Full Length Research Article

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Research Journal of Agricultural Sciences
Volume 15; Issue 03 (May–Jun 2024); pp 674–681

ISSN: 0976-1675 (P)
ISSN: 2249-4538 (E)

Diving into the Genetic Pool and Molecular Treasury of *Sphagneticola trilobata*: A Unique Journey with ISSR Markers

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Received: 16 Feb 2024; Revised accepted: 21 Apr 2024

Abstract

The research aimed to explore the genetic diversity of *Sphagneticola trilobata* populations sourced from Thiruvananthapuram and Alleppey, two distinct locations in Kerala, India, utilizing ISSR (Inter Simple Sequence Repeat) markers, a robust tool in population genetics for assessing genetic variation. Subsequently, DNA extraction was carried out following established molecular biology protocols to obtain high-quality genomic DNA from the collected samples. ISSR analysis was then conducted, employing PCR amplification targeting regions between microsatellite sequences, with primers designed to anneal at these sites. The resulting amplification patterns served as fingerprints, reflecting the genetic variation within the samples. For comprehensive data analysis, Nei's gene diversity and Shannon's Information index were computed to quantitatively assess genetic diversity within each population. The study unveiled significant disparities in genetic diversity metrics between the two locations, Polymorphic diversity analysis further supported this, highlighting heightened genetic variation in Sample 2 from Thiruvananthapuram. Overall, Sample 2 consistently displayed elevated genetic diversity, suggesting a more robust genetic makeup compared to Sample 1 from Alleppey. These findings not only contribute to our understanding of the genetic variation, effective allele numbers, and information content within *Sphagneticola trilobata* populations but also hold implications for conservation strategies and evolutionary studies in the studied regions.

***Key words*:** ISSR markers, Genetic diversity, DNA isolation, Extraction, Phytochemicals

A medicinal plant or herb is a natural resource valued for its fragrance, taste, or therapeutic properties, traditionally used for treating various illnesses. In modern society, these plants are frequently consumed as dietary supplements and wellness enhancers. Herbal remedies offer a holistic approach to maintaining or improving well-being and come in various forms, including tablets, capsules, powders, teas, extracts, as well as fresh or dried plant material. Exploring the ethno dermatological applications of medicinal plants in India necessitates further scientific investigation to establish chemical, microbiological, and clinical evidence supporting their efficacy in treating skin disorders [1]. Traditional medicine, distinct from allopathic practices, relies on theories, beliefs, and accumulated experiences to maintain health, prevent, diagnose, and treat physical and mental ailments. These practices, contributing significantly to global healthcare, particularly in community-level primary healthcare, remain popular worldwide [2]. Throughout history, plants have played pivotal roles in art, intellect, and scientific study, enriching our understanding of life processes. Research on fundamental plant life processes not only enhances our intellectual landscape but also informs approaches to challenges in agriculture, health, and the environment. Despite advancements in synthetic drugs, medicinal plants continue to offer diverse and potent remedies for infections and diseases [3].

Approximately 80% of the world's population relies primarily on traditional medicine, often involving plant extracts or their active constituents [4-5]. The increasing global demand for medicinal herbs raises safety concerns, emphasizing the necessity for standardization, quality control, and evaluation of herbal materials to ensure their safety and efficacy. While medicinal plants show potential as alternative cancer therapy, more clinical trials are crucial to validate their benefits for cancer patients [6-7]. This study focuses on *Sphagneticola trilobata* (L.) Pruski, a member of the Asteraceae family native to South America and the West Indies. This creeping evergreen herb, known as Wedelia, features rounded stems, fleshy leaves, and bright yellow flowers. Propagation is mainly vegetative due to limited seed fertility, and it thrives in various ecological conditions, tolerating diverse soil types, inundation, and high salinity [8]. The plant exhibits anti-inflammatory, antimicrobial, analgesic, larvicidal, anti-diabetic, and anti-turmeric properties.

Assessing genetic diversity is crucial for various research applications. Molecular markers, including RAPD, DAF, APPCR, ISSR, and AFLP, allow precise evaluation of genetic diversity. Genetic diversity in Sphagneticola trilobata genotypes was assessed using agro-morphological traits, ISSR, and SCOT profiling, revealing substantial genetic variation. Cluster analysis grouped genotypes into eight main clusters,

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Citation: Sofia S, Majeed A. 2024. Diving into the genetic pool and molecular treasury of *Sphagneticola trilobata*: A unique journey with ISSR Markers. *Res. Jr. Agril. Sci.* 15(3): 674-681.

highlighting genetic variability. ISSR markers detected high polymorphism (100%) [9-10].

