivars belonging to numerous species of Withania have been conducted, but these efforts did not include a molecular diversity evaluation and consideration for parent selection. Knowledge of molecular marker-assisted genetic variability characteristics, in conjunction with morphological and biochemical relatedness and differences among *W. somnifera*, could provide advantages in terms of strategic trait combinations and germplasm exploitation.

A study was carried out by Bhat et al. (2012) to evaluate genetic variations among 5 accessions of *W. somnifera* using RAPD, morphological and biochemical markers. It was noted that on the basis of morphological characterization the differences were seen between cultivated and wild type accessions in their height, color of berries, fruit calyx and leaf shape. On the other hand, biochemical markers revealed considerable variations among 5 genotypes of *W. somnifera*. In this, wild accessions contain more chlorophyll, starch, protein and sugar content than cultivated accessions. Moreover, maximum genetic diversity was present in wild accessions than cultivated ones.

Udayakumar et al. (2013) studied the genetic diversity and relationships among 20 accessions of *W. somnifera* collected across southern India, including Maharashtra, Tamil Nadu, Karnataka, Puducherry, Andhra Pradesh, Kerala and Gujarat, as well as two commercial varieties from Delhi and Uttar Pradesh. The findings reveal a significant level of genetic variability among *W. somnifera* accessions, which could have an impact on phytochemical constituents in *W. somnifera* populations. One of the reasons for this could be that human degradation of natural habitat causes geographical isolation, which reduces gene flow. It's important to note that *W. somnifera* populations from the same region show genetic variance, whereas populations from different regions show genetic similarity.

Sairkar et al. (2013) studied genetic diversity in two important medicinal plants of Madhya Pradesh. In this study, total 14 accessions were selected, out of which 7 were of *W. somnifera* and 7 of *Rauwolfia serpentina*. The findings of RAPD revealed that we need to include members from a wider range of populations in attempt to preserve their biodiversity in the future. The research confirms RAPD's appropriateness for molecular diagnosis of distinct accessions of a medicinally important plant as a reliable, easy-to-handle, quick, and simple technique. Moreover, it has been proven that the entries that were considered to be identical in taxonomical classifications based on morphological features, exhibit DNA variance. The findings suggested that *W. somnifera* has a low diversity, which is concerning for the species' conservation, while *R. serpentine* has a larger diversity, which is encouraging for the species' survival. Low gene pool in *W. somnifera* is attributed to some plant producers' poor breeding programes or inappropriate plant conservation. Intraspecific hybridization, which uses the same parent's plants to produce new offspring, may be the cause of poor genetic diversity.

Khatak et al. (2013) checked genetic diversity among 30 genotypes of *W. somnifera* which were collected from different states of India. In this report, high degree of polymorphism was revealed among 30 genotypes of *W. somnifera*. The information obtained from this research was highly useful, as it was able to demonstrate good polymorphism and establish a unique DNA fingerprint. The findings of this study showed RAPD patterns can be used as a valuable input to the traditional method of varietal identification, long term gene banks maintenance, and marker great addition to optimize the effectiveness of new cultivar improvement in breeding strategies that rely entirely on morphological traits. Surprisingly, samples from all over the country did not form well-defined different groups and were interspersed with one another, showing that there was no link between both the RAPD pattern as well as the geographic origin of genotypes.

Khanna et al. (2013) carried out a study to find genetic diversity by using morphological and RAPD markers among 14 genotypes of *W. somnifera* which were collected from different origin. In this study, genetic diversity was observed in both cultivated or wild type accessions of *W. somnifera*. These variations occur due to the difference in origin and environmental factors. Mutations were also observed in morphological characters of *W. somnifera*. Morphological traits play important role in accessing genetic diversity. RAPD markers depicts high discrimination between wild and cultivated genotypes of *W. somnifera* with high degree of polymorphism.

Khan and Shah (2016) also used RAPD and ISSR molecular markers to check genetic variations across 16 *W. somnifera* accessions obtained from multiple sites, including Nimuch, Mumbai, Lucknow, and Karnataka. The majority of the variations were discovered to be somewhere between populations. Within the population, there was 90 percent homogeneity. The species' self-pollinating nature could be one factor.

Tiwari and Shrivastava (2016) examined the efficacy of RAPD markers for detection of genetic diversity among 11 accessions of *W. somnifera*. They recorded 100 percent polymorphism in four accessions, which was shown to be higher than in other threatened species.

Chauhan et al. (2017) did a study on genetic diversity between 25 accessions of *W. somnifera* collected from Mandsaur, Anand, Hisar and Udaipur using RAPD markers. High level of genetic diversity was reported among all *W. somnifera* accessions.

Tripathi and Awasthi (2018) employed RAPD markers to examine genetic diversity within 21 *W. somnifera* accessions gathered from various climatic zones across Central India. All genotypes formed different clusters based on RAPD analyses, apart from their respective collection sites. According to their findings, there was a lot of genetic variability among 21 genotypes of *W. somnifera*. The biologically distinct genotypes could be employed to improve the Ashwagandha crop in the future.

Ganaie et al. (2021) conducted a study on *W. somnifera* genetic diversity, which included 32 genotypes collected from different places. It was discovered that all accessions of *W. somnifera* had a high level of polymorphism, indicating that *W. somnifera* was closer to *W. frutescence* than to *W. coagulans.*

Plant material

The plants of *Withania somnifera* growing at Thapar Institute of Engineering and Technology were selected to study genetic variations among them. A total of 12 plants were selected and tagged as WS 1 to WS 12. For morphological analysis, different parameters were measured in three replicates i.e., leaf length, leaf width, number of berries in a cluster and inter-nodal distance by calculating mean value and standard error (SE) for statistical data. By looking at their patterns Leaf shape, leaf arrangement and leaf venation were scored.

