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Research Note

Decoding genetic diversity in *Withania somnifera*: A molecular approach using ISSR primers and multivariate analysis

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Abstract
Withania somnifera (L.) Dunal is a medicinally significant plant that is gaining commercial and therapeutic relevance. This study evaluates the efficacy of various primers in detecting genetic polymorphisms by examining the genetic diversity of *W. somnifera* accessions using a variety of molecular markers. Nine of the 12 ISSR primers exhibited noticeable, repeatable, and highly polymorphic banding patterns. The maximum level of genetic variation was demonstrated by UBC 840, while the least diversity was revealed by UEC 8 and UEC 856. In general, the loci analyzed showed a restricted degree of polymorphism, suggesting that the potential for improved resolution may require the use of alternative marker systems or additional primers. The Principal Coordinate Analysis (PCoA) identified two distinct genetic clusters: a compact group of NAS 11–20 and a more dispersed group of NAS 1–8. It is important to note that NAS 9 and NAS 10 were genetically distinct samples, which implies the existence of distinctive traits that require additional investigation. The study underscores the significance of selecting effective primers for accurate diversity assessment and emphasizes the moderate genetic diversity among the accessions. These findings establish a basis for evolutionary investigations, conservation initiatives, and breeding programs.

Keywords: *W. somnifera*, Genetic diversity, ISSR markers, PCoA.

Withania somnifera (L.) Dunal, commonly known as Ashwagandha or Indian ginseng, is one of the most valued medicinal plants in traditional Indian medicine, particularly Ayurveda. Belonging to the Solanaceae family, *W. somnifera* is renowned for its diverse pharmacological properties, including adaptogenic, anti-inflammatory, immunomodulatory, antistress, neuroprotective, and anticancer effects (Dar *et al.,* 2015). These therapeutic benefits are largely attributed to a group of steroidal lactones known as Withanolides, which represent the plant's primary bioactive constituents.

The increasing global demand for *W. somnifera* in the pharmaceutical, nutraceutical, and herbal industries has led to its extensive cultivation and exploitation, raising concerns about genetic erosion in wild populations and variability in bioactive compound content (Ganaie *et al.,* 2016). Understanding the genetic diversity within and among *W. somnifera* populations is critical for developing effective conservation strategies, ensuring sustainable utilization, and breeding superior genotypes with enhanced therapeutic potential.

Molecular markers have emerged as powerful tools for assessing genetic diversity in medicinal plants. Techniques such as Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSR), Simple Sequence Repeats (SSR), and Amplified Fragment Length

Polymorphism (AFLP) have been successfully applied to evaluate the genetic variation in *Withania somnifera* (Khanna *et al.,* 2014). These markers offer advantages such as high reproducibility, genome-wide coverage, and the ability to detect subtle genetic differences among accessions. Such molecular approaches are instrumental in identifying elite genotypes, maintaining genetic integrity, and guiding breeding and conservation programs.

Recent studies have demonstrated significant genetic variability within *W. somnifera* populations. Khabiya *et al*. (2024) reported substantial polymorphism in 39 accessions, including both commercial cultivars and wild types, using RAPD and ISSR markers. Their findings revealed 96.9% polymorphism with RAPD and 91.75% with ISSR markers, underscoring the utility of these markers in detecting genetic differentiation. Similarly, Khan and Shah (2016) analyzed 16 genotypes from different regions of India and found a strong correlation between genetic diversity and geographic origin, with distinct clustering patterns reflecting regional differentiation. Bamhania *et al*. (2013) highlighted the influence of geographic factors on genetic diversity, demonstrating broad variability among 12 internationally sourced genotypes. Additionally, Udayakumar *et al.* (2013) observed significant genetic variation among 20 South Indian populations, with clear associations between genetic structure, environmental conditions, and geographic isolation.

