MOLECULAR PROFILING FOR GENETIC VARIABILITY IN *HYGROPHILA RINGENS* (L.) R. BR. EX STEUD. IN KERALA USING RAPD AND ISSR MARKER SYSTEM

Dissertation submitted in partial fulfillment of the requirement for the award of the degree of "**Masters of Science**" in

BOTANY

By

ANNCHRISTY JAMES

Reg. No: AM21BOT004

DEPARTMENT OF BOTANY AND CENTER FOR RESEARCH

ST. TERESA'S COLLEGE (AUTONOMOUS),

ERNAKULAM

MAY 2023

CERTIFICATE

This is to certify that the dissertation entitled "**MOLECULAR PROFILING FOR GENETIC VARIABILITY IN *HYGROPHILA RINGENS* (L.) R. BR. EX STEUD. IN KERALA USING RAPD AND ISSR MARKER SYSTEM**" an authentic record of work carried out by **Annchristy James** under my supervision and guidance in the partial fulfillment of the requirement of the M.Sc. Degree Mahatma Gandhi University, Kottayam. I, further certify that no part of the work embodied in this dissertation work has been submitted for the award of any other degree or diploma.

12/04/22

Ms. Merin Alice George
Supervising Teacher
Department of Botany
St. Teresa's College,
(Autonomous)

Dr.Liza Jacob
Head of Department of Botany
St. Teresa's College,(Autonomous)
Ernakulam

St. Teresa's College (Autonomous) Ernakulam DEPARTMENT OF BOTANY

Place: Ernakulam
Date: 12/04/2023

External Examiner Valued.

1. Dr. Stephy Sequeira 18/4/2023.
2. Dr. Justin K. Nayagam 18/04/2023

KSCSTE-MBGIPS

KSCSTE - MALABAR BOTANICAL GARDEN AND INSTITUTE FOR PLANT SCIENCES

(An Institution under Kerala State Council for Science, Technology & Environment)

Post Box No. 1, Kozhikode - 673 014, Kerala, India. Phone: +91 495 2430939

Email: malabarbot.garden@gmail.com, www.mbgs.in

CERTIFICATE

This is to certify that the dissertation entitled **"MOLECULAR PROFILING FOR GENETIC VARIABILITY IN *HYGROPHILA RINGENS* (L.) R. BR. EX STEUD. IN KERALA USING RAPD AND ISSR MARKER SYSTEM"** submitted by **Ms. ANN CHRISTY JAMES** in partial fulfilment for the M.Sc. Degree in Botany, is the bonafide record of the research work under taken by her in this institution under my guidance and supervision.

Dr. N S Pradeep
Principal Scientist
KSCSTE-MBGIPS

KSCSTE - MBGIPS
KOZHIKODE-14
KERALA

KSCSTE-MBGIPS

KSCSTE - MALABAR BOTANICAL GARDEN AND INSTITUTE FOR PLANT SCIENCES

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KSCSTE

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Scientist-in-charge
KSCSTE- MBGIPS

KSCSTE - MBGIPS
KOZHIKODE-14
KERALA

ST.TERESA'S COLLEGE (AUTONOMOUS) ERNAKULAM

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I hereby declare that the work which is being presented in the dissertation, entitled **"Assessment of genetic diversity in *Hygrophila ringens* (L.) R. BR. EX. Steud using Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) Markers"**, in fulfillment of the requirements for the award of the degree of Master of Science in Botany and submitted to St. Teresa's College (Autonomous), Ernakulam is an authentic record of my own work carried out during M.Sc. period under the supervision of Ms. Merin Alice George.

The matter embodied in this dissertation has not been submitted by me for the award of any other degree of this or any other University/ Institute.

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Date: 12-04-23 Signature of the Candidate

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ANNCHRISTY JAMES

CONTENTS

LIST OF TABLES

LIST OF PLATES AND FIGURES

ABSTRACT

Hygrophila ringens (L.) R. BR. EX. Steud is an under-exploited plant of the Acanthaceae family. Genetic diversity of *Hygrophila ringens* was evaluated using Random amplified polymorphic DNA (RAPD) and Inter simple sequence repeats (ISSR) markers. A total of 10 genotypes of *Hygrophila ringens* were collected from different regions of Kerala. Plant populations are named HR1 to HR 10. Numerical Taxonomy and Multivariate Analysis System (NTSYS) was used to estimate phylogenetic tree using the UPGMA methods for constructing dendrograms. ISSR markers were found to be more efficient than the RAPD markers with regards to polymorphism detection. It was observed that percentage of ISSR polymorphism bands (80.2 %) was higher than RAPD (77.7%). RAPD and ISSR marker system were found to be useful for the genetic diversity studies in *Hygrophila ringens* (L.) R. BR. EX. Steud and to identify the variation.

Key Words: *Hygrophila ringens,* Random amplified polymorphic DNA (RAPD), Inter simple sequence repeats (ISSR) markers, Numerical Taxonomy and Multivariate Analysis System (NTSYS)

INTRODUCTION

Hygrophila ringens (L.) R. BR. EX. Steud. is a herbaceous plant in the Acanthaceae family that grows in marshy places and is native to tropical and subtropical regions. The plant is widely distributed in Indonesia, Malaysia, Africa, Brazil, India, and Central America. It is commonly called swamp weed in English, "Itkata" in Sanskrit, "Nir chulli" in Malayalam, "Nir-c-culli" in Tamil, and "Sadu gobbi" in Telugu. Plants in Acanthaceae including *Hygrophila ringens* showed the antiplasmodial property and cytotoxic activities. The study of medicinal plants of Wayanad noted that young twigs and leaves of hygrophila are used by native peoples as medicine for diabetics. So it is an important medicinal plant in the Acanthaceae family which is widely used in Ayurveda. (Anil Kumar *et al*. 2001)

1.1 Taxonomy of *H.ringens*

Hygrophila ringens is commonly known as swamp weeds. The plant belongs to the family Acanthaceae. It is distributed in tropical and subtropical regions of the world. There are about 100 species of Hygrophila recorded, most of which are aquatic plants, and 4 are found in Kerala. The plant is an Erect subshrub, rooting at lower nodes; stems are quadrangular. Leaves to 10 x 1 cm, linear-lanceolate, strigose along the nerves. Flowers in axillary clusters, sessile; bracts 6 mm long, linear; bracteoles minute; calyx 12 mm long, tubular; lobes oblong, acuminate, hairy within; corolla pale pink, 15 mm long, bilabiate; tube slender, ventricose above; stamens 4, filaments united into a sheath, ciliate. Capsule 14 x 2 mm, terete, glabrous; seeds many, densely hairy. (Dr.N Sasidharan. 2013).

1.2 Medicinal properties

- Young twigs and leaves have anti-diabetic properties
- Antiplasmodial properties
- Cytotoxic activity
- Galactagogue
- Diuretic properties

1.3 Importance of Genetic diversity study

Genetic diversity is defined here as any measure that quantifies the magnitude of genetic variability within a population. Understanding the molecular underpinnings of fundamental biological processes in plants is critical for the successful management, conservation, and application of plant genetic resources (PGR). For basic science and practical aspects like the effective management of agricultural genetic resources, a sufficient understanding of the genetic variety that currently exists, where it is distributed in plant populations, and how to best utilize it, is essential. Crop genetic resources must be continuously improved through the application of current breeding techniques, traditional varieties, and wild relatives. Each of these procedures calls for some sort of diversity assessment in order to choose resilient, highly productive types.

As an important plant in nature with some important medicinal properties, the study of the genetic diversity of *H.ringens* get attention.For the effective conservation, management, and effective utilization of plant genetic resources (PGR) it is crucial to understand the molecular basis of essential biological phenomena in plants. Advanced molecular techniques such as Molecular markers, PCR etc. Have enabled the study of genetic variability at the DNA level which has significantly increased the accuracy in assessing genetic diversity and identifying cultivars. The importance of plant genetics (PGD) is now being recognized as a specific area since exploding population with urbanization and decreasing cultivable lands are the critical factors contributing to food insecurity in the developing world. Agricultural scientists realized that PGD can be captured and stored in the form of plant genetic resources (PGR) such as a Gene bank, DNA library, and so forth, in the biorepository which preserves genetic material for a long period. The conserved PGR could be utilized for crop/plant improvement in order to meet future global challenges in relation to food medicinal and nutritional security.

1.4 Molecular markers

A gene whose phenotypic expression can often be recognized and used to identify an individual is a molecular marker, as is a sequence of DNA with a known position on the chromosome, as well as a probe to designate chromosomes, nuclei, or loci Markers enable the identification of genetic variations between particular organisms or species by

demonstrating polymorphism, which can result from nucleotide change or mutation at specific genomic loci. Genetic markers are used to determine genetic variation among particular species or creatures, and the genetic markers are used to create linkage maps.