Genomic DNA isolation

The total genomic DNA of all the selected plants (WS 1-WS 12) was isolated using Cetyl trimethyl ammonium bromide (CTAB) method (Annexure) given by Doyle and Doyle (1990). Samples of 2 grams of fresh leaf was frozen in liquid nitrogen and grinded to a fine slurry. The mixture is transferred to 50 mL of Oakridge tube followed by addition of 10mL prewarmed extraction buffer, 0.2% (w/v) β-mercaptoethanol and incubation at 60 ℃ for 1 hour. After incubation, 10 mL of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 10000 g, 15 minutes, 4ºC. After centrifugation, aqueous phase was transferred to clean tube and DNA was precipitated by adding 0.66 volume of cold isopropanol followed by incubation at -20 ℃ for 1 hour. Again, centrifugation was done at 10000 g, 15 min, 4ºC and pellet was dissolved in 1 mL of TE buffer (Annexure).

The purification of isolated DNA was performed by addition of 10µg/mL RNAase enzyme followed by incubation at 37 ℃ for 1 hour. Equal volume of phenol: chloroform: isoamyl alcohol (25: 24:1) was added and centrifuged at 10,000 g for 10 min, 4ºC. After this, aqueous layer was pipette out into new vial and 0.3 volume of 3M sodium acetate and 0.6 volume of chilled isopropanol was added followed by incubation at -20 ℃ for 1 hour. Centrifugation was done at 10000 rpm for 10 min and pellet was dissolved in 30 µL TE buffer.

Qualitative and Quantitative analysis

The quality of isolated DNA was checked on 0.8% (w/v) agarose gel stained with 0.5 µg/mL ethidium bromide (EtBr) and the quantity was measured with the help of Nanodrop 1000^{TM} UV/VIS spectrophotometer respectively.

Testing of primers

A total of 20 RAPD primers were tested and amplification of each primer was performed with DNA of *W. somnifera* plant 1 (WS 1). The primers 15 producing maximum and scorable bands were selected for further study (Table 3).

PCR amplification

The amplifications of 12 DNA samples of *W. somnifera* (WS 1-WS 12) with each 15 selected RAPD primers was carried out in PCR-Veriti 96 Well thermocycler (Applied Biosystem). PCR reaction mixture consisted of 2 µL of 10x Taq buffer, 200 µM dNTPs, 1.5 µM $MgCl_2$, 10 µM primer, 50 ng/µL template, 1.0 U Taq polymerase and final volume makeup upto 20 µL with sterile MilliQ water. Amplification conditions followed were; initial denaturation at 94 °C for 4 minutes, denaturation at 94 °C for 1 minute, annealing 32 °C for 45 seconds, extension at 72 °C for 2 minutes and final extension at 72 °C for 5 minutes, followed by 41 cycles. Amplified PCR products were separated on 1.2% agarose gel prepared in 0.5 X TAE buffer (Tris-acetate-EDTA) (Annexure) and stained in 0.5 µg/mL EtBr. Later on, the gel was visualized and photographed under UV-transilluminator (Gel Doc Mega: Biosystematics USA). Number of bands produced by selected primers and size of the bands were analyzed with respect to 1kbp (kilogram base pair) ladder.

Data scoring and Statistical analysis

Scoring of bands obtained from different RAPD primers was done on the basis of presence (taken as 1) or absence (taken as 0) of bands with respect to molecular weight. The only clear and unambiguous bands were scored for each primer. The binary data was analyzed by Jaccard's similarity coefficient to generate matrix by using Multivariate Statistical Package 3.2.1 (MVSP; Kovach Computing Services, Anglesey, Wales). The value similarity indices between 0 to 1. The value closer to 1 indicates higher similarity present in the population. By using Jaccard's similarity coefficient data a dendrogram was constructed by unweighted pair group method (UPGMA) using Multivariate Statistical Package 3.2.1 (MVSP; Kovach Computing Services, Anglesey, Wales). A scatter plot of these accessions was plotted by using above data by PCA (Principal Coordinate Analysis) using SPSS (Statistical Package for Social Sciences) software.

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

where, a = presence of bands in sample A and sample B

b = presence of bands in sample A

c = presence of bands in sample B

Selection of plants: Twelve plants of *W. somnifera* growing at different locations were selected randomly from Thapar Institute of Engineering and Technology (Figure 3). DNA was isolated from all selected plants using CTAB method.

Figure 3: The figure represents the plants of *Withania somnifera* growing in different locations. The alphabets a-l represents plants 1 to 12.

The morphological data of 12 *W. somnifera* accessions was collected from various parameters. Leaf shape, arrangement, venation, length, width, number of berries in a cluster, and inter-nodal distance is represented in Table 2. A little difference was observed in leaf length and width, shape, internodal distance and number of berries in a cluster. The data acquired with all the parameters appeared to be almost identical. As a result, morphological data alone would not be sufficient to identify across groups.

Table 2: These are some parameters used to analyze morphological data of 12 accessions *W. somnifera*. by calculating mean and standard error.