Building upon these findings, the present study aimed to assess the genetic diversity and phylogenetic relationships among 20 *W. somnifera* accessions, representing both cultivated and wild types, using Inter Simple Sequence Repeat (ISSR) markers. The study also sought to evaluate the effectiveness of different ISSR primers in detecting polymorphism and uncovering genetic structure, with implications for conservation, breeding, and sustainable resource management of this medicinally important species.

Young, healthy leaves from 20 genetically distinct *W. somnifera* genotypes were collected from various regions across India, including Gujarat, Madhya Pradesh, Uttar Pradesh, Andhra Pradesh, Karnataka, and Tamil Nadu (**Table 1**). The collection was performed following Good Agricultural and Collection Practices (GACP) for medicinal plants to ensure sample quality and authenticity.

The freshly collected leaves were thoroughly rinsed with distilled water, surface-dried using sterile tissue paper, and immediately ground in liquid nitrogen to preserve DNA integrity. Genomic DNA was extracted using the Cetyl Trimethyl Ammonium Bromide (CTAB) method as described by Doyle and Doyle (1990), with minor modifications to optimize yield and purity. The quality and integrity of the extracted DNA were verified by electrophoresis on a 1% agarose gel, and DNA concentration was adjusted using TE buffer to the working concentration required for downstream applications.

PCR amplification was performed in a total reaction volume of 9 µL, containing 50 ng of genomic DNA, 0.8 µM primer, 10 mM dNTPs, 1 U of Taq DNA polymerase, 3X Taq buffer, 25 mM $MgCl_2$, and sterile nuclease-free water. Amplifications were carried out using a Prima 96 PCR thermal cycler (HiMedia) under the following cycling

Table 1. Samples were collected from various locations, detailing their distinct varieties and specific characteristics.

Sample code	Variety	Location
NAS 1	RVA 100	Jawaharlal Nehru Krishi Vishwavidyalaya (JNKVV) Jabalpur (Madhya Pradesh)
NAS 2	Jawahar 134	JNKVV Jabalpur (Madhya Pradesh)
NAS 3	Wild variety	Topslip (Tamil Nadu)
NAS 4	Wild variety	Aliyar, Coimbatore (T.N)
NAS 5	Wild variety	Arumbakkam ,Chennai (Tamil Nadu)
NAS 6	Wild variety	Jabalpur (Madhya Pradesh)
NAS 7	RVA 20	Krishi Vigyan Kendra (KVK) Neemach Madhya Pradesh
NAS 8	RVA 100	KVK Neemach (Madhya Pradesh)
NAS 9	RAS 134	KVK Neemach Madhya Pradesh
NAS 10	APH-6	Indian Institute of Horticultural Research (IIHR), Hessarghatta, Bangalore (Karnataka)
NAS 11	Anand Ashwagandha 1	Anand , (Gujarat)
NAS 12	Nimitli	Central institute of Medicinal and Aromatic Plants (CIMAP) Lucknow (Uttar Pradesh)
NAS 13	Chetak	CSIR-CIMAP Lucknow (Uttar Pradesh)
NAS 14	Pratap	CSIR-CIMAP Lucknow (Uttar Pradesh)
NAS 15	Poshita	CSIR-CIMAP Lucknow (Uttar Pradesh)
NAS 16	Wild variety	Mettur M.P.C.A , Salem district, Tamil Nadu
NAS 17	Wild variety	Near Podukottai Dam, Pudukottai District (Tamil Nadu)
NAS 18	Wild variety	Aliyar Dam Research Nursery Coimbatore District (Tamil Nadu)
NAS 19	Wild variety	Tiruchirapalli District (Tamil Nadu)
NAS 20	Wild variety	Anantapur, Andhra Pradesh

conditions: initial denaturation at 94°C for 4 minutes; 30 cycles of denaturation at 94°C for 30 seconds, annealing at 51°C for 45 seconds, and extension at 72°C for 45 seconds; followed by a final extension at 72°C for 10 minutes to ensure complete product synthesis.