Molecular markers are also employed in a wide range of other fields, such as epidemiology, genetic mapping, the detection of defective genes, and population research. Molecular markers are employed in a wide range of applications, including genetic mapping, paternity testing, cultivar identification, marker-assisted crop breeding, population history, epidemiology, and food safety.

Ideal Desirable Features of Molecular Markers:

Accessibility, simplicity, reproducibility, and high polymorphism, as well as co-dominant inheritance and recurrent occurrence in the genome Data sharing across various laboratories should be simple and selectively indifferent to environmental factors.

With the development of Polymerase Chain Reaction (PCR), numerous molecular technologies have been. Still, they are being developed which can be used for the detection, characterization, and evaluation of genetic diversity. The invention of molecular markers in advanced molecular technologies has become an important tool in the studies of genetic diversity. Markers show polymorphism, which may arise due to alteration of nucleotides or mutation in the genome loci and make it possible to identify genetic differences between individual organisms or species.

1.5 Random Amplified Polymorphic DNA

Molecular markers are grouped after their different abilities to show homozygosity (dominant marker) or heterozygosity (co-dominant marker), RAPD and ISSR are commonly using dominant markers in plant diversity studies small oligo nucleotide primers with arbitrary sequences, typically 10 bp in length, are used as RAPD markers to create band profiles. When the sections between the opposing primer sites are within amplifiable distances, PCR amplification takes place. These primers bind to the complementary sequences throughout the genome. The fundamental method of RAPD entails I very pure DNA separation, (ii) insertion of a single arbitrary primer, (iii) polymerase chain reaction (PCR) (iv) The PCR products that are produced are typically resolved on 1.5–2.0% agarose

gels and stained with ethidium bromide (EtBr) or polyacrylamide gels combined with either AgNO3 staining or fluorescently labeled primers or nucleotides or radioactivity are occasionally utilized. Although agarose gel electrophoresis has a low resolving power, it is simple to use, and it is inexpensive, which is more rapid and popular than both AP-PCR and DAF.

1.6 Inter-Simple Sequence Repeat (ISSR)

Inter-Simple Sequence Repeat (ISSR) is the process of amplifying a DNA segment that lies between two identical microsatellite repeat sections that are orientated in opposition to one another. Inter-Simple Sequence Repeat primers are typically 16–25 bp long and used in PCR reactions that target numerous genomic loci in order to amplify inter-SSR sequences of various lengths. Either dinucleotides or trinucleotides can be utilized as the primer for the microsatellite repeats. High polymorphism ISSR markers are utilized for gene tagging and genetic diversity.

Simple sequence repeats (SSRs), also known as microsatellites, are found in all eukaryotic genomes. There is a significant bottleneck in producing SSR markers since flanking sequences must be known in order to design 5′-anchors for polymerase chain reaction (PCR) primers.

ISSR PCR is a method that resolves issues like AFLP's high cost, RAPD's poor reproducibility, and the flanking sequences used to create species-specific SSR polymorphism primers. ISSR primarily segregates as dominant markers in the context of straightforward Mendelian inheritance. They have also been demonstrated to segregate as codominant markers in a few instances, allowing for the differentiation of homozygotes and heterozygotes. Since 1994, a variety of organisms have been analyzed using ISSR for genome mapping, diversity analysis, and DNA fingerprinting. The benefits of ISSR ISSR is rapid, straightforward, and highly reproducible, and radioactivity is not required. The main benefit of ISSR markers is that they typically exhibit high levels of polymorphism and do not require knowledge of the genome sequence.

1.7 NTSYS

A set of software tools called NTSYSpc is used to identify and present structure in multivariate data. For instance, it would be of interest to learn that a sample of data points indicates that the samples may have originated from two or more different populations. Equally intriguing is the discovery of the high correlation between several categories of variables The program was initially created for use

in the field of numerical taxonomy in biology, which is why it is called NTSYS (for Numerical Taxonomy SYSTEM). However, morphometrics, ecology, and numerous other fields in the natural sciences, engineering, and humanities have also made extensive use of the programs additionally, the concepts of automatic classification and mathematical taxonomy have been utilized to describe this field of application. These techniques are frequently applied in morphometric research. By employing cladistics, one can infer the evolutionary history of the creatures they are studying and use that information to classify them. The idea that the underlying model is of a branching evolutionary tree has led to the development of specialized approaches. It is anticipated that the evolutionary history of a group of organisms will provide the best biological explanation for the observed diversity. Given a collection of descriptive information about a group of organisms, the approaches are designed to produce the most accurate estimates of the evolutionary tree. The philosophical principle of parsimony, states that (the shortest tree that can be fitted to a set of data should be the best estimate of the true tree), although statistically more potent techniques based on the maximization of likelihood are becoming more and more common. The neighbor-joining approach is also frequently employed.

Performing various sorts of agglomerative cluster analyses of any type of similarity or dissimilarity matrix is probably the most frequent use of NTSYSpc. In a batch file that will standardize a data matrix, compute distance coefficients between the columns of the standardized data matrix (there are other coefficient options), cluster the distance matrix using the single-link clustering method (there are other clustering options), and standardize a second data matrix.generate a cophenetic-value (ultrametric) matrix, compute other options (such as UPGMA), the cophenetic correlation as a gauge of fit quality, and finally graph the outcomes as a phenogram. Additionally, the distance matrix is visible.

1. OBJECTIVES

1. Survey and collection of *Hygrophila ringens* (L.) R. Br. ex Steurd. from different parts of India
2. Isolation of genomic DNA from collected plant sample
3. PCR amplification of isolated genomic DNA using Random Amplified Polymorphic DNA and Inter Simple Sequence Repeats
4. Analysis of genetic diversity in *Hygrophila ringens* (L.) R. Br. ex Steurd.

2. REVIEW OF LITERATURE

Hygrophila ringens (L.) R. Br.ex Steud of the family Acanthaceae is an erect or ascending annual or perennial herb that grows primarily in the wet tropical biome. The plant is native to tropical and subtropical Asia. There are about 100 species under the genus hygrophila, of which many of which are aquatic plants. In Kerala, the genus is represented by 4 species. Plants having synonyms like *Dipteracanthus ringens* (L.) Abeywickr, *Hygrophila angustifolia* (Poir.)R.Br, *Hygrophila assurgens* Nees, *Hygrophila barbata* Nees ex Steud, *Hygrophila malabarica* Raf, *Ruellia ringens* (L). *Ruellia longifolia* Roth etc. It is commonly called swamp weed in English, "Itkata" in Sanskrit, "Nir chulli" in Malayalam, "Nir-c-cull" in Tamil, and "Sadu gobbi" in Telugu. Panarat charoencha *et al* in their study of plants in Acanthaceae including showed the antiplasmodial property and cytotoxic activity. The study of Anil Kumar *et al.* (2001) on medicinal plants of Wayanad, noted that young twigs and leaves of hygrophila are used by native peoples as medicine for diabetics. So it is an important medicinal plant in the Acanthaceae family which is widely used in Ayurveda.

3.1 Taxonomy

Acanthaceae, also known as the acanthus family, is a family of dicotyledonous flowering plants, of order lamiales comprising nearly 250 genera and about 2500 species, mostly tropical herbs, shrubs, twining vines or epiphytes, most of them are found in tropical regions only a few species distributed in temperate regions. Indonesia and Malaysia, Africa, Brazil, and Central America are major centers of distribution. Leaves are usually opposite and stipules are absent. Flowers are bisexual, zygomorphic to sub-actinomorphic, usually arranged in terminal or axillary spikes, and racemes or panicles. Zhenghao Xu & Le Chang. (2017)

A detailed study of this had been done by Dr. N Sasidharan. (2013). He observed that the plant is an erect shrub, rooting at lower nodes; with a quadrangular stem. Leaves to 10 x 1 cm, linear-lanceolate, strigose along the nerves. Flowers in axillary clusters, sessile; bracts 6 mm long, linear; minute bracteoles; calyx 12 mm long, tubular; lobes oblong, acuminate, hairy within;

corolla pale pink, 15 mm long, bilabiate; tube slender, ventricose above; stamens 4, filaments united into a sheath, ciliate. Capsule 14 x 2 mm, terete, smooth & glabrous; seeds many, densely hairy. Mostly seen in moist localities and flowering and fruiting from October to March.

3.2 **Medicinal value**

The study of Anil Kumar *et al.* (2001) on medicinal plants of Wayanad, noted that young twigs and leaves of hygrophila are used by native peoples as medicine for diabetics. So is an important medicinal plant in the Acanthaceae family which is widely used in Ayurveda. Panarat charoencha *et al.* in their study of plants in Acanthaceae including *Hygrophila ringens* showed the antiplasmodial property and cytotoxic activity.