Isolating and purifying DNA is a crucial step in plant molecular techniques, essential for identifying genotypes, studying economic traits linked to specific genes, and exploring genetic diversity. Understanding the genetic diversity within and among populations is particularly important for enhancing medicinal plant traits. Throughout the process of clonal reproduction, plants undergo intricate adaptations, developing defense mechanisms in response to environmental stressors. These adaptations are facilitated by potential epigenetic traits, which mediate genetic responses leading to phenotypic changes, a phenomenon known as phenotypic plasticity [11]. Adaptation pressures from diverse habitats are influencing the genetic and epigenetic diversity within the species. Environmental factors exert varying degrees of influence on both genetic and epigenetic levels, with a notably stronger impact on epigenetics [12].

Simple Sequence Repeats (SSR) is highly polymorphic tandem repeats of 2-6 base pairs, variable in the number of repeats at specific loci. Several studies have emphasized the importance of employing multiple molecular markers to assess the genetic diversity of various species. Characterizing germplasm is a crucial step in supporting breeding programs, particularly in angiosperms. Molecular markers, such as ISSR (Inter Simple Sequence Repeat), play a vital role in this process. By utilizing ISSR markers, can effectively analyze the genetic diversity and variability present within the germplasm. This characterization provides valuable information that can aid in the selection of diverse and desirable traits for breeding purposes, ultimately contributing to the improvement and sustainable cultivation [13]. It is evident that combined molecular investigations yield more comprehensive results than individual analysis systems. Therefore, this study aims to evaluate the molecular-level genetic variation and population structure in *Sphagneticola trilobata* collected from diverse locations in Kerala, using a multifaceted approach.

This study holds significant importance in several aspects. Firstly, it contributes to the scientific understanding of genetic diversity within *Sphagneticola trilobata* populations, providing insights into the species' evolutionary dynamics and adaptive potential. By utilizing ISSR markers, the research offers a comprehensive analysis of genetic variation, shedding light on the genetic makeup of populations from distinct geographic locations. Secondly, the findings have practical implications for conservation strategies, as understanding genetic diversity is crucial for effective management and preservation of plant species. By identifying regions with higher genetic diversity, conservation efforts can be targeted to prioritize the protection of valuable genetic resources. Additionally, the study highlights the role of environmental factors in shaping genetic diversity, emphasizing the need for sustainable land management practices to mitigate the impact of habitat degradation on plant populations. Overall, this research contributes valuable knowledge that can inform conservation practices, enhance biodiversity conservation efforts, and guide future studies on the evolutionary ecology of *S. trilobata*.

MATERIALS AND METHODS

Description of collection spots

The selected plant for the study was *Sphagneticola trilobata* (L.) Pruski. The research involved the collection of *S. trilobata* specimens from Thiruvananthapuram and Alleppey districts of Kerala. The primary objectives were to investigate the phytochemical constituents and assess molecular variability among these plants.

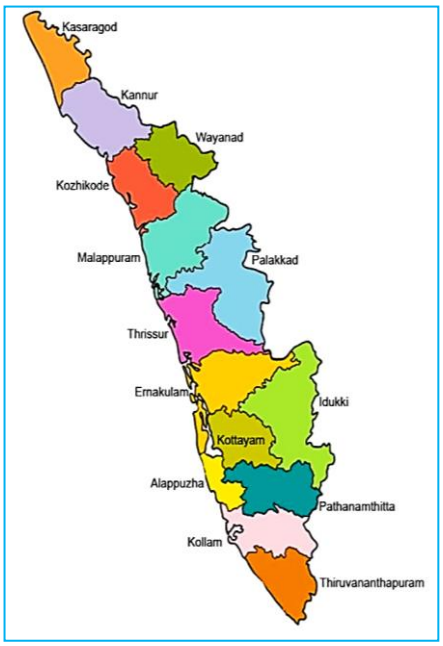


Fig 1 Locations from the plants collected

A - Thiruvananthapuram
B - Aleppy

Thiruvananthapuram, situated in the southern part of Kerala, became a distinct district in 1957. The city of Thiruvananthapuram serves as both its administrative center and the capital of Kerala. Known for its rich biodiversity, the district features a diverse array of plant life, encompassing rare orchids, medicinal herbs, spices, hedges, tuber crops, fruit-bearing trees, and fiber-yielding plants. Alappuzha, often referred to as Aleppy, is a coastal city located in Kerala, India, along the Laccadive Sea. Positioned at a distance of 55 km from Kochi and 155 km north of Thiruvananthapuram, Alappuzha district was established on August 17, 1957.