PCR products were resolved on 2.5% agarose gels stained with ethidium bromide (0.5 µg/mL) for visualization. Molecular size determination was performed using a HiMedia 100 bp DNA ladder (MBT 130-50 LN) and a BioHelix RTU 100 bp DNA ladder (DM 015-R500) as references. Gel images were captured and documented using an Alpha Innotech gel documentation system equipped with a GeneDireX UV transilluminator (USA).

An initial screening of 12 ISSR primers revealed that nine produced clear, consistent, and highly polymorphic banding patterns. These were selected for detailed analysis (**Table 2**). The ISSR-PCR generated well-defined bands, which were scored as binary data—presence (1) or absence (0)—to construct a matrix for genetic diversity assessment.

Genetic diversity parameters including observed number of alleles (Na), effective number of alleles (Ne), Nei's gene diversity (h), Shannon's Information Index (I), and the percentage of polymorphic loci (P) were calculated using Gen AlEx version 6.5. Additionally, polymorphism information content (PIC), heterozygosity (He), marker index (MI), resolving power (R), and discriminating power (D) were computed to evaluate the informativeness and efficiency of each primer.

Genetic diversity and primer efficiency: PIC values, which indicate the discriminatory power of markers, ranged from 0.008 (UBC 840) to 0.131 (UEC 856), with an average of 0.094. Higher PIC values in UEC 856 (0.131) and UEC 8 (0.128) suggest these primers are highly informative, whereas UBC 840, despite low PIC, demonstrated the highest effective allele count (Ne = 1.707) and expected heterozygosity (He = 0.414), indicating significant allelic richness.

The mean heterozygosity was 0.208, with effective allele numbers ranging from 1.052 to 4.158. UBC 825 showed the highest marker index (MI = 4.158), resolving power (R = 8.316), and discriminating power (D = 0.999), establishing it as the most effective primer for assessing genetic variation. Conversely, UEC 8 and UEC 856 exhibited the lowest Ne (1.052) and He (0.118), indicating limited diversity detection capability.

Shannon's Index (I) ranged from 1.052 (UEC 8, UEC 856) to 1.707 (UBC 840), reinforcing the high variation potential of UBC 840. Band frequency (0.050–0.500) and allele frequencies further corroborated the genetic richness and informativeness of UBC 840, while UEC 8 and UEC 856 had the lowest minor allele frequency (p = 0.025), suggesting restricted polymorphism detection.

Clustering and multivariate analysis: Principal Coordinates Analysis (PCoA) was employed to visualize genetic relationships among the 20 accessions. The first three axes accounted for 30.07% of the total variance, with axis 1 contributing 16.33%, followed by axis 2 (7.39%) and axis 3 (6.35%) (**Table 6**). Although no single axis dominated the variance, the spread across dimensions suggests complex patterns of genetic differentiation.

The PCoA plot (**Graph 1)** revealed two major genetic clusters. A compact cluster (NAS 11–20) in the lower left quadrant indicated a high degree of similarity, potentially due to shared genetic lineage or ecological conditions. A moderately dispersed cluster (NAS 1–8) suggested some genetic overlap with the compact group, but with distinct substructure. NAS 9 and NAS 10 appeared as clear outliers, suggesting the presence of unique alleles or divergent evolutionary histories.

Table 2. Details of the primers used for the amplification with their sequences.

S. No	Oligo Name	Primer sequence
1	UBC 840	5'- GAGAGAGAGAGAGAGAY- 3'
2	UBC 7	5'-TCTCTCTCTCTCTCTCG- 3'
3	UEC 6	5'- ACACACACACACACACG- 3'
4	UEC 8	5' -AGAGAGAGAGAGAGAGC- 3'
5	UEC 4	5'-CACACACACACACACAA-3'
6	UBC 6	5'- TCTCTCTCTCTCTCTCA- 3'
7	UBC 825	5'- ACACACACACACACACT-3'
8	UBC 2	5'- GAGAGAGAGAGAGAGAA- 3'
9	UEC 856	5'-ACACACACACACACACCTA- 3'
10	UEC 842	5'- GAGAGAGAGAGAGAGAYG-3'
11	UBC 811	5' GAGAGAGAGAGAGAGAC-3'
12	UBC 827	5'-ACACACACACACACACG-3'