Plant sample	**Leaf shape**	**Leaf arrangement**	**Leaf venation**	**Average leaf length**	**Average leaf width**	**Average internodal distance**	**Average number of berries in a cluster**
WS 1	Ovate	Spiral	Pinnate	8.94±0.36	5.43±0.59	4.77±0.11	5.33±0.33
WS 2	Ovate	Spiral	Pinnate	9.24±0.41	5.17±0.09	3.77±0.11	5.22±0.55
WS 3	Ovate	Spiral	Pinnate	8.72±0.32	5.13±0.11	3.55±0.31	5.55±0.61
WS 4	Ovate	Spiral	Pinnate	8.81±0.08	5.38±0.28	3.11±0.24	5.11±0.61
WS 5	Ovate	Spiral	Pinnate	6.53±0.05	4.33±0.03	3.16±0.16	4.66±0.51
WS 6	Ovate Oval	Spiral	Pinnate	9.07±0.34	5.65±0.37	5.66±0.34	5.44±0.22
WS 7	Ovate, Oval	Spiral	Pinnate	7.23±0.03	4.28±0.02	5.38±0.43	5.33±0.51
WS 8	Ovate	Spiral	Pinnate	8.97±0.15	4.86±0.05	3.42±0.20	5.66±0.19
WS 9	Ovate	Spiral	Pinnate	8.61±0.18	4.61±0.11	3.51±0.21	5.88±0.40
WS 10	Ovate, Oval	Spiral	Pinnate	10.06±0.14	5.27±0.15	4.22±0.29	4.44±0.40
WS 11	Ovate	Spiral	Pinnate	6.56±0.16	3.62±0.16	4.55±0.11	5.44±0.2
WS 12	Oval	Spiral	pinnate	5.23±0.08	3.35±0.07	3.88±0.56	5.44±0.40

PCR amplifications: For RAPD analyses, 20 RAPD primers were tested initially. Out of 20 RAPD primers, 15 primers were selected for final amplification (Table 3). All selected primers were producing clear and scorable bands. The DNA fragments of all 12 genotypes of *Withania somnifera* was amplified with selected primers, one at a time and finally the agarose gel electrophoresis was performed.

Table 3: Sequence of various RAPD primers used, data profile of amplifications

RAPD Primer	**Sequence (5'→3')**	**No of fragment**	**Size range (bp)**	**Polymorphic bands**	**Polymorphism (%)**	**PIC value**
RAPD 1	AGCGCCATTG	6	500-2500 bp	4	66.66	0.159
RAPD 2	CTTCCCCAAG	4	750-4000 bp	3	75	0.201
RAPD 3	AGGGCGTAAG	7	1000-6000 bp	7	100	0.400
RAPD 5	ACCGCGAAGG	8	750-3000 bp	7	87.5	0.277
RAPD 6	GGACCCAACC	5	500-4000 bp	4	80	0.180
RAPD 7	GTCGCCGTCA	5	500-4000 bp	3	60	0.202
RAPD 9	TGAGCGGACA	6	500-4000 bp	5	83.33	0.335
RAPD 10	ACCTGAACGG	7	1000-4000 bp	7	100	0.271
RAPD 11	TTGCACGGG	10	250-6000 bp	10	100	0.293
RAPD 16	CATCCGTGCT	5	500-2000 bp	5	100	0.197
RAPD 18	GAGAGCCAAC	5	500-3000 bp	4	80	0.291
RAPD 19	CTGGGGACTT	5	1000-3000 bp	5	100	0.35
RAPD 20	ACCCGGTCAC	6	750-4000 bp	4	66.66	0.310
RAPD 25	AGGGGTCTTG	5	500-5000 bp	4	80	0.391
RAPD 32	TCGGCGATAG	5	750-3000 bp	5	100	0.358
	Total number of Bands	**89**	**Total number Polymorphic bands**	**77**		

In total 89 bands were scored. The size of the amplified bands ranged in between 250-6000 bp. Maximum number of bands were produced by RAPD primer 11 giving a total of 10 amplified bands with a size range in between 250-6000 bp. Minimum amplified bands were produced by

RAPD primer 2 giving a total of 4 bands with a size range in between 750-4000bp. Furthermore, 100% polymorphism was recorded in case of RAPD primer 3, 10, 11, 16, 19, 32 whereas, 60% polymorphism was recorded in case of RAPD primer 7. The highest PIC (Polymorphic Information Content) value (0.400) calculated was in RAPD 3 and lowest PIC value (0.159) was recorded in RAPD 1.