Fig 2 Plants from Thiruvananthapuram and Alappuzha

Systematic position

Kingdom	:	Plantae
Clade	:	Asterids
Order	:	Asterales
Family	:	Asteraceae
Genus	:	*Sphagneticola*
Species	:	*trilobata*

Description

Sphagneticola trilobata is a perennial plant characterized by green, rounded stems that can grow to a height of 45-60 cm. These stems root at nodes and can extend to lengths of 10-30 cm. The flowering portions of the plant exhibit a coarse, bristly, or hairy texture, though they may occasionally appear nearly

hairless. The leaves are typically medium-textured, fleshy, measuring approximately 4-9 cm in length and 2-5 cm in width. They are simple, obovate in shape, with irregularly toothed or serrated edges. The leaves often feature a pair of lateral lobes and are arranged opposite to sub opposite. *S. trilobata* produces flowers throughout the year, with solitary blossoms emerging in the leaf axils at various heights. The peduncles, measuring 3-10 cm long, support the campanulate-hemispherical involucre, about 1 cm in height. Chaffy bracts are lanceolate and rigid, while the ray florets are typically yellow, measuring 6-15 mm in length and the pappus consists of a crown of short fimbriate scales. The achenes are tuberculate, about 4-5 mm long, with a brown, dry, and hard fruit covering, resulting in inconspicuous fruits.

Genetic diversity analysis of Sphagneticola trilobata (L.) Pruski

DNA isolation from collected leaf samples

To initiate the polymerase chain reaction (PCR) for CMG detection, the initial crucial step involved the isolation of total genomic DNA [14]. The CTAB method was employed for this purpose. Subsequently, the collected samples were appropriately labeled for identification and stored at a temperature of 4°C until they were ready to be utilized in the PCR analysis.

CTAB method of DNA isolation

In the process of extracting DNA using the CTAB method, fresh β-mercaptoethanol was incorporated into the CTAB extraction buffer to achieve a final concentration of 2% (v/v). The buffer was heated to 65°C using a water bath. Samples weighing 200 mg each were ground with a sterile mortar and pestle. Subsequently, warm extraction buffer (1 ml) was added to the ground samples, and the mixture was transferred to sterile 2 ml centrifuge tubes. Gentle inversion was used to homogenize the contents, followed by incubation in a water bath at 65°C for 30 minutes with intermittent shaking. After incubation, the mixture underwent centrifugation at 6,000 rpm for 15 minutes at room temperature. The supernatant was carefully transferred to another sterile centrifuge tube using a sterile pipette tip.

The homogenate was subjected to extraction with an equal volume of a phenol/chloroform/isoamyl alcohol solution (25:24:1, v/v). After thorough mixing by inversion 6-8 times, the mixture underwent centrifugation at 6,000 rpm for 15 minutes, and this extraction step was repeated until the aqueous phase became clear. To the clear aqueous phase, an equal volume of chloroform: isoamyl alcohol solution (24:1, v/v) was added. After mixing by inversion 6-8 times, the mixture was centrifuged at 10,000 rpm for 5 minutes.

To the resulting aqueous phase, chilled isopropanol and 40 µl of 3 M sodium acetate were added. The mixture was gently mixed by inversion and then incubated at room temperature for 15 minutes or overnight at 4°C to precipitate the nucleic acid. Following incubation, the precipitated DNA was pelleted by centrifugation at 12,000 rpm for 15 minutes. The supernatant was carefully decanted, and the DNA pellet underwent washing with 0.5 ml of 80% ethanol, followed by centrifugation at 10,000 rpm for 10 minutes at room temperature. The supernatant was discarded, and the pellet was washed with 0.5 ml of 75% ethanol, followed by centrifugation at 10,000 rpm for 10 minutes at room temperature. The supernatant was discarded, and the DNA pellet was air-dried for 10-15 minutes and then dissolved in 35 µl of nuclease-free water. The DNA underwent incubation at 65°C for 10 minutes to ensure complete dissolution. Finally, the isolated DNA samples were stored at 4°C.