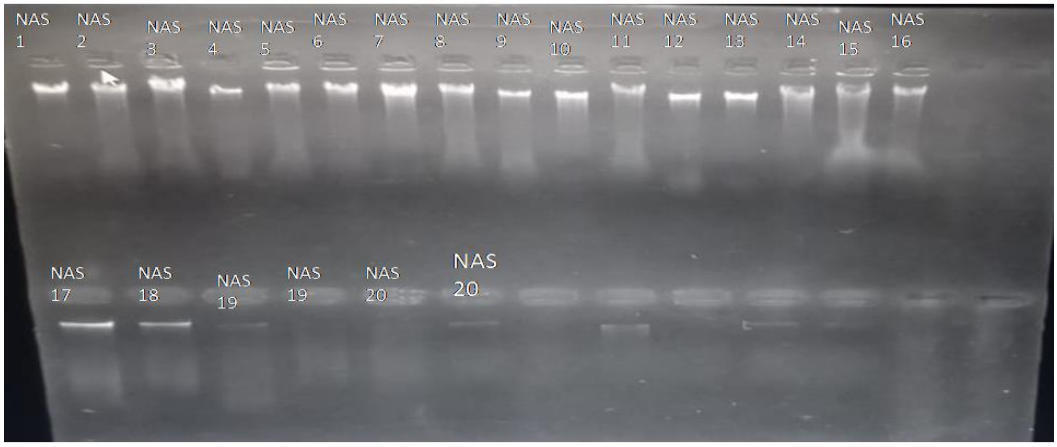


Fig. 1. Genomic testing of isolated DNA samples

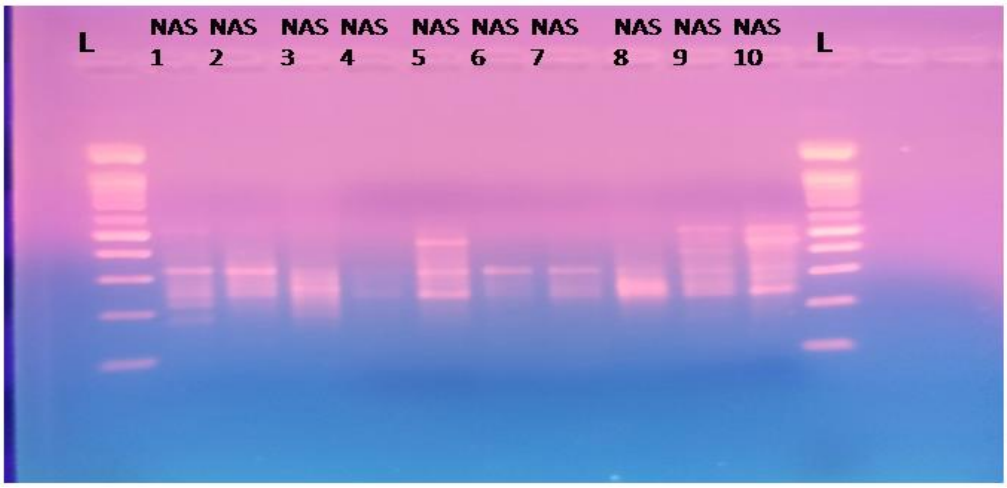


Fig. 2. Primer UEC 4 with set 1 (Sample 1-10) and HiMedia 100 bp DNA ladder (MBT 130-50 LN).