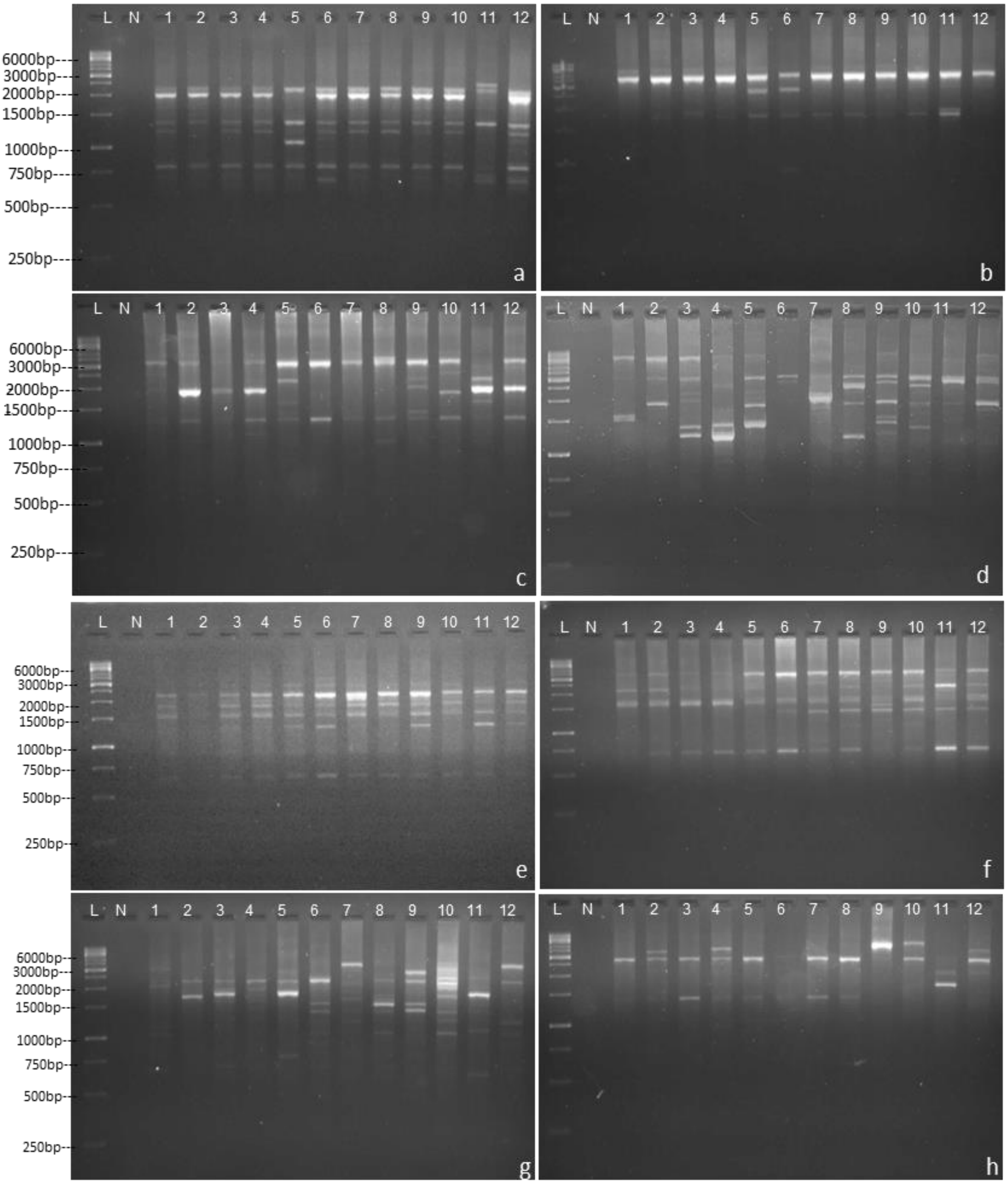


Figure 4: RAPD amplification profiles of 12 populations of *Withania somnifera* with **(a)** RAPD 1 (**b**) RAPD 2 (**c**) RAPD 3 (**d**) RAPD 5 (**e**) RAPD 6 (**f**) RAPD 7 (**g**) RAPD 9 (**h**) RAPD 10, where L is 1kbp ladder, N is negative control and Lane 1-12 is selected plants (WS 1-WS 12)