Analysis of the extracted DNA

Agarose gel electrophoresis

To assess the quality and integrity of the extracted DNA, agarose gel electrophoresis was employed. A 0.8% agarose gel was prepared in 1X TAE buffer, and ethidium bromide (EtBr) was added at a concentration of 0.5 µl/l. Two microliters of each DNA sample, mixed with loading dye, were loaded into separate wells on the gel. Electrophoresis was conducted at a rate of 5 V/cm for duration of 30 minutes. Following electrophoresis, the gel was visualized under UV light to evaluate the quality of the DNA.

Molecular variation analysis of isolates from different locations using polymerase chain reaction using ISSR primers

Polymerase chain reaction (PCR) was carried out using the total DNA extracted from the plant samples to evaluate genetic variation among samples collected from different locations in Kerala. Three ISSR primers, specifically ISSR2 and ISSR3, were utilized in the PCR. Among these, ISSR2 and ISSR3 were identified to produce satisfactory results, leading to their selection for further analysis.

ISSR primers (Inter simple sequence repeat)

The preservation of genetic diversity is essential for the successful adaptation of plant populations to changing environmental conditions, making it a crucial aspect of biological conservation programs. Molecular markers play a significant role in assessing genetic diversity in both natural and cultivated plant populations. One such marker is ISSR (Inter-Simple Sequence Repeat), a PCR-based technique developed by Zietkiewicz and colleagues. ISSR markers offer several advantages, including ease of use, rapidity, simplicity, and cost-effectiveness.

In comparison to other markers like AFLPs (Amplified Fragment Length Polymorphisms), RAPDs (Random Amplified Polymorphic DNA), and more specific SSRs (Simple Sequence Repeats), ISSR markers stand out due to their reproducibility, attributed to their high annealing temperature stringency. Notably, ISSR markers do not require prior knowledge of gene sequences or genetic studies before analysis. They have proven effective in assessing genetic diversity and determining the gene pool origin of plant species.

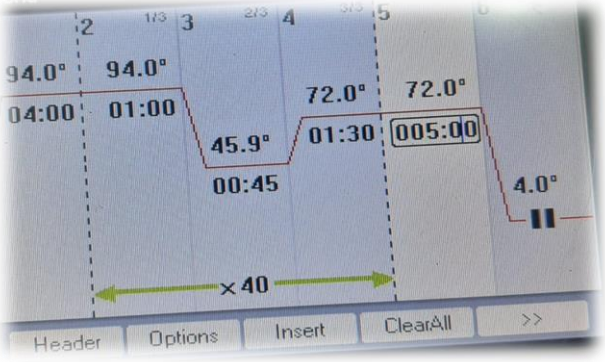
It's important to highlight that the selection of suitable ISSR primers is a crucial initial step in genetic diversity analysis. The success of ISSR markers depends on their ability to amplify genetic fragments with sufficient polymorphism. Since ISSR primers recognize sequences scattered throughout the genome, careful primer selection and testing are essential before conducting comprehensive analyses on the target population samples.

Gradient PCR analysis with ISSR primers

Initially a gradient PCR was performed using a single sample to detect the annealing temperature of ISSR2 and ISSR3 primers.

The components of the mixture were optimized as listed below:

Emerald-Amp PCR master mix	:	10 µl
ISSR2/ ISSR 3primer	:	0.8 µl
Nuclease-free water	:	7.2 µl
Template DNA	:	2 µl
Total volume	:	20 µl



The PCR was conducted using the Eppendorf Nexus Mastercycler. The PCR program was established with an initial denaturation step at 94°C for 4 minutes. Subsequently, 40 cycles were performed, consisting of denaturation at 94°C for 1 minute, annealing at a temperature range of 45°C to 55°C for 45 seconds, and extension at 72°C for 1 minute and 30 seconds. A final extension was carried out at 72°C for 5 minutes. To distinguish target products from non-target products and primer dimers, control reactions were included.

The amplified products, along with a PCR Marker (1 kb plus) from 'Thermo Scientific,' were separated by electrophoresis on a 1% agarose gel. The gel was visualized under a UV transilluminator. The specific annealing temperature at which the maximum number of bands was obtained was selected, and subsequent PCRs were conducted using that particular annealing temperature. This step ensures optimal conditions for amplifying the genetic fragments of interest.