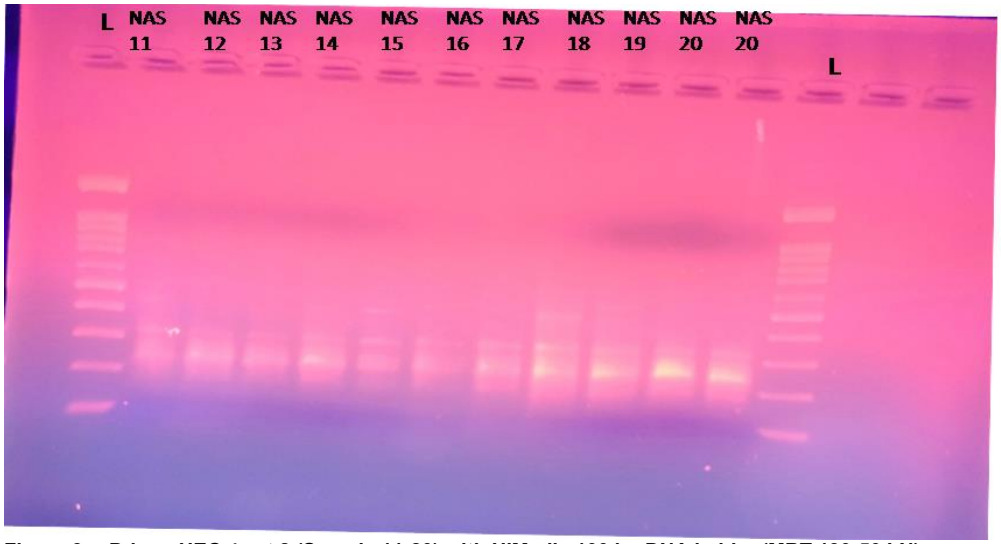


Fig. 3. Primer UEC 4 set 2 (Sample 11-20) with HiMedia 100 bp DNA ladder (MBT 130-50 LN)

Hierarchical cluster analysis supported the PCoA findings. Dendrogram analysis based on genetic distance (**Table 5**) separated the accessions into two primary clusters, with additional sub-clusters revealing fine-scale genetic structure. Sub-clusters A (NAS 1–4) and B (NAS 5–8) displayed moderate divergence, while sub-cluster C (NAS 11–20) was more cohesive, albeit with some internal variation (e.g., NAS 17–20 showing a distinct lineage). Outlier samples NAS 9 and NAS 10 formed separate branches, reflecting their high genetic distances from other samples.

Pairwise genetic distances indicated strong similarity between some accessions (e.g., NAS 12–13 with a distance of 15), and notable divergence in others (e.g., NAS 9–17 with a distance of 31), suggesting both close genetic relationships and substantial differentiation among accessions from different geographical origins.

Collectively, the findings indicate moderate genetic diversity within *W. somnifera* accessions. Among the primers tested, UBC 825 was the most effective for detecting polymorphism, while UBC 840 showed high genetic variation despite low PIC. UEC 8 and UEC 856 were less informative, with low PIC, heterozygosity, and effective allele numbers. The results emphasize the importance of selecting primers with high PIC, MI, and resolving power to ensure reliable genetic differentiation. The observed genetic structure aligns with geographic and potential evolutionary divergence, particularly evident in the differentiation of NAS 9 and NAS 10. These outlier genotypes may harbour valuable traits for breeding and conservation, warranting further investigation.

This study assessed the genetic diversity of *W. somnifera* accessions using ISSR markers and identified considerable variation among accessions. UBC 825

Table 3. Polymorphic information content and genetic diversity indices of various primers

	H	PIC	E	H. av.	MI	D	R
UBC 840	0.500	0.008	1.000	0.013	0.013	0.757	1.895
UBC 7	0.387	0.058	0.789	0.007	0.005	0.934	1.579
UEC 6	0.361	0.068	0.947	0.005	0.005	0.946	1.789
UEC 8	0.100	0.128	0.474	0.001	0.000	0.998	0.947
UEC 4	0.102	0.128	2.105	0.000	0.000	0.997	4.211
UBC 6	0.104	0.128	1.211	0.000	0.000	0.997	2.421
UBC 825	0.123	0.126	4.158	0.000	0.000	0.996	8.316
UBC 2	0.114	0.127	1.947	0.000	0.000	0.996	3.895
UEC 856	0.076	0.131	0.158	0.001	0.000	0.999	0.316
Average	0.208	0.100	1.421	0.003	0.003	0.958	2.819

(H – Heterozygosity, PIC – Polymorphism Information Content, E – Effective Number of Alleles, H. av. – Average Heterozygosity, MI – Marker Index, D – Discriminating Power, R – Resolving Power.)