Lemmens R.H.M.J. (2016) in his study of plant resources in South Asia observed that leaves are used as poultices to treat skin irritation, wounds, swellings, and also for toothache in Peninsular Malaysia. Seeds have skin rejuvenating capacity so it is widely used in cosmetic and pharmaceutical industries, mainly in central Europe. In some places, leaves may be eaten as vegetables. In India and Burma, *Hygrophila schulli* is used as traditional medicine for the treatment of liver diseases and spermatorrhea. Roots of *H.schulli* is used in the treatment of dropsy, hepatic obstruction, and rheumatism and also it has diuretic properties

The detailed study of Ramana *et al.* on the anti-diabetic and acute toxicity of aqueous leaf extracts of in experimental mice showed that there is no toxicity in the leaf extract and it contains anti-diabetic properties. They observed mice after administration of a dosage of 2000mg/kg body weight and compared body weight and sugar level after 21 days. The preliminary studies suggest that the leaf extract of *H.ringens* contains safe principles and thus it is a plant containing great potential for medicinal purposes

Plants under the genus Hygrophila have various medicinal properties *Hygrophila spinosa* has been appreciated in the ancient medical literature. Studies by A.D kshiesagar *et al.* (2010) noted that the plant is cultivated throughout India. all plant parts are used in ayurvedic medicine. *H.spinosa* is a rich source of phytoconstituents mainly alkaloids, fatty acids, phytosterols, polyphenols, vitamins, flavonoids, etc .plant is widely used in folk medicines for the treatment of various disorders like anasarca, urinogenital tract diseases, rheumatism, asthma, leukorrhea, etc

Neeraj k. sethiya *et al.* (2017) in their study on ethnomedical, phytochemical, and pharmaceutical updates of *Hygrophila auriculata*, shows that plant has various medicinal properties against jaundice, oedema, gastrointestinal ailment, gallstone, anemia, tuberculosis, skin diseases, etc. The plant contains flavonoids (apigenin, luteolin, ellagic acid, and quercetin), alkaloids (asteracanthine), triterpenes (lupeol, lupenone, hentricontane and betulin), sterols (stigmasterol and asterol), minerals, amino acids, fatty acids, open-chain esters, and essential oils. Extracts and bioactive compounds from the plant are found to possess antimicrobial, anthelmintic, anti-termite, nephroprotective, hepatoprotective, central systema nervosum protecting, antitumor, anticataract, inhibitor, haematogenic, diuretic, antinociceptive, antipyretic, aphrodisiac, neuroprotection, anti-endotoxin and anti-urolithiasis activities.

In the study of Gomes.A *et a.l.* (2001) using ethanolic extract of the aerial elements of *H spinosa* a semi-woody herb was examined on experimental rats they show some hematological changes. The extract (100 & two hundred mg/kg, po) considerably multiplied the hemoprotein, hematocrit, RBC, and total white corpuscle, as compared with vehicle-treated control rat haemogram. In anemic male albino rats, the extract considerably multiplied hemoprotein, hematocrit and RBC count in blood and serum, and total iron binding capability was considerably reduced in *H.spinosa* extract-treated anemic rats as compared with those within the vehicle-treated anemic management rats. These findings incontestible the medicinal drug result of *H. spinosa* extract on experimental animals.

In the experimental evaluation of *Hygrophila schulli* extract for antistress activity by Dayanand Kannur *et al.* (2017), The ethanolic and alkane series extracts of *Hygrophila schulli* were subjected to qualitative analysis to notice the presence of assorted phytoconstituents. The extracts were subjected to HPLC analysis. The natural action analysis was distributed that revealing the multi-component and sophisticated nature of the extracts. The seed extracts of *H. schulli* were screened for antistress activity using the Swim Endurance test in mice and the Cold-Immobilization Stress model in rats to determine the Adaptogenic potential. HPLC analysis confirmed the presence of flavonoid Quercetin, the ethanolic and alkane series extracts were found to extend the swim endurance time, each extract lowered the elevated glucose, and sterol in addition to lipid levels in cold immobilization stress model and maintained the traditional physiological state. The seeds of Hygrophila schulli, therefore, possess adaptogenic properties.

Parvathy G.Nair *et al.* (2021) in their study based on source plant identification for ayurvedic polyherbal formulations, mentioned that *H.ringens* (Itkata) is included by Charaka in group of Stanya ganana (galactagogue) and Moothra virechana(diuretic).

3.3 **Molecular markers**

A molecular marker is a DNA sequence that is readily detected and whose inheritance can easily be monitored. In the report of Atul grover and P.C Sharma (2014) on the development and use of molecular markers: past and present pointed out that molecular markers, due to their stability, cost-effectiveness, and ease of use, molecular markers are very popular tools for various applications such as genome mapping, gene tagging, genetic diversity, phylogenetic analysis, and forensic investigation. Over the last 30 years, many molecular labeling techniques have been developed and used in various systems around the world. However, of these techniques, only RFLP, RAPD, AFLP, ISSR, SSR, and SNP are globally accepted. Recent revolutions in DNA sequencing technology have brought the discovery and application of molecular markers to high-throughput and ultra-high-throughput levels. The choice of marker obviously depends on the intended use, but genotyping by microsatellites, SNPs, and sequencing (GBS) will most likely meet the needs of most users. Additionally, modern transcriptome and functional markers guide efforts in combination with other high-throughput techniques for building high-density genetic maps, identifying QTLs, breeding, and conservation strategies.

Genetic markers are one of the advances which have occurred in the genomics era. Mojtaba Kordrostami *et al.* (2015) in their study of molecular markers in plants noted that among genetic markers, molecular markers mainly because of their abundance, are the most widely used them. The development of molecular markers has greatly altered genetics and plant breeding. Genetic markers indicate the genetic differences between different organs or species. Some studies which were conducted during the last decade of the 20^{th} century reported numerous DNA markers that have been utilized in plant breeding programs. Apart from the application of molecular markers in the construction of linkage maps, they have numerous applications in plant breeding such as assessing the genetic variations within cultivars and germplasms. The most interesting application of molecular markers is marker-assisted selection (MAS). Suitable DNA markers should be polymorphic in the DNA level and can be expressed in all tissues, organs, and various developmental stages. Compared with traditional breeding programs, molecular markers can increase the efficiency and effectiveness of breeding programs

Molecular markers have many advantages including

1) Time-saving: genomic DNA may be removed from any part of the plant tissue at each degree of its development, and its goal trait records may be acquired with related DNA markers earlier than pollination, as a result permitting breeders to perform extra knowledgeable genetic crosses.

2) Balance and reliability: phenotypic assessment of genetic traits is regularly complex with the aid of using environmental factors. However, DNA markers are normally impartial actors in environmental variation. The breeder can evaluate their material independently of the environmental factors (environmental situations may be favorable or unfavorable for morphologic and/or biochemical marker expression).

3) Biosafety: diagnostic exams for the presence or absence of traits for disease resistance may be conducted with the aid of using DNA markers tightly related to the linked gene without resorting to pathogen inoculation in the field or greenhouse. Additionally, molecular markers facilitate the introgression of genes into elite cultivars for an increase of the prevalence of certain races of illnesses or biotypes of insects.

4)Performance: assessment of breeding strains in early generations of the breeding technique with DNA markers can permit breeders to reject progenies from the program and enhance the genetic quality of breeding materials. reading materials.

5) Precise selection of the complex traits: polygenic traits are often difficult to select for using conventional breeding approaches. DNA markers linked to QTL allow them to be treated with single Mendelian factors. Besides analysing and selecting the interesting characters, molecular markers allow the researchers also to analyse the wild species with potential interest for the breeding program

3.3.1 **RAPD Marker**

Bardakci and fevzi. (2001), due to advances in biology techniques, giant numbers of extremely informative DNA markers are developed for the identification of genetic polymorphism. within the last decade, the random amplified polymorphic DNA (RAPD) technique supporting the polymerase chain reaction (PCR) has been one of the foremost ordinarily used molecular techniques to develop DNA markers. RAPD markers are amplification products of anonymous DNA sequences using single, short (10 bp), and impulsive oligonucleotide primers, and therefore don't need previous data of a DNA sequence. Low expense, potency in developing a large number of DNA markers during a short time, and demand for fewer refined instrumentation have created the RAPD technique valuable though the reliability of the RAPD profile remains the center of discussion.

The basic technique of RAPD involves,

i. Isolation of highly pure DNA
ii. Addition of a single arbitrary primer
iii. Polymerase chain reaction (PCR)
iv. The resulting PCR products are generally resolved in 1.5-2.0% agarose gels and stained with ethidium bromide or polyacrylamide gel in combination with either AgNO3 staining

Despite its low resolving power, simplicity and low cost of agarose gel electrophoresis has made RAPD more rapid and popular (Idress M.U *et al.*2014)

To analyze genetic linkages and genetic diversity, molecular genetic markers have evolved into an effective tool. The Random Amplified Polymorphic DNA (RAPD) technique may be used in molecular ecology to ascertain the taxonomic identity, evaluate family links, analyze mixed genome samples, and build particular probes as an expansion to the range of existing techniques using

polymorphic DNA markers. The RAPD technology has several key benefits, including (i) its suitability for work on anonymous genomes, (ii) its applicability to issues where only little amounts of DNA are accessible, (iii) efficiency, and (iv) its low cost.