PCR analysis with ISSR primers

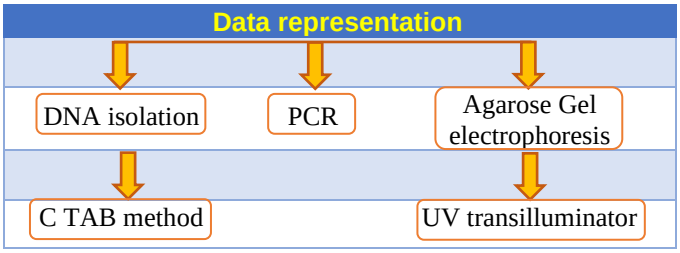
Moreover, PCR was conducted using ISSR 2 and ISSR 3 primers with specific annealing temperatures determined through gradient PCR. This approach enabled the detection of molecular variations among the different isolates of *Sphagneticola* collected from various locations in Kerala. Gradient PCR is a valuable technique for optimizing annealing temperatures, ensuring the specificity and efficiency of the PCR reactions for the particular primers used in the study.

Table 1 Primer details

S. No.	Genes	Primer Sequence	Oligo base types
1.	ISSR 2	5'-GAG AGA GAG AGA GAG ACG -3'	18-DNA bases
2.	ISSR 3	5'-GAG AGA GAG AGA GAG ATC -3'	

Analysis of molecular variability using popgene software

To analyze the variability, the bands obtained from PCR using ISSR2 and ISSR3 primers were scored separately, and the scoring patterns are provided in (Table 1-2). These scoring patterns were then input into the POPGENE software to calculate the molecular variability among the various isolates of Wedelia that were collected. The results can be found in Table 5.3. This analysis likely involved assessing parameters such as genetic diversity, polymorphism, or other relevant measures to understand the genetic variation among the collected Wedelia isolates [15-16].



RESULTS AND DISCUSSION

Genetic diversity analysis

Genetic diversity plays a pivotal role in comprehending the taxonomy, origin, and evolution of plant taxa. Additionally, it holds substantial implications for germ plasm resource conservation, development, utilization, and breeding. The objective of this study was to identify ISSR primers capable of discerning genetic polymorphism among *Sphagneticola* individuals, facilitating the assessment of the species' genetic diversity. ISSR markers are useful tools for assessing and differentiating the genetic diversity within *Sphagneticola trilobata* populations. Inadequate genetic diversity levels can constrain a population's adaptive capacity to changing environmental conditions, thereby diminishing plant fitness. Information regarding genetic diversity is also instrumental in discerning evolutionary relationships within a taxon [17]. The specific patterns observed, with Sample 2 displaying greater polymorphism, contribute valuable insights into the genetic makeup and variability of these samples.

The genetic diversity analysis of *Sphagneticola trilobata* samples using ISSR markers suggests that these markers are effective in distinguishing between the samples. Notably, there is a discernible difference between Sample 1 and Sample 2. Sample 2 exhibits a higher level of polymorphism, as indicated by the elevated values of both Shannon's index and Nei's gene diversity. The increased polymorphism in Sample 2 implies a greater variety of genetic traits or variations within that sample compared to Sample 1. This heightened diversity is often indicative of a more complex genetic composition, possibly resulting from factors such as a larger population size, historical or ecological influences, or other genetic dynamics.

DNA isolation

The quality and quantity of isolated DNA are influenced by the handling procedures employed by researchers. If the material is not promptly placed in cell lysis buffer for further processing, a decline in DNA quality and quantity is observed [18]. In DNA-typing studies, fresh plant material is the primary source of DNA 'fingerprint' for comparison. The findings demonstrate the potential use of plant extracts as alternative DNA extraction sources. PCR products of similar base-pair sizes for the target mitochondrial gene were obtained from the sample.

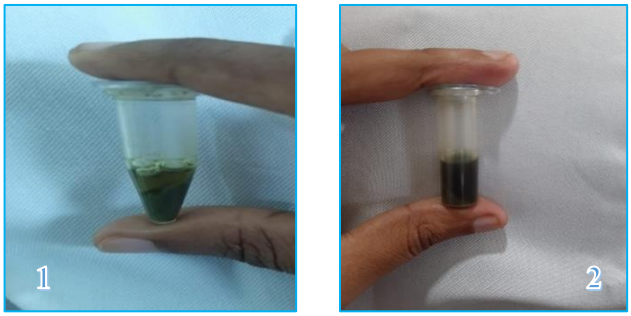


Fig 5.1 DNA isolation of samples
(1 – Thiruvananthapuram, 2 – Alleppey)

The centrifugation process at 10,000 rpm for 10 minutes resulted in the separation of distinct supernatant and pellet fractions for both Sample 1 and Sample 2. An interesting observation is that the supernatant obtained from Sample 1 displayed a more pronounced color density compared to that of Sample 2. This difference in color density suggests variations in the composition or concentration of the suspended particles or solutes in the supernatant between the two samples.