Table 4. Band frequency, allelic diversity, and heterozygosity assessments

	Band Freq.	p	q	N	Na	Ne	I	He	uHe
UBC 840	0.500	0.293	0.707	20.000	2.000	1.707	0.605	0.414	0.425
UBC 7	0.267	0.147	0.853	20.000	2.000	1.340	0.394	0.240	0.246
UEC 6	0.238	0.134	0.866	20.000	2.000	1.316	0.333	0.206	0.212
UEC 8	0.050	0.025	0.975	20.000	2.000	1.052	0.118	0.049	0.051
UEC 4	0.055	0.028	0.972	20.000	1.949	1.058	0.126	0.054	0.056
UBC 6	0.055	0.028	0.972	20.000	2.000	1.057	0.126	0.054	0.055
UBC 825	0.066	0.034	0.966	20.000	2.000	1.070	0.144	0.065	0.066
UBC 2	0.061	0.031	0.969	20.000	2.000	1.064	0.136	0.060	0.061
UEC 856	0.050	0.025	0.975	20.000	2.000	1.052	0.118	0.049	0.051
Average	0.149	0.083	0.917	20.000	1.994	1.191	0.233	0.132	0.136

(Band Freq. ─ Band Frequency, p – Minor Allele Frequency, q – Major Allele Frequency, N – Sample Size, Na – Observed Number of Alleles, Ne – Effective Number of Alleles, I – Shannon's Information Index, He – Expected Heterozygosity, uHe – Unbiased Expected Heterozygosity.)

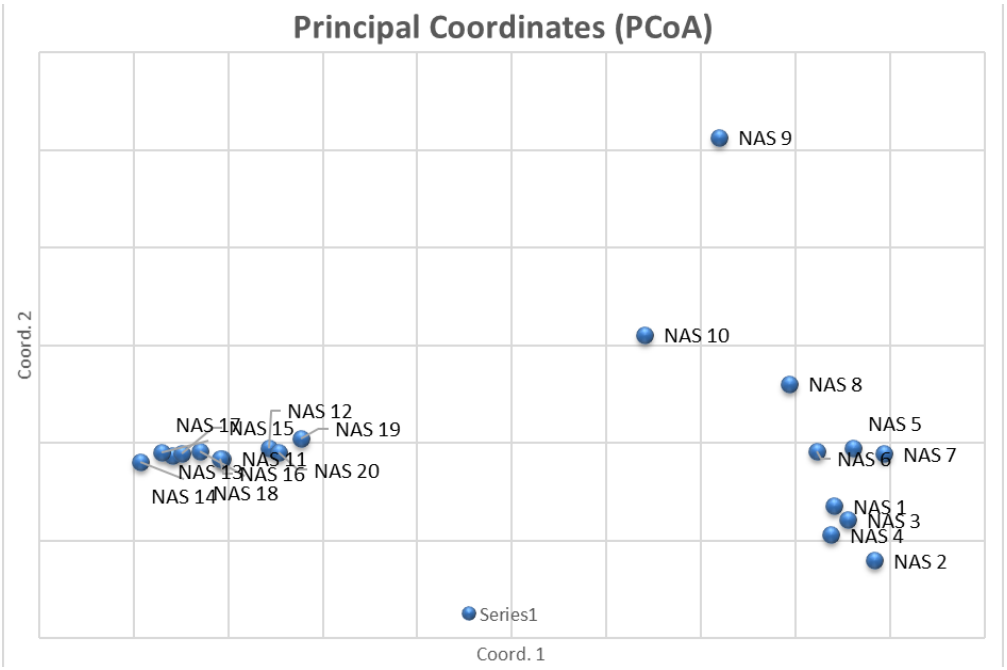
Fig. 4. Genetic distance dendrogram of 20 *W. somnifera* Samples (NAS 1- NAS 20)

Table 5. Genetic distance matrix among 20 *W. somnifera* samples from various sites and varieties.