In the study of H.Hadrys *et al.* (1992) on the topic of applications of random amplified polymorphic DNA (RAPD) in molecular ecology to analyze genetic linkages and genetic diversity, molecular genetic markers have evolved into an effective tool The Random Amplified Polymorphic DNA (RAPD) technique may be used in molecular ecology to ascertain the taxonomic identity, evaluate family links, analyze mixed genome samples, and build particular probes as an expansion to the range of existing techniques using polymorphic DNA markers. The RAPD technology has several key benefits, including (i) its suitability for work on anonymous genomes, (ii) its applicability to issues where only little amounts of DNA are accessible, (iii) efficiency, and (iv) its low cost.

3.3.2 **ISSR MARKER**

Many molecular marker techniques are available today. PCR-based approaches are in demand because of their simplicity and requirement for only small quantities of sample DNA. Unanchored inter simple sequence repeats (ISSRs) are arbitrary multilocus markers produced by PCR amplification with a microsatellite primer. They are advantageous because no prior genomic information is required for their use. the technique is stable across a wide range of PCR parameters.

Polymorphisms were abundant among most of the species. Thus, non-anchored ISSR markers are a good choice for DNA fingerprinting. Bornet. B and Branchard.M(2001)

For most genetic variation studies, a good genetic marker is defined by high genetic variability and the ability to generate multilocus data from the genome under study. The generation of ISSR markers makes use of microsatellite sequences that are highly variable and ubiquitously distributed across the genome, at the same time achieving higher reproducibility compared to using RAPDs and costs less in terms of time and money compared to using AFLPs. All these make ISSR an ideal genetic marker for various studies, most notably on genetic variation/diversity, DNA fingerprinting, and phylogenetics

The basic procedure to conduct an ISSR genotyping experiment is simple:

1. PCR, using an ISSR primer, with genomic DNA (gDNA) as its template

2. Use of agarose or polyacrylamide gel electrophoresis of PCR amplification products

3. Scoring of ISSR bands

4. Data analysis.

In the study of S. C. Debnath *et al.* in 2008 using inter simple sequence repeat (ISSR) markers, strawberry genotypes' genetic diversity and relatedness are evaluated. This study used Inter Simple Sequence Repeat (ISSR) markers to assess the degree of genetic diversity and relatedness among 16 strawberries. cultivars and 11 breeding lines created in Canada. There were 225 polymorphic ISSR-PCR bands produced by 17 primers. The genotypes had a high degree of genetic similarity, ranging from 63 to 77%, according to cluster analysis using the unweighted pair-group method with arithmetic averages (UPGMA), which was in agreement with the principal coordinate (PCO) analysis. Just 1.4% of the total variation was explained by the breeding program's geographic dispersion, as revealed by a molecular variance analysis (AMOVA). The method is useful for cultivar identification and for the more effective selection of parents in current strawberry breeding projects since the ISSR markers revealed a sufficient level of variability to differentiate between strawberry genotypes.

The study of Dr. Ian D. Godwin *et al.* on the application of inter-simple sequence repeat (ISSR) markers to plant genetics concluded that, Single-locus SSR markers have been developed for a number of species. The development of Inter SSR (ISSR) fingerprinting eliminated the need for sequence knowledge. It is possible to create primers with a degenerate 3′-anchor, such as (CA)8RG

or (AGC)6TY, based on repetitive sequences, such as (CA)n. A multilocus marker system helpful for fingerprinting, diversity analysis, and genome mapping is produced by the ensuing PCR process, which amplifies the sequence between two SSRs. By end-labeling or PCR incorporation, PCR products are radiolabelled with 32P or 33P before being separated on a polyacrylamide sequencing gel. in advance of autoradiographic visualization. 20–100 bands per lane are typically produced by a normal reaction, depending on the species and primer. Many plant species have been subjected to ISSR fingerprinting, and we present some findings on two significant tropical species, sorghum,and banana. Prior research has shown that ISSR analysis typically reveals a higher amount of polymorphism than that obtained with RFLP or random amplified polymorphic DNA (RAPD) assays. According to our findings, this is not due to a higher level of genetic polymorphism but rather to technical issues with the detection methods utilized for ISSR analysis.

3.4 **Genetic Diversity Studies**

In the study of comparative analysis of genetic diversity in blackgram genotypes using RAPD and ISSR markers by Souframanein. J *et al.* (2004). A total of 25 random and 16 ISSR primers were used. Amplification of genomic DNA of the 18 genotypes, using RAPD analysis, yielded 104 fragments that could be scored, of which 44 were polymorphic, with an average of 1.8 polymorphic fragments per primer. A number of amplified fragments with random primers ranged from two (OPA-13) to nine (OPK-4) and varied in size from 200 bp to 2,500 bp. Percentage polymorphism ranged from 16.6% (OPK-7) to a maximum of 66.6% (OPE-5, OPH-2, and OPK-8), with an average of 42.7%. The 16 ISSR primers used in the study produced 101 bands across 18 genotypes, of which 55 were polymorphic. The number of amplified bands varied from two (ISSR 858) to ten (ISSR 810), with a size range of 200–2,200 bp. The average numbers of bands per primer and polymorphic bands per primer were 6.3 and 3.4, respectively. Percentage polymorphism ranged from 25% (ISSR 885) to 100% (ISSR 858), with an average percentage polymorphism of 57.5% across all the genotypes. The 3′-anchored primers based on poly (GA) and poly (AG) motifs produced high average polymorphisms of 54.98% and 58.32%, respectively. ISSR markers were more efficient than the RAPD assay, as they detected 57.4% polymorphic DNA markers in *Vigna mungo* as compared to 42.7% for RAPD markers. The Mantel test between the two Jaccard's similarity matrices gave r =0.32, showing a low correlation between RAPD- and ISSR-based similarities. The clustering of genotypes within groups was not similar when RAPD and ISSR-derived dendrograms were

compared, whereas the pattern of clustering of the genotypes remained more or less the same in ISSR and combined data of RAPD and ISSR.

4.MATERIALS AND METHODS

4.1 PLANT MATERIALS

A total of 10 genotypes were collected from different regions of Kerala (Table 1). These plants were successfully maintained in the aquatic plant conservatory at KSCSTE- Malabar Botanical Garden and Institute for Plant Science, Calicut, Kerala, India. Plant populations are named HR 1 to HR10. The germplasm was well maintained with proper irrigation and under favorable conditions of growth. Thus, a healthy plant population of all 10 samples was available for study at any time. Pictures of *Hygrophila ringens* are given in Figures 1&2.

Figure 1: *Hygrophila ringens* habitat

Figure 2: *Hygrphila ringens* single plant

Table 1: Details of accession collected from different regions of India for genetic diversity analysis

Sl.No	Lattitude	Longitude	Location	Altitude (M)
HR 1	11.240561	75.834034	IRINGALLUR, KKD	53
HR 2	12.123862	75. 239641	PAYYANNUR, KNR	12
HR 3	11. 524203	76.044442	BEGUR, WND	801
HR 4	11.73277	75.611289	KADAVATHUR, KNR	32
HR 5	8.805825	76.668563	PARAVUR, KLM	1
HR 6	10.290286	76.165349	MATHILAKAM, THRISSUR	9
HR 7	12.269732	75.350432	CHERUPUZHA, KNR	52
HR 8	11.195294	76. 213375	MUTHANGA, WND	54
HR 9	12.411329	75.098804	PERIYA, KZD	90
HR 10	11.110437	75.883991	CHELARI, MPM	14

4.2 PREPARATION OF GENOMIC DNA FROM PLANT TISSUE

Collect healthy and tender leaf samples from each accession. Wash thoroughly and dry using sterile filter paper. Weigh 1gm leaf sample and cut it into pieces into a pre-cooled mortar. Pre-warm the

CTAB extraction buffer and add Polyvinylpyrrolidone (PVP) prior to extraction. Stock solutions required for Genomic DNA extraction are mentioned below (Table 2). Grind the tissue into powder using a mortar and pestle and add 500-1000 µl extraction buffer, mix thoroughly to make it into a slurry, and transfer into a 2ml centrifuge tube. Incubate the tubes at 65℃ for 45 minutes in a water bath with occasional mixing at regular intervals. Cool the mixture to room temperature and add 70 µl Chloroform: Isoamyl alcohol mixture (24:1), mix thoroughly to form an emulsion, and centrifuge at 12000 rpm for 15 minutes at 25℃. Collect the upper aqueous phase to a fresh tube and add 1/10 volume of CTAB/ NaCl solution. Gently shake the mixture and add an equal volume of chloroform and centrifuge at 12000 rpm for 10 minutes at 4℃.