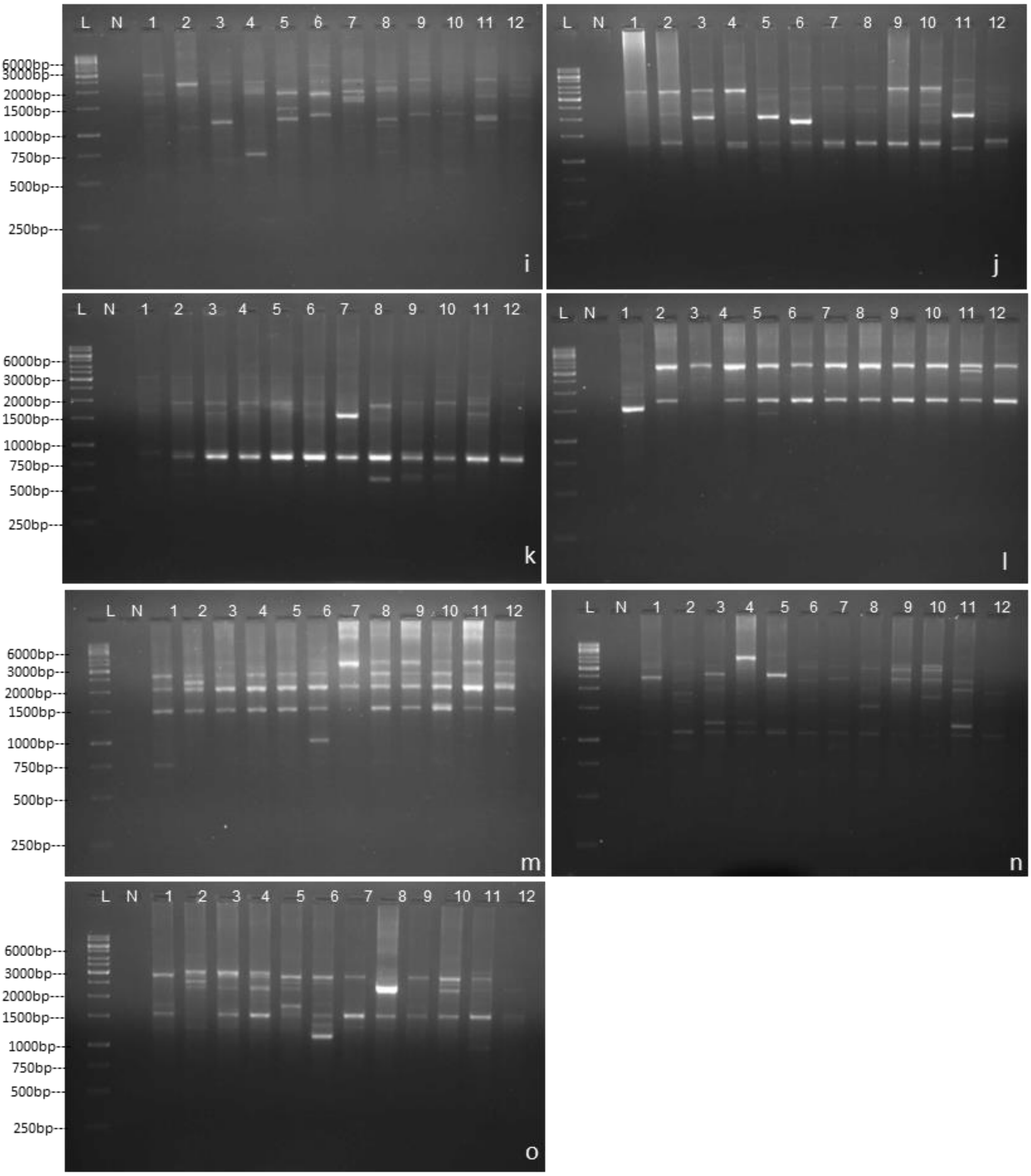
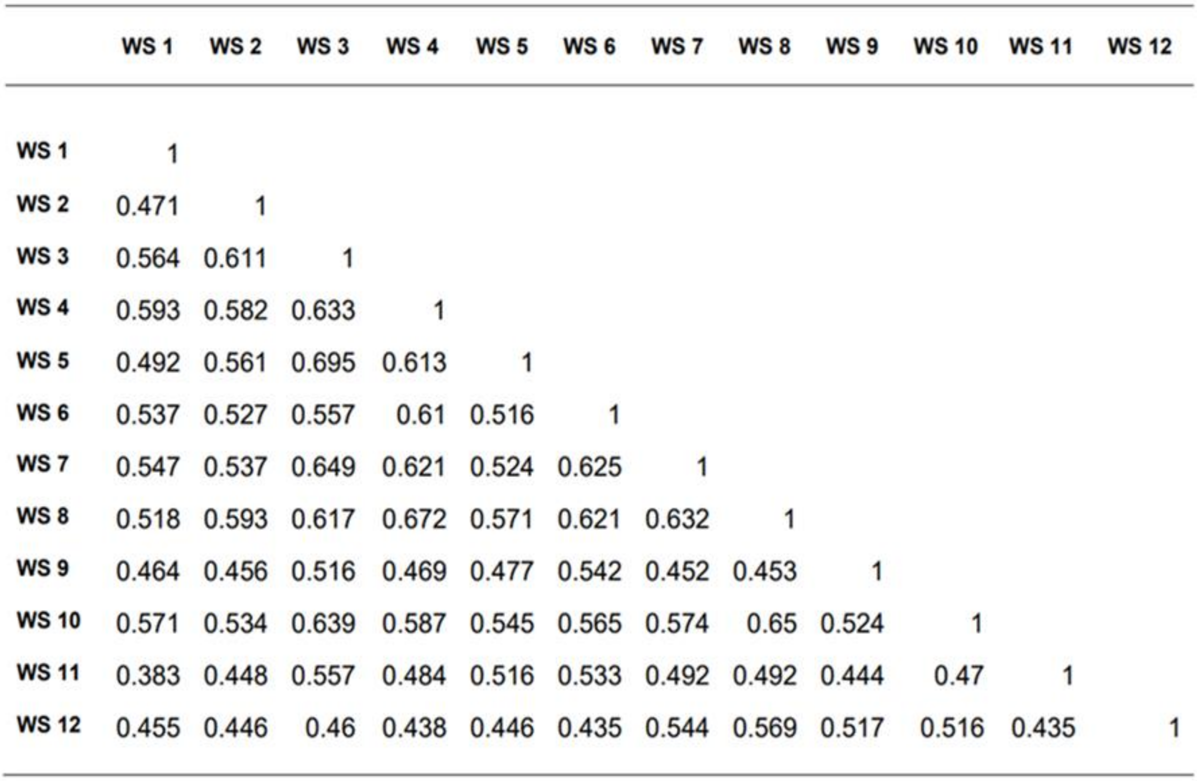


Figure 5: RAPD amplification profiles of 12 populations of *Withania somnifera* with (**i**) RAPD 11 (**j**) RAPD 16 (**k**) RAPD 18 (**l**) RAPD 19 (**m**) RAPD 20 (**n**) RAPD 25 (**o**) RAPD 32, where L is 1kbp ladder, N is negative control and Lane 1-12 is selected plants (WS 1-WS 12)

Jaccard's similarity matrix: Binary data was used to generate Jaccard's similarity matrix. The values of Jaccard's similarity coefficient ranges from 0.383 to 0.695 (Table 4). These values indicating that significant genetic diversity was present among the *W. somnifera* genotypes. Maximum genetic similarity was observed in genotypes WS 3 and WS 5, with similarity coefficient 0.695, followed by genotypes WS 4 and WS 8 with genetic coefficient 0.672. Moreover, least genetic similarity (0.383) was observed in genotype WS 1 and WS11 with similarity coefficient 0.383.