NAS 1	NAS 2	NAS 3	NAS 4	NAS 5	NAS 6	NAS 7	NAS 8	NAS 9	NAS 10	NAS 11	NAS 12	NAS 13	NAS 14	NAS 15	NAS 16	NAS 17	NAS 18	NAS 19	NAS 20	
0	23	22	22	22	20	24	24	28	26	26	24	25	27	26	24	29	25	23	25	NAS 1
23	0	21	21	23	21	25	25	29	27	27	25	26	28	27	25	30	26	24	26	NAS 2
22	21	0	22	22	20	24	22	28	26	24	24	25	27	26	24	29	25	23	25	NAS 3
22	21	22	0	20	20	24	24	28	26	26	22	25	27	26	22	29	25	23	23	NAS 4
22	23	22	20	0	20	24	20	26	26	26	24	25	25	26	24	29	25	23	23	NAS 5
20	21	20	20	20	0	20	22	24	24	24	22	23	23	24	22	27	23	21	23	NAS 6
24	25	24	24	24	20	0	26	28	28	28	26	27	29	28	26	31	27	25	27	NAS 7
24	25	22	24	20	22	26	0	26	26	26	22	25	27	26	24	29	25	23	25	NAS 8
28	29	28	28	26	24	28	26	0	28	28	26	27	29	28	26	31	27	25	27	NAS 9
26	27	26	26	26	24	28	26	28	0	26	24	25	27	26	24	29	25	23	25	NAS 10
26	27	24	26	26	24	28	26	28	26	0	18	17	19	18	16	21	17	17	19	NAS 11
24	25	24	22	24	22	26	22	26	24	18	0	15	17	18	16	19	17	15	13	NAS 12
25	26	25	25	25	23	27	25	27	25	17	15	0	12	17	15	20	16	16	18	NAS 13
27	28	27	27	25	23	29	27	29	27	19	17	12	0	15	17	22	18	18	18	NAS 14
26	27	26	26	26	24	28	26	28	26	18	18	17	15	0	16	21	17	17	19	NAS 15
24	25	24	22	24	22	26	24	26	24	16	16	15	17	16	0	19	11	15	17	NAS 16
29	30	29	29	29	27	31	29	31	29	21	19	20	22	21	19	0	20	20	22	NAS 17
25	26	25	25	25	23	27	25	27	25	17	17	16	18	17	11	20	0	16	18	NAS 18
23	24	23	23	23	21	25	23	25	23	17	15	16	18	17	15	20	16	0	14	NAS 19
25	26	25	23	23	23	27	25	27	25	19	13	18	18	19	17	22	18	14	0	NAS 20

Table 6. PCoA via covariance matrix with data standardization

Percentage of variation explained by the first 3 axes			
Axis	**1**	**2**	**3**
%	16.33	7.39	6.35
Cum %	16.33	23.72	30.07



Graph 1. PCoA via covariance matrix with data standardization analysis

emerged as the most effective primer based on marker index, heterozygosity, and resolving power, while UEC 8 and UEC 856 demonstrated limited/ least genetic diversity. Multivariate analyses, including PCoA and hierarchical clustering, revealed two principal genetic clusters: one compact group (NAS 11–20) and one more dispersed group (NAS 1–8), with NAS 9 and NAS 10 standing out as genetically distinct. These results reflect both regional differentiation and underlying genetic diversity within the species.

These findings provide valuable insights into the genetic architecture of *W. somnifera*, with implications for breeding, conservation, and selection of superior genotypes. To further enhance resolution, future studies should expand sample size, incorporate more molecular marker systems (e.g., SSR, SNP), and integrate chemotypic or ecological data to better understand the evolutionary dynamics and trait associations within this important medicinal plant.

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