Collect the upper aqueous phase to a fresh Eppendorf tube and an equal amount of chloroform was added and centrifuged at 12000 rpm for 10 minutes at 4℃. Transfer the supernatant to a fresh Eppendorf tube and add 100% chilled isopropanol through the sides, gently mix by inverting the tubes. Keep the mixture at -20℃ for 1 hour. Centrifuge at 7850 rpm for 15 minutes at 4 ℃. Collect the pellets twice with cold 70% ethanol at 1000 rpm for 5 min and air dry the pellets. Suspend the pellets in 200µl TE buffer. Add 4µl RNase and incubate at 37 ℃ for 1 to 2 hours in a water bath.

Add 500 µl Phenol: Chloroform: Isoamyl mixture (24:25:1), mix gently, and centrifuge at 10000 rpm for 10 minutes at 4℃. Collect the supernatant in a fresh Eppendorf tube and add 500µl chloroform, mix gently, and centrifuge at 1000 rpm for 10 minutes. Collect the supernatant in a fresh 1.5 ml Eppendorf tube and add double volume chilled ethanol and 0.1 volume 7.5 M Sodium acetate. Keep the sample overnight at -20 ℃. Collect the pellets by centrifugation at 10000 rpm for 15 minutes at 4℃. Wash the pellet in cold 75% ethanol and air dry it. Resuspend the pellet in about 50µl TE buffer. The resuspended pellets were collected and stored at -20℃. (Murray and Thomson 1980).

Table 2: Stock solution required for Genomic DNA extraction

Solutions	Composition	Quantity
Tris buffer pH 8	Tris 1M H_2O	12.11 gm 100 ml
EDTA	Na_2EDTA H_2O	18.61 gm 100 ml
CTAB Extraction buffer pH 8.0 (Stored at room temperature)	CTAB 2% W/V Tris buffer 100 mM Na_2EDTA 20 ml PVP 1% NaCl 1.4 M H_2O	2 gm 10 ml 4 ml 1 gm 8.2 gm 100 ml
TE buffer	Tris buffer 10mM Na_2EDTA 1M H_2O	1 ml 0.2 ml 100 ml
Sodium acetate	Sodium acetate 3 M H_2O	24.61 gm 100 ml

4.3 QUANTIFICATION AND VISUALIZATION OF DNA

DNA was quantified by measuring optical density (O.D) at A_{260} and A_{280} with a nanodrop spectrophotometer (Multiskan sky). The purity and concentration of the samples were noted using the Nanodrop (Thermo Scientific, USA) software. The DNA samples were diluted to the concentration of 50 ng/µl and stored at +20℃ for use. For DNA visualization, stock solutions mentioned in table 3 collected DNA is subjected to gel electrophoresis. DNA samples were prepared by mixing 6X loading dye up to a final concentration of 1X. 1µl loading dye and 5µl isolated genomic DNA were loaded on 0.8% agarose gel stained with ethidium bromide and subjected to electrophoresis in 1X TAE buffer for 30 minutes at 100 V to check the quality of isolated DNA. The gels were photographed under a Gel Documentation system (Bio-Rad).

Table 3: Stock solution required for Agarose gel electrophoresis

Solution	Composition	Quantity
TAE Buffer (10x) pH 8	Tris base	21.6 gm
	acetic acid	11 gm
	Na_2EDTA 0.5Mm	8 ml
	H_2O	100 ml
Gel loading buffer	Bromo phenol blue 0.25%	250 gm
	Xylene cyanole 0.25 %	250 gm
	Sucrose 40%(w/v)	40 gm
	H_2O	100 ml
Ethidium bromide	Ethidium bromide	1gm
	H_2O	100 ml

Table 4: List of Equipment used

No	Equipment	Make/ Model
1	Autoclave	Trueklav, India
2	Deep freezer (-20℃)	Cellfrost, India
	Deep freezer (-80℃)	New Brunswick, U101, Germany
4	Electronic balance	Sartorius, Germany
5	Electrophoresis Power Unit	Bio-Rad Laboratories, USA
6	Gel Documentation	Bio-Rad gel doc XR+ System with image lab software,Bio-Rad Laboratories, Hercules,California, US
7	Gel Electrophoresis	Bio-Rad Laboratories, USA
8	Hot Air Oven	Beston; Universal, India
9	Micro Centrifuge	Eppendorf, Germany

10	Micro Wave Oven	LG, India
11	Micropipettes	Eppendorf, Germany
12	pH Meter	Horiba, LAQUA pH -1100
13	Refrigerated Micro Centrifuge	Eppendorf, Germany
14	Thermal cycler (PCR)	Bio-Rad S1000
15	Nanodrop Spectrophotometer	Multiskan sky, Thermo scientific
16	Water bath	Julabo, Germany
17	Water purification System	Lab sil instruments, India

4.4 PCR AMPLIFICATION

PCR amplification of 10 accessions of genomic DNA of was carried out using Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) primers, which were synthesized by Bioserve Biotechnologies as per the sequence of Operon technologies, Inc. USA, were used for amplification. PCR amplification reactions were carried out in 25 µl volume containing 1µl of template DNA, 12.5 µl Master Mix (Takara),1µl of 10 pmol primers (Name and sequence details of each primer is mentioned in Table 5 and 6), and Milli Q water to make it to 25 µl. All the reagents were mixed by giving a short spin. Amplification was performed by using a thermal cycler (Bio-Rad). Details of equipment are mentioned in Table 4.

RAPD amplification was performed using 10-mer short oligonucleotide primers randomly from the operon series. RAPD amplification sequential steps involved, 1 cycle of 2 min at 95℃, 2 min at

35℃ and 2 min at 75℃ followed by 39 cycles of 1min at 36℃ and 2 min at 72℃. The last cycle was followed by 7 min extension at 72℃. samples were held at 12℃.

ISSRs are amplified by PCR using microsatellite core sequences as primers with a few selective nucleotides as anchors into the non-repeat adjacent regions (16-18 bp). The sequential steps involved, 1 cycle of 2 min at 95 ◦C, 2 min at 53 ◦C, and 2 min at 72◦C followed by 39 cycles of 0.30 min at 94 ◦C, 1 min at 53 ◦C, and 2 min at 72 ◦C. The last cycle was followed by 10 min extension at 72 ◦C. Conditions for PCR reaction are detailed in Table 7.

Table 5: List of RAPD primers used in the study

Primer name	**Primer sequence (5 ' -3 ')**	**G C content (%)**
OPA 02	TGCCGAGCTG	70
OPA 5	AGGGGTCTTG	60
OPB 01	GTTTCGCTCC	60
OPB 04	GGACTGGAGT	60
OPG 03	GAGCCCTCCA	80

Table 6: List of ISSR Primers used in the study

Primer name	**Primer sequence (5 ' -3 ')**	**G C content (%)**
ISSR 1	AGAGAGAGAGAGAGAGT	47.05
ISSR2	AGAGAGAGAGAGAGAGC	52.2
ISSR2	AGAGAGAGAGAGAGAGG	52.2

ISSR4	GAGAGAGAGAGAGAGAT	47.05
ISSR5	GAGAGAGAGAGAGAGAC	52.2

Table 7: Conditions for the PCR reaction

PCR steps	**Temperature**	**Time**
Initial denaturation	94℃	2 min
Denaturation	95℃	2 min
Annealing	35℃	2min
Extension	72℃	2min
No. of cycles of denaturation, annealing, extension	39 cycles at 93℃,36℃,72℃	1min,1min, and 2min
Final extension	72℃	7 min

4.5 DATA ANALYSIS

Qualitative scoring of bands was done from gel photographs obtained from RAPD and ISSR analysis. Clearly reproducible bands from each accession were scored as unit characters and dates were recorded in a binary matrix (‘0’ for absence and ‘1’ for presence) sheet. Both monomorphic and polymorphic bands were included in the data set to prove an unbiased estimation of genetic variation. Qualitative scoring of bands was done from gel photographs obtained from RAPD and ISSR analysis. Clearly reproducible bands from each accession were scored as unit characters and dates were recorded in a binary matrix (‘0’ for absence and ‘1’ for presence) sheet. Both monomorphic and polymorphic bands were included in the data set to prove an unbiased estimation of genetic variation. The binary data obtained from the marker systems were analyzed using NTSYS (version 1.32) software package The coefficient selected in NTSYS is the ‘J’ coefficient, and for clustering, we

used UPGMA (Unweighted Pair Group Method with Arithmetic Mean) for generating a dendrogram for data interpretation and to diversity patterns.