Table 4: Using Jaccard's similarity coefficient from RAPD binary data, a similarity connection between 12 *W. somnifera* accessions was calculated.



	WS 1	WS 2	WS 3	WS 4	WS 5	WS 6	WS 7	WS 8	WS 9	WS 10	WS 11	WS 12
WS 1	1											
WS 2	0.471	1										
WS 3	0.564	0.611	1									
WS 4	0.593	0.582	0.633	1								
WS 5	0.492	0.561	0.695	0.613	1							
WS 6	0.537	0.527	0.557	0.61	0.516	1						
WS 7	0.547	0.537	0.649	0.621	0.524	0.625	1					
WS 8	0.518	0.593	0.617	0.672	0.571	0.621	0.632	1				
WS 9	0.464	0.456	0.516	0.469	0.477	0.542	0.452	0.453	1			
WS 10	0.571	0.534	0.639	0.587	0.545	0.565	0.574	0.65	0.524	1		
WS 11	0.383	0.448	0.557	0.484	0.516	0.533	0.492	0.492	0.444	0.47	1	
WS 12	0.455	0.446	0.46	0.438	0.446	0.435	0.544	0.569	0.517	0.516	0.435	1

*WS 1- 12 refers to as *Withania somnifera* genotype 1 to 12.

UPGMA dendrogram: A dendrogram was constructed by using Jaccard's coefficient similarity values between 12 genotypes of *W. somnifera*. In this dendrogram, two clusters were formed namely Cluster I and Cluster II which further divided into sub cluster groups (Figure 6).

- Cluster I is supported by 40% bootstrap value, only two accessions were present in this cluster i.e., WS 9 and WS 12. Hence, WS 9 and WS 12 depicts more similarity among each other.
- Cluster II is supported by 25% bootstrap value, two sub clusters were formed namely Sub cluster-I and Subcluster II. Sub-cluster I contained only single accession namely WS 11 whereas, sub cluster-II is further divided into three subgroups consisted of 9 accessions. Among these 9 accessions WS 1 and WS 2 formed separate sub-groups and remaining 7 accessions (WS 3, WS 4, WS 5, WS 6, WS 7, WS 8, WS 10) were grouped together. High diversity was seen in accession number WS 1 and WS 11 of *W. somnifera* because they possess lowest similarity values index as 0.383.

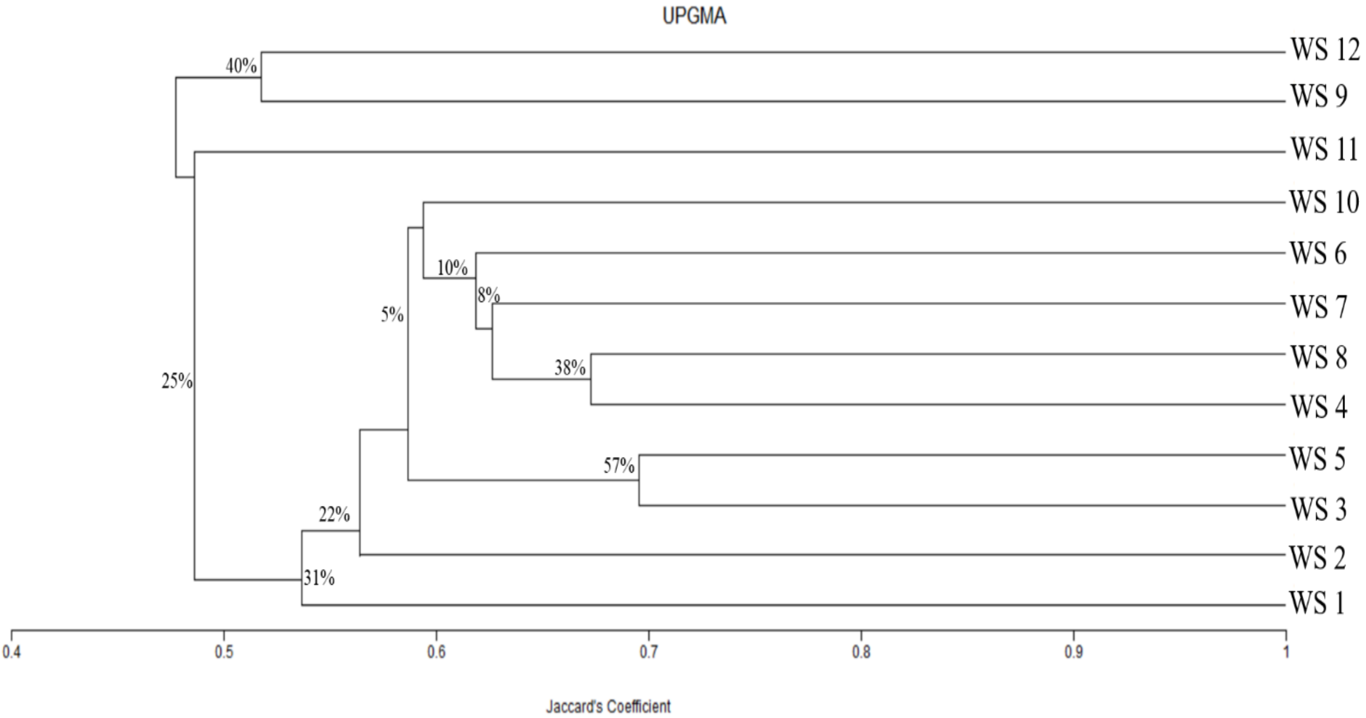


Figure 6: A dendrogram of different accessions of *W. somnifera* was constructed from RAPD data, using UPGMA (unweighted pair group method with arithmetic mean). The bootstrap values are indicated by the values present on each node.