Due to the more intense color in the supernatant of Sample 1, special attention was given to this fraction. The denser supernatant from Sample 1 was carefully transferred to a new tube, indicating a selective approach to furthering the precipitation process. This meticulous transfer suggests that the researcher prioritized the potentially enriched or concentrated components in the supernatant of Sample 1, aiming to isolate and analyze them more precisely.

Total genomic DNA extraction from leaf samples

Microsatellite markers stand out as potent tools for population genetic analyses owing to their high variability. An alternative strategy to expedite the process and enhance yield involves the use of ISSR-PCR. ISSR-PCR holds advantages as it generates more intricate marker patterns compared to the RAPD approach, proving particularly effective in distinguishing closely related cultivars. The reproducibility of ISSR markers surpasses that of RAPD markers, primarily because ISSR primers possess greater length, facilitating higher annealing temperatures [19-20].

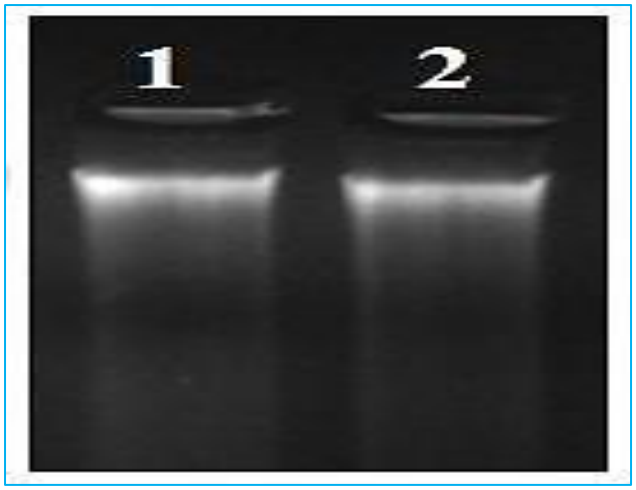


Fig 5.2 DNA isolated
(1 – Thiruvananthapuram, 2 – Alleppey)

The quality of DNA was checked in Agarose gel electrophoresis.

Variation analysis using ISSR2 and ISSR3 primers

ISSR (Inter Simple Sequence Repeat) markers play a crucial role in genetic diversity analysis, serving as an effective tool for evaluating genetic variation within and among populations. Specifically designed to target microsatellite regions - short, repetitive DNA sequences distributed across the genome - ISSR primers contribute valuable insights due to the polymorphic nature of microsatellites. This polymorphism, characterized by variations in the number of repeat units among individuals, enhances the markers' utility in assessing genetic diversity. The DNA profiles or banding patterns generated by ISSR markers are distinct for each individual or population. The specific presence or absence of bands at designated positions on electrophoretic gels serves as a direct indicator of the genetic variation present within the sampled individuals. This method provides a reliable means to characterize and compare genetic diversity across populations [21-22].

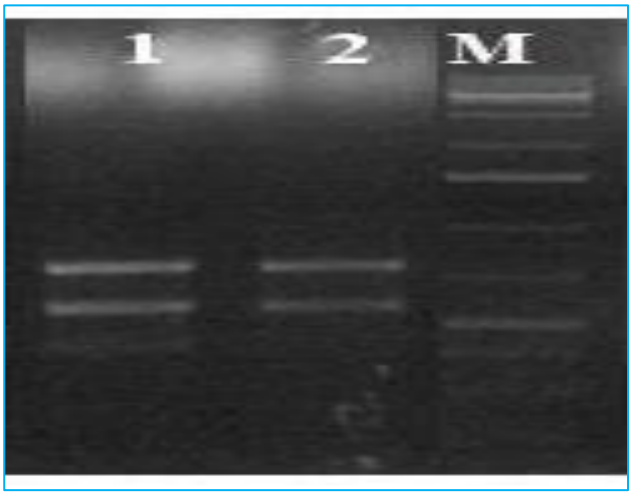


Fig 5.3 PCR using ISSR 2 Primer
lane 1-sample 1, lane 2- sample 2, M- 1 kb plus DNA ladder

Variation analysis using ISSR2 and ISSR3 primers

This indicates that the experiment focuses on assessing genetic variation using two specific primers, ISSR2 and ISSR3. Primers are short DNA sequences used to initiate DNA replication in PCR (Polymerase Chain Reaction) reactions.