4.5.1 PIC (Polymorphism Information Content)

In dominant markers, we elaborate on a binary matrix of the presence and absence of bands as a result of the polymorphism revealed by the marker. If we consider the frequency of bands present as *P,* and the frequency of absents as *q,* we can establish the following general equation to estimate the Polymorphism Information Content (PIC)

PIC 1-(P2+Q2)

(Lemos *et.* al,2019). The frequency of an allele was obtained by dividing the number of alleles of isolates where the band was found by the total number of isolates.PIC is a good index for genetic diversity evaluation.PIC value is often used to measure the information of a genetic marker for linkage studies. The PIC index can be used to evaluate the level of gene variation where.PIC >0.5 indicates that the locus is of high diversity

PIC <0.25 indicates the locus is of low diversity

PIC between 0.25 and 0.5 indicates the locus is an intermediate diversity

PIC analyses can be used to evaluate markers so that the most appropriate marker can be selected for genetic mapping and phylogenetic analysis (Anderson *et al*. 1993. Powell *et al* 1996)

4.5.2 EMR (Effective Multiplex Ratio)

EMR of a primer is defined as “the product of the fraction of polymorphic loci and the number of polymorphic loci of the individual assay” (Milbourne *et al*. 1997).it is the product of the total number of fragments per primer (n) and the fraction of polymorphic fragments β(Prevost *et al.* 1999)

E=nβ

where n =total number of bands and β =total number of polymorphic DNA

4.5.3 MARKER INDEX

The marker index MI was calculated for all ten markers to determine the overall utility of a given marker system. The high MI reflects the marker's efficiency in simultaneously analyzing the more

significant number of bands rather than the level of polymorphism detected (Powell *et.al.* 1996).MI is the product of PIC and EMR (Varshney *et.al* .2005)

MI=PIC *EMR

Where PIC =Polymorphism information content and EMR =Effective Multiplex Ratio

5. RESULTS

In this study, 10 samples of *Hygrophila ringens* were subjected to amplification using RAPD and ISSR primers in PCR thermal cycler. The banding pattern thus obtained by RAPD and ISSR markers clearly distinguished varieties into different clusters showing genetic diversity. Diversity estimates provide useful information to understand the genetic structure of the plant using RAPD and ISSR markers. In the present study, 5 RAPD and 5 ISSR primers were screened for polymorphism survey in pooled DNA accessions of *Hygrophila ringens*

5.1 ISSR BAND ANALYSIS

The PCR analysis of these 10 samples taken in the study with 5 Inter Simple Sequence Repeat markers generated 54 scorable bands. An average of 10.8 bands per primer was generated. Among 5 ISSR markers, ISSR 3 produced a maximum number of bands 13, followed by ISSR 2 produced 12 bands ISSR 1 produced 11 bands and ISSR 5 produces 9 bands and ISSR 4 has 7 bands. ISSR 4 and ISSR 5 generated a minimum number of bands in the genomic pool.

Out of 54 bands, 42 bands were found polymorphic. They show 80.2% polymorphism and the average number of the polymorphic band per primer was 10.8. Different primers produced different levels of polymorphisms. The primer ISSR1 and 5 showed 100% whereas ISSR 2 showed 75 %. ISSR3 shows 69% and ISSR 4 exhibited 57% polymorphism. The average polymorphism per primer was noted as 80.2% (Mentioned in table 9).

PIC values obtained for the primers range from 0.36 for ISSR 2 and 4 to 0.46 for ISSR1 and 5. Intermediate PIC values are shown in Table 8. The PIC value of ISSR 1 and 5 is found to be more convenient for studies as the value 0.46 is closest to 0.5.

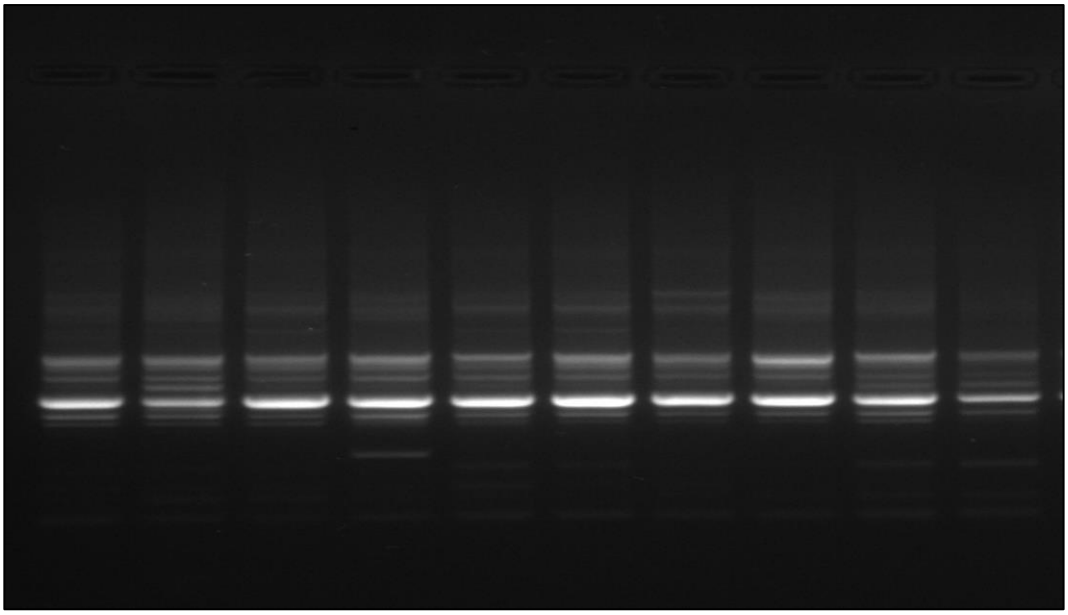

Figure 3: ISSR fingerprint obtained with ISSR1 primer in different *Hygrophila ringens*

Table 8: Data for 5 ISSR primers used for analyzing 10 accessions of *Hygrophila ringens*

ISSR PRIMER	NUMBER OF BANDS	NO. OF POLYMORPHIC BAND	% OF POLYMORPHISM	PIC	EMR	MI
ISSR 1	11	11	100%	0.46	121	55.66
ISSR 2	12	9	75%	0.36	108	38.88
ISSR3	13	9	69%	0.41	117	47.97
ISSR4	7	4	57%	0.36	28	10.08
ISSR5	9	9	100%	0.46	99	46
TOTAL	54	42				
AVERAGE	10.8	8.2	80.2%			

Table 9: List showing ISSR markers details obtained from 10 accessions of *H. ringens*

PRIMER	ISSR
No. of primers used	5
Total no. of polymorphic band	42
Total no of monomorphic band	12
Total no. of bands	54
Percentage of polymorphism	80.2%
Average no. of bands/primer	10.8
Average no. of polymorphic bands/bands	8.2

5.1.1 EMR AND MI

EMR or Effective Multiplex Ratio is the product of the total number of fragments per primer (n) and the fraction of polymorphic fragments β(Prevost *et, al.* 1999). According to the calculations, the EMR is highest for ISSR 1 marker which is 121, and lower for ISSR 4 marker which is 28. For Marker Index (MI), as per calculations, ISSR 1 showed the highest MI at 55.66 and ISSR 4 showed the least value which was 10.08.

5.1.2 GENETIC IDENTITY AND GENETIC DISTANCE USING ISSR

NTSYS were used to estimate a phylogenetic tree using the UPGMA methods for constructing dendrograms. Genetic distance and Genetic identity between 10 samples were able to relate. Genetic identity is shown above the diagonal of table 10 and genetic distance is shown below the diagonal of it.

According to the table, maximum genetic diversity is shown by population HR1 and HR8 by showing a maximum identity value of 0.8387. The genetic identity ranges from 0.3182 shared by population HR9 and shared by population HR8 to 0.8387

	HR1	HR2	HR3	HR4	HR5	HR6	HR7	HR8	HR9	HR10
HR1	1.0000									
HR2	0.2432	1.0000								
HR3	0.2500	0.2051	1.0000							
HR4	0.2500	0.3256	0.2432	1.0000						
HR5	0.3571	0.2500	0.2581	0.4000	1.0000					
HR6	0.3667	0.2927	0.2353	0.4054	0.6071	1.0000				
HR7	0.2619	0.4444	0.2273	0.6410	0.3571	0.3636	1.0000			
HR8	0.1613	0.2308	0.5417	0.3429	0.2903	0.2286	0.2500	1.0000		
HR9	0.3415	0.6750	0.2174	0.5000	0.3111	0.3778	0.6818	0.2955	1.0000	
HR10	0.6364	0.2973	0.2333	0.3056	0.2903	0.3030	0.3415	0.2258	0.4250	1.0000

Table 10: similarity table of ISSR markers

5.2 RAPD BAND ANALYSIS

The PCR analysis of these 10 samples taken in the study with 5 Random Amplified Polymorphic markers generated 48 scorable bands. An average of 9.86 bands per primer was generated. Among 5RAPD markers, OPA 4 produced a maximum number of bands 13, followed by OPA 2 produced 11 bands OPG 3, OPB 1, and OPA 5 produced 8 bands ie, the minimum number of bands in the genomic pool.