Principle Component analysis (PCA): A scatter plot was created (Figure 7) of all 12 *W. somnifera* accessions using PCA. The overall variations in component 1 and 2 were 33.47 percent and 19.6 percent, respectively, in this plot. The accessions were divided into two groups. Only two accessions, WS 9 and WS 12, were found in one cluster. Another cluster, which is further subdivided into two groups. WS 2, WS 3, WS 4, and WS 5 accessions were found in sub-group I. In sub-group II, the remaining accessions, WS 1, WS 6, WS 7, WS 8, WS 10, and WS 11, were present.

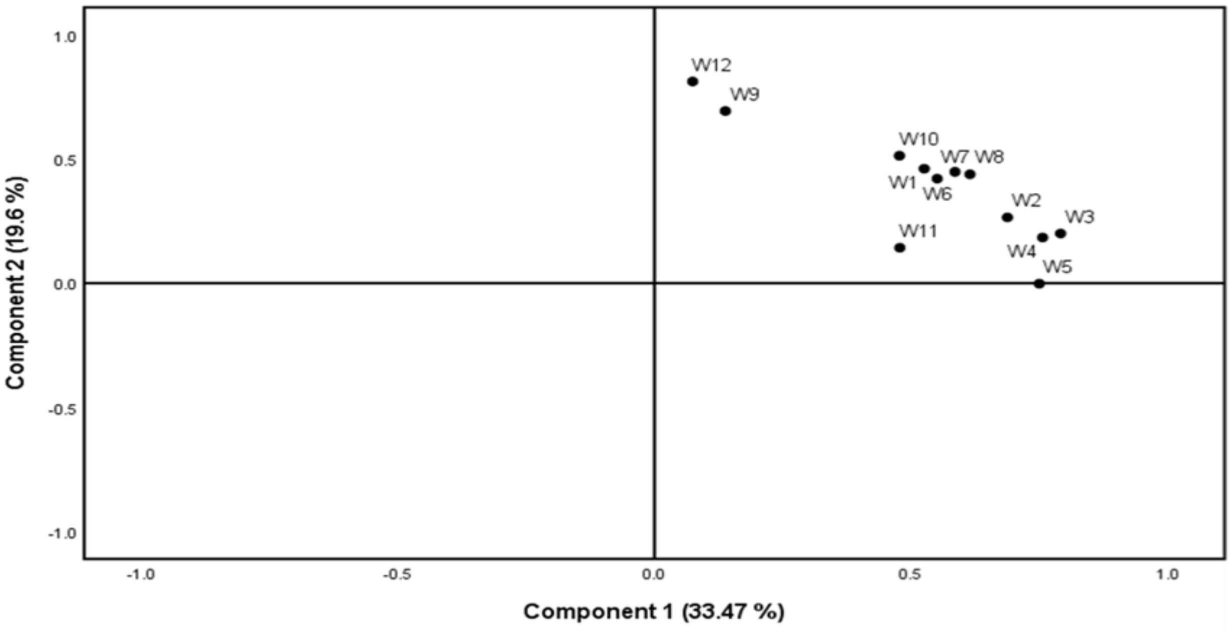


Figure 7: A scatter plot of 12 accessions of *W. somnifera* based on RAPD data generated by SPSS software using PCA analysis.

W. somnifera is one of the important medicinal plants in ayurveda. It is a rich source of many bioactive compounds which attributes to its medicinal properties. In ayurveda, it has been used as rasayana. As a consequence, it is essential to preserve genetic diversity of such plants for future use (Gupta and Rana 2007). The evaluation of genetic diversity is essential for selection, genetic improvement, preservation, and maintenance. The most significant aspect of improving medicinal plant productivity is genetic assessment (Tiwari and Shrivastava 2016). It's also essential to understand which kind of markers, and how many of them, accurately depict variation over the genetic material and could be utilized to make accurate diversity estimates (Tripathi et al. 2012).

Diversity within the Solanaceae family, as well as within *Withania*, is not uncommon (Knapp et al. 2004). The large geographical distribution of *W. somnifera* allows for morphological and chemical diversity (Atal and Schwarting 1962; Lavie et al. 1975). Our findings support the idea that *W. somnifera* has a lot of diversity at genomic level (Negi et al. 2000) as compared to morphological data. However, (Kaur 1957; Atal and Scharting 1962) found that withania has a lot morphological diversity.

In present study, 12 accessions were taken from different locations at Thapar institute of engineering and technology (Patiala). Morphological and RAPD markers were used for analyses of genetic variations among them. In morphological markers, different parameters were studied such as leaf shape, arrangement, venation, length, width, number of berries in a cluster and inter-nodal distance. The data obtained from morphological markers was not enough to characterized into distinct groups. As a result, the data obtained by using these parameters were almost identical with little variations. In contrast, morphological data from other studies revealed a wide range of differences in their morphological characteristics (Khanna et al. 2013; Ganaie et al. 2021). However, Environmental factors and traditional practices have a big impact on physiological and morphological characteristics (Bruschi et al. 2003). Morphological traits are the plant's visible expression, and it is influenced by the environment and traditional practises as well as gene-environment interactions. Considering this, physical features are useful for measuring genetic variability even though they are quick, easy, and universal approach. The second option will be morphological diversity assessment, which also offers estimate variations those are comparable to molecular diversity (Khanna et al. 2013).