Lane 1 - Sample 1, Lane 2 - Sample 2: The samples derived from the genetic material of interest are loaded into separate lanes on the gel. In this case, Sample 1 is loaded in Lane 1, and Sample 2 is loaded in Lane 2. Each lane represents a separate experimental condition or genetic sample.

M - 1 kb plus DNA ladder: The "M" denotes the marker lane, and a "1 kb plus DNA ladder" serves as a molecular weight marker. This ladder contains DNA fragments of known sizes, allowing researchers to estimate the sizes of the DNA fragments in the experimental lanes by comparison.

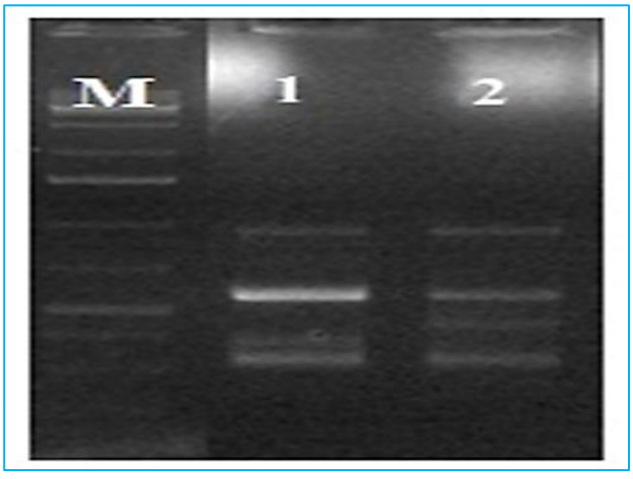


Fig 5.4 PCR using ISSR 2 Primer
lane 1-sample 1, lane 2- sample 2, M- 1 kb plus DNA ladder

This figure and table show the results of DNA bands obtained from PCR using ISSR 2 primer. DNA of Thiruvananthapuram sample has 3 bands and DNA of Alappuzha sample has 2 bands.

The centrifugation process revealed distinguishable characteristics in the supernatant and pellet fractions of both samples, with particular emphasis on the more vivid color

density in the supernatant of Sample 1, prompting a focused continuation of the experimental procedure. The content indicates that the gel electrophoresis is being used to analyze genetic variation in two samples (Sample 1 and Sample 2) using ISSR2 and ISSR3 primers. The DNA ladder in the marker lane provides a reference for estimating the sizes of the DNA fragments in the experimental samples. This technique is commonly employed in molecular biology to visualize and analyze genetic differences among samples. Molecular techniques play a pivotal role in gene analysis, genetic mapping, and gene transfer technologies, contributing to studies on phylogeny, species evolution, and genetic variation within and between species [23].

ISSR technology serves as an exceptionally sensitive tool for detecting substantial levels of genetic variation, making it invaluable for investigating population genetics across diverse plant species. Its applicability extends to the identification of distinct species, cultivars, or populations within the same species. The present study underscores the effectiveness of ISSR markers in assessing genetic diversity, providing a clear molecular differentiation among the investigated Wedelia accessions. In phylogenetic studies, ISSR has demonstrated superiority over RAPD [24].

Band scoring of ISSR markers

Scoring ISSR (Inter Simple Sequence Repeat) markers involves the interpretation of banding patterns generated through electrophoresis of PCR-amplified DNA fragments. The process of band scoring is crucial for assessing genetic diversity within and among populations. It's important to note that consistency and accuracy in band scoring are crucial for reliable results. The banding patterns can be influenced by factors such as primer specificity, PCR conditions, and gel electrophoresis parameters. Validation and replication of results can help ensure the robustness of your genetic diversity analysis using ISSR markers.

Table 51 Scoring of ISSR 2 marker in different samples

Sample No.	Band 1	Band 2	Band 3
1	1	1	1
2	1	1	0

This table showing the results of DNA bands when we used ISSR2. The scoring suggests a genetic difference between the two samples, with Sample 2 lacking Band 3, which is present in Sample 1. This information is valuable for assessing genetic diversity or specific variations between the samples in the context of ISSR2 markers.

We can explore the similarity between the samples using a measure called the Jaccard similarity coefficient, which is commonly used for binary data. The Jaccard coefficient measures the proportion of shared elements between two sets.