Out of 48 bands, 37 bands were found polymorphic. They show 77.7% polymorphism and the average number of the polymorphic band per primer was 7.4. Different primers produced different levels of polymorphisms. The primer OPG 3 showed 100% whereas OPA 2 showed 81.8 %. OPA 5 shows 75% and OPB 4 exhibited 69.23% polymorphism and the least polymorphism ie, 62.5% is shown by OPB 1 (Mentioned in table 12).

PIC values obtained for the primers range from 0.39 for OPB1 and 0.48 for OPA 2 and three Intermediate PIC values are shown in the table11. The PIC value of OPA 2 is found to be more convenient for studies as the value 0.48 is closest to 0.5.

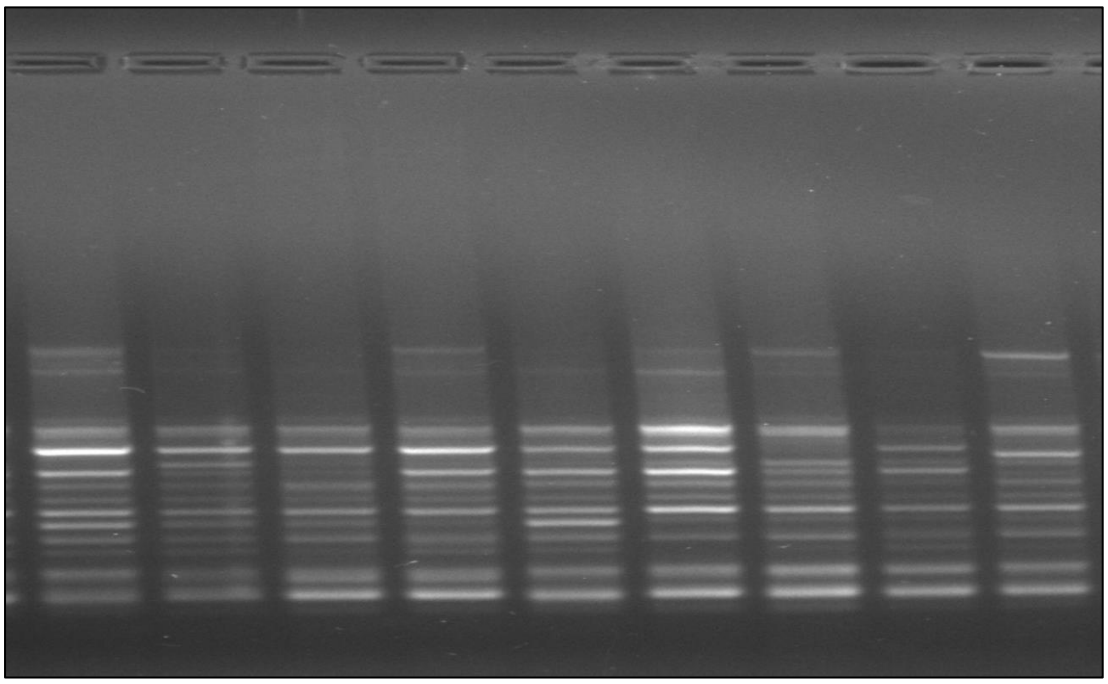

Figure 4: RAPD fingerprint obtained in different *H.ringens*

5.2.1 EMR AND MI

EMR or Effective Multiplex Ratio is the product of the total number of fragments per primer (n) and the fraction of polymorphic fragments β (Prevost *et, al* 1999). According to the calculations, the EMR is highest for OPB 4 marker which is 117, and lower for OPB 1 marker which is 40. For Marker Index (MI), as per calculations, OPB 4 showed the highest MI at 49.14 and OPB 1 showed the least value which was 15.6.

Table 11: Data for 5 RAPD primers used for analyzing 10 accessions of *H. ringens*

RAPD PRIMER	NUMBER OF BANDS	NO. OF POLYMORPHISM	% OF POLYMORPHISM	PIC	EMR	MI
OPA 2	11	9	81.8%	0.48	99	47.52
OPB 4	13	9	69.23%	0.42	117	49.14
0PG3	8	8	100%	0.42	64	26.88
OPB 1	8	5	62.5%	0.39	40	15.6
OPA5	8	6	75%	0.42	48	20.16
TOTAL	48	37				
	9.6	7.4	77.7%			

Table 12: List showing RAPD markers details obtained from 10 accessions of *H. ringens*

PRIMER	RAPD
No. of primers used	5
Total no. of polymorphic band	37
Total no of monomorphic band	11
Total no. of bands	48
Percentage of polymorphism	77.7%
Average no. of bands/primer	9.6
Average no. of polymorphic bands/bands	7.4

5.2.2 GENETIC IDENTITY AND GENETIC DISTANCE USING RAPD

NTSYS were used to estimate a phylogenetic tree using the UPGMA methods for constructing dendrograms. Genetic distance and Genetic identity between 10 samples were able to relate. Genetic identity is shown above the diagonal of table 13 and genetic distance is shown below the diagonal of it.

According to the table, maximum genetic diversity is shown by population HR1 and HR4, HR1 and HR7 by showing a maximum identity value of 0.7692. The genetic identity ranges from 0.1842 shared by populations HR8, HR4 & HR7 to 0.7692.

	HR1	HR2	HR3	HR4	HR5	HR6	HR7	HR8	HR9	HR10
HR1	1.0000									
HR2	0.3333	1.0000								
HR3	0.4516	0.5405	1.0000							
HR4	**0.2308**	0.5789	0.3590	1.0000						
HR5	0.3667	0.3250	0.2432	0.2564	1.0000					
HR6	0.3667	0.3590	0.3939	0.2564	0.4483	1.0000				
HR7	**0.2308**	0.4634	0.3947	0.5556	0.2250	0.2250	1.0000			
HR8	0.5000	0.5000	0.7407	0.2821	0.2286	0.3871	0.3514	1.0000		
HR9	0.3256	**0.8158**	0.5897	0.5854	0.3182	0.3488	0.5116	0.5128	1.0000	
HR 10	0.6000	0.3000	0.4063	0.2632	0.4138	0.3226	0.2632	0.3548	0.3571	1.0000

Table 13: Similarity table of RAPD markers

5.3 CLUSTER ANALYSIS

Cluster analysis refers to "a group of multivariate techniques whose primary purpose is to group individuals or objects based on the characteristics they possess so that individuals with similarity descriptions are mathematically gathered into the same cluster" The resulting clusters of individuals should then exhibit high internal (within the cluster) homogeneity and high external(between cluster) heterogeneity. Thus, if the classification is successful, individuals within a cluster shall be closer when plotted geometrically and different clusters shall be farther apart (Muhammadi *et al*. 2003).

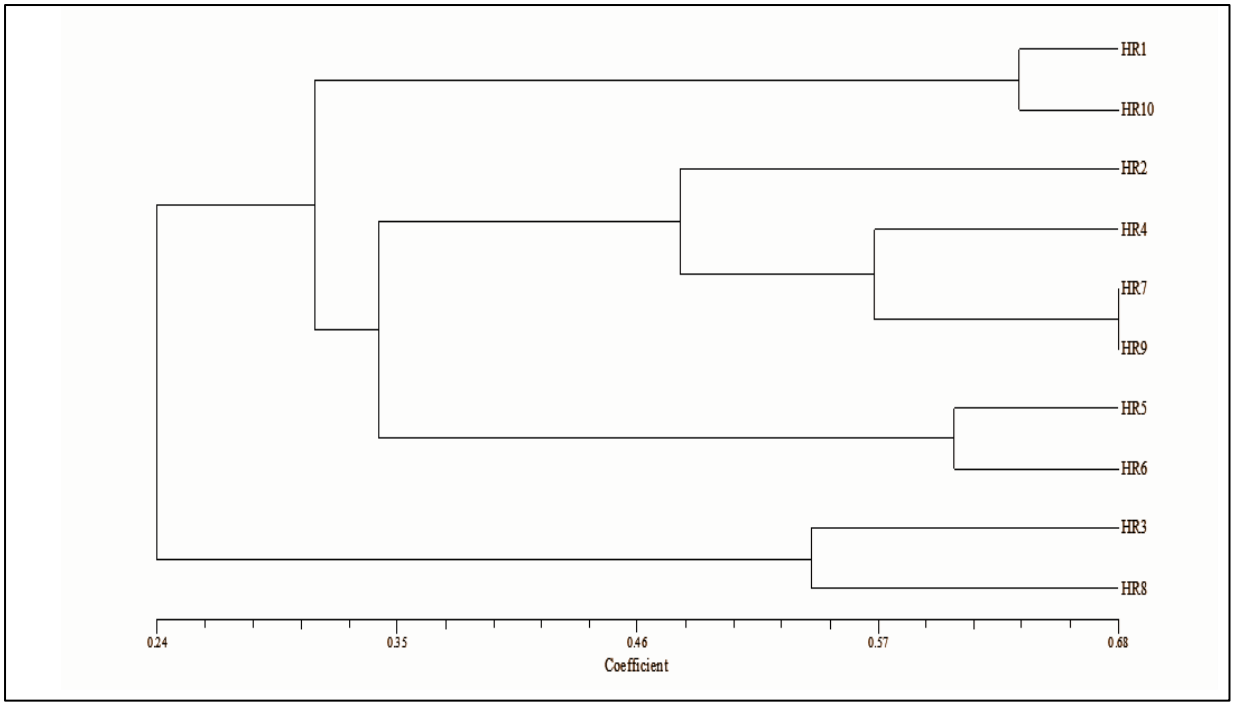

Figure 5: Dendrogram based on ISSR analysis for the estimation of genetic diversity in different accessions of *H. ringens* from different localities.