Then, molecular data analysis was performed to obtain a remarkable difference between theses accessions of *W. somnifera*. PCR-based RAPD markers were used. In earlier studies, it has been reported that RAPD markers are useful for accessing genetic diversity and were produced good amplifications at genomic level and successfully find out polymorphism in *W. somnifera* species (Tiwari and Shrivastava 2016; Khanna et al. 2014; Bhatt et al. 2012). In the present study, 89 molecular markers were scored in 12 accessions of *W. somnifera* by using RAPD markers. Out of which 77 markers were polymorphic in nature and remaining 13 markers were monomorphic. In RAPD analysis, mainly two clusters were formed and these two clusters were further divided into sub clusters. In one cluster, only two accessions were placed i.e., WS 9 and WS 12. Whereas, in another cluster 10 genotypes were grouped of which one genotypes (WS 11) did not form any other sub group. There are two primary clusters, one in which WS 9 and WS 12 are connected together to create an outgroup. This signifies that these two accessions are more similar to one another and different from other accessions, which is why they are grouped together. The highest PIC value (0.400) was observed with RAPD 3 and the lowest PIC value (0.159) was noticed with RAPD 1. The findings of this study are consistent with other reports on *W. somnifera*, which indicate significant variance across the same and different collection sites (Udayakumar et al. 2013; Khanna et al. 2013).

In this analysis, high degree of polymorphism was detected among all the accessions of *W. somnifera*. High genetic diversity can emerge as a result of mutations caused by environmental and geographical conditions, resulting in a vast gene pool within a species' population (Oleszek et al. 2002). In contrary (Mir et al. 2009), detected low level of polymorphism by RAPD and AFLP markers. They predicted two possible reasons for this. First and foremost, for the previous three years, all of the genotypes have been grown in the same habitat. The species' self-pollinating habit can be the second important cause. Our data, however, demonstrate a higher level of polymorphism than those found in melon and oat genotypes. (Garcia-Mas et al. 2000). On the other hand, Udayakumar et al. (2013) observed significant genetic diversity across different *W. somnifera* collections which could be attributed to a reduction in gene flow as a result of greater geographical distance induced by the loss of native or natural habitat by humans. Therefore, a number of uncommon species have been shown to have significant genetic heterogeneity among populations. This present study reveals that all the accessions of *W. somnifera* belongs to the common origin. However, the presence of high level of genetic diversity depicts plants are able to adapt itself as per their needs to survive.

Several studies have been carried out using molecular techniques to evaluate genetic variability in seedlings, showcasing the co-relation between Jaccard's genetic similarity index and geographic location difference (Mir et al. 2009; Khanna et al. 2013). A contrary study was carried by Bhatt et al. (2012) they reported that there has been no correlation among geographical origins and genetic divergence. This lack of connection was most likely caused by the free flow of seeds through one location to the another. Also, changes within the same ecological zone may have resulted from genetic forces such as selection and genetic drifting.

The RAPD method used in this research demonstrated intra-population variability or molecular polymorphism that has already been present in numerous datasets. Considering their morphological similarities, the genotypes studied were highly polymorphic. This study also reveals that RAPD polymorphisms can be used to identify *W. somnifera* similarity and evolutionary relationships. Various *W. somnifera* genotypes or accessions can also be identified using the RAPD method. The data acquired here could aid in the development of management and conservation strategies for this populations. The RAPD molecular marker approach, according to Gurudeeban et al. (2011), may identify significantly lower level of variability and can be applied as a diagnosis DNA fingerprinting system for accessions or genotypes assessment, genomic modeling, and genetic variability evaluation (Martin et al. 1991; Reiter et al. 1992). RAPD is the easiest method for developing molecular markers (randomly amplified polymorphic DNA). The technique can generate more than single band due to the combination of short sequence primers & low annealing temperature, making it more suitable for rapid polymorphism detection among populations (Scott et al. 1993). The RAPD is quick (Colombo et al. 1998), inexpensive (Fugang et al. 2003), and can analyse data without any need for DNA sequencing (Huff et al. 1993).

In conclusion, it can be concluded that genetic diversity was exhibited in *Withania somnifera* accessions collected from Thapar Institute of Engineering and Technology (Patiala). It was recorded that morphologically plants were very similar and it was very difficult to record any phenotypic changes. Moreover, in our case twelve accessions of *Withania somnifera* from same geographical regions showed the presence of genetic variations.

- It's worth noting that *W. somnifera* populations taken from the same area show genetic vaiability.
- It is reasonable to speculate that such genetic diversity of *W. somnifera* accessions studied that was caused by sexual reproduction among population and selection pressure throughout time.
- *W. somnifera* is believed to have evolved from one of the places and then moved to considerable distance and adapted within the area by associating with environment conditions. This could be one of the factors influencing and result in changes in phytochemical composition in *W. somnifera* populations.
- The RAPD markers has shown potential in revealing patterns of genetic diversity amongst *W. somnifera* populations, which could be important in *W. somnifera* breeding programs and management.
- This research shows, how RAPD polymorphisms can be exploited to determine similarities and evolutionary relationships among *W. somnifera*. The data collected here may be useful in developing conservation and maintenance plans for such population.

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1) Preparation of extraction buffer for DNA isolation

Components	Quantity (1L)
2% CTAB	20 grams
20 mM EDTA	40 mL EDTA (0.5M)
100 mM Tris HCL	100 mL Tris HCL (1M)
1.4 M NaCl	81.82 grams

2) Preparation of 50X TAE buffer

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