Jaccard (Sample 1, Sample 2) = (Intersection: 3) / (Union: 5) = 0.6

The Jaccard similarity coefficient of 0.6 suggests a moderate level of similarity between Sample 1 and Sample 2 regarding the presence or absence of bands. This indicates that 60% of the bands are shared between the two samples, implying a certain degree of similarity in their band patterns.

Table 5.2 Scoring of ISSR 3 marker in different samples

Sample No.	Band 1	Band 2	Band 3	Band 4	Band 5
1	1	1	0	1	1
2	1	1	1	0	1

Table showing the results of DNA bands when we used ISSR 3. The scoring reveals different genetic profiles between Sample 1 and Sample 2, with variations in the presence or absence of specific ISSR bands. This information is useful for assessing and comparing the genetic diversity or specific variations in the context of these ISSR markers between the two samples.

Upon thorough comparison of the results, it becomes apparent that sample no. 2 manifests a notably higher Nei's gene diversity and Shannon's Information index in contrast to sample 1. This statistical scrutiny of the ISSR results allows us to draw a conclusive inference: sample no. 2 showcases superior polymorphism and diversity when juxtaposed with sample 1.

ISSR technology stands out as a highly sensitive method capable of detecting substantial genetic variation, proving to be an invaluable molecular tool for delving into population genetics across a diverse array of plant species. Notably, ISSR analysis, being a PCR-based approach, offers the advantages of cost-effectiveness and efficiency in comparison to alternative DNA genotyping techniques [25-26]. The outcomes of the study underscore the successful application of ISSR markers in evaluating genetic diversity, unveiling a noteworthy molecular differentiation among the studied Wedelia accessions.

diversity analysis using popgene software

Table 5.4 Results obtained after uploading ISSR band score into POPGENE software

Sample No.	Average (na)	Average (ne)	Average (h)	Average (I)
1	1.5000	1.4890	0.2472	0.3438
2	2.0000	1.9656	0.4912	0.6843

na = Observed number of alleles, ne = Effective number of alleles, h = Nei's gene diversity, I = Shannon's Information index

In this study, two samples of *Sphagneticola trilobata* were collected from different districts of Kerala to investigate molecular variability using ISSR markers. The results indicated that sample no. 2 (Alappuzha sample) exhibited higher Nei's gene diversity and Shannon's Information index compared to sample no. 1 (Thiruvananthapuram sample). Therefore, based on the statistical analysis of ISSR results, it can be concluded that samples collected from Alappuzha are highly polymorphic and diverse compared to the Thiruvananthapuram sample. Additionally, qualitative analysis of phytochemicals revealed variations between the two samples, further supporting the conclusion that the samples from Thiruvananthapuram and Alleppey differ from each other.

ISSR analysis has been previously utilized to explore relationships among various stevia accessions and collections [27-30]. However, it's worth noting that while ISSR markers are valuable for molecular identification due to their reliability compared to morphological characterization, their application has primarily focused on assessing relationships between accessions rather than revealing genetic diversity within individual accessions or collections. In contrast, other techniques such as esterase isozymes and expressed sequence tags (ESTs) in simple sequence repeats (SSRs) have been employed to investigate molecular polymorphism within landraces and cultivated populations of stevia [31]. These approaches offer complementary insights into the genetic diversity present within stevia cultivars, enhancing our understanding of their genetic makeup and potentially informing breeding and conservation efforts.

It’s important to recognize that the abundance of potential ISSR markers hinges on the microsatellite frequency,

a variable trait among different species [32]. This underscores the immense potential of integrating ISSR–PCR into plant improvement programs, with broad applications across diverse crop species [33]. Recent research has highlighted the immunomodulatory effects of plant-derived compounds, with potential applications in vaccine development.

CONCLUSION

This study investigated the genetic diversity of *Sphagneticola trilobata* populations from Thiruvananthapuram and Alleppey in Kerala, India, using Inter-Simple Sequence Repeat (ISSR) markers. DNA extraction and ISSR analysis revealed significant differences in genetic diversity metrics between the two locations, with Thiruvananthapuram consistently showing higher values, indicating greater genetic diversity. Sample 2 from Thiruvananthapuram exhibited heightened genetic variation, reinforcing its distinctiveness compared to Sample 1 from Alleppey. Overall, Sample 2 displayed superior genetic diversity across all measured parameters, suggesting a more robust genetic makeup. These findings deepen our understanding of genetic variation within *Sphagneticola trilobata* populations and have implications for conservation strategies and evolutionary studies in the region. The research provides valuable insights into the mechanisms driving genetic diversity and adaptation in this species, informing future conservation efforts and management strategies.

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