A dendrogram generated from UPGMA cluster analysis of ISSR primer is shown in figure 5. this dendrogram clearly separates the whole genotype into 2 major clusters, cluster1, and cluster2. In cluster1, comprising 8 populations, HR1, HR10, HR2, HR4, HR7, HR9, HR5, HR6. Cluster 1 is divided into two subclusters, sub cluster1, and sub cluster 2. Sub-cluster 1 has a single clade with two populations, HR1 and HR10. Subcluster 2 has two clades, clade 1 and clade 2. clad 1 is subdivided into two sub-clades in which subclade 1 has only one population, HR2. Subclade 2 has three populations, in which HR7 and HR9 are in a group and are closely related whereas HA4 is

placed separately, originating directly from subclade 2. In clade 2 there are two populations, HR5 and HR6. Cluster 2 has 2 populations, HR3 and HR8, which are placed in a single sub-cluster.

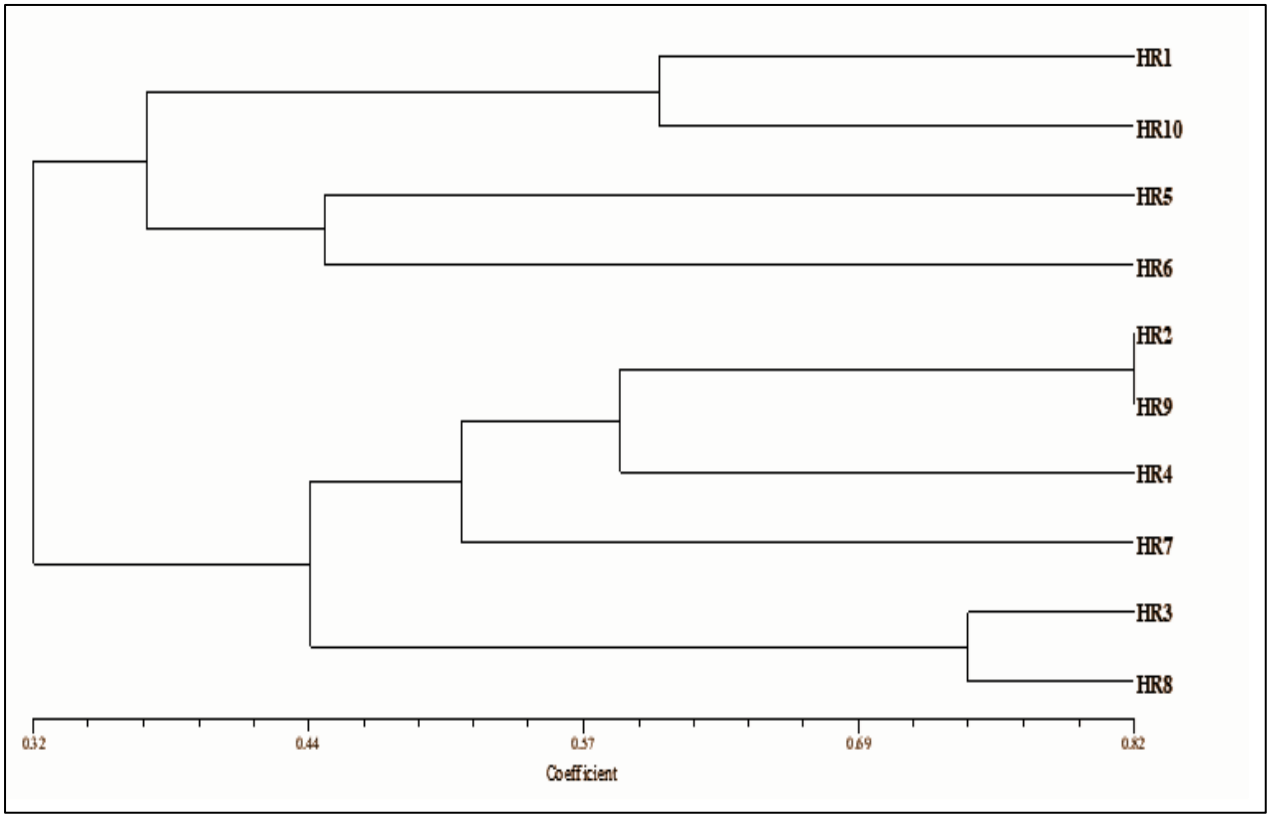

Figure 6: Dendrogram based on RAPD analysis for the estimation of genetic diversity in different accessions of *H. ringens* from different localities

A dendrogram generated from UPGMA cluster analysis of RAPD primer is shown in Figure 6. This Dendrogram clearly separates the whole genotype into two major clusters, cluster1, and cluster2. In cluster1, two sub clusters consisting of HR1, HR10, HR5, and HR6. Cluster2 is divided into two sub-clusters, sub cluster1, and sub cluster2. Sub-cluster 1 comprises clades with populations, HR2, HR9, HR4, and HR7 in which HR2 and HR9 are closely related. Subcluster 2 consists of 2 populations HR3 and HR8. In this Cladogram. HR2 and HR9 show the least diversity with a similarity value of 0.8158, and HR4, HR7and HR1 show high diversity with a similarity value of

6. DISCUSSION

Hygrophila ringens are tracheophytes within the family Acanthaceae family that grows in muddy places and are native to Asia. The plant may be an Erect subshrub, with maturation at lower nodes; stems polygonal shape. Leaves to 10x1cm, linear-lanceolate, strigose on the nerves. Flowers in axillary clusters, sessile; bracts 6mm long, linear; bracteoles minute; calyx 12mm long, tubular; lobes rectangular, acuminate, bushy within; curl pale pink, 15 mm long, bilabiate; tube slender, ventricous above; stamens four, filaments united into a sheath, ciliate. Capsule 14×2 mm, terete, glabrous; seeds several, densely bushy.

Hygrophila ringens is commonly known as swamp weeds, there are roughly 100 species, many of which are water plants. The world's tropical and subtropical regions are home to the genus. The genus is represented in Kerala by 4 species (Sasidharan, 2013). Herbs that are annual or perennial. Oval to linear, complete, sessile, opposite leaves. Bisexual, zygomorphic, solitary, or in axillary clusters of flowers. the leaves-like bracts. Deeply 5-lobed calyx, occasionally with one lobe that is larger and wider than the rest. Corolla with two lips, cylindric tube, recurved lobes on upper and lower lips, and lower lip. 3-lobed, with occasionally reflexed lobes. Didynamous with 4 stamens. Fruit in a capsule shape. ecosystems of *H. ringens* are southern Western forests and open areas that are wet or swampy.

With the development of Polymerase Chain Reaction (PCR), various molecular technologies are still area units being developed that might be used for the detection, characterization, and analysis of genetic diversity. The invention of molecular markers in advanced molecular technologies became a vital tool in the studies of genetic diversity. Markers show polymorphism, which can arise thanks to alteration of nucleotides or mutation within the ordination loci and build it doable to spot genetic variations between individual organisms or species.

Population genetic studies are essential for the conservation and restoration of threatened species. The massive loss of valuable plant species over the past centuries and the negative impact on their ecological and socioeconomic values has caused the conservation of plant resources. Proper identification and characterization of the plant material is essential to ensure that recently developed molecular tools provide a simple and non-tedious means of assigning known and unknown plant taxa. These techniques answer many new evolutionary and taxonomic questions that were previously

not possible with phenotypic methods alone. Recently, molecular techniques such as DNA barcoding, randomly amplified DNA polymorphism (RAPD), amplified fragment length polymorphism (AFLP), microsatellites, and single nucleotide polymorphism (SNP) have been used for plant diversity studies. Each technique has its own advantages and limitations. These techniques differ in their resolution to detect genetic differences, the types of data generated, and their applicability at specific taxonomic levels. Ibrahim A. Arif *et al.* (2010)

Recent advances withinside the utility of the polymerase chain response makes it viable to attain people at a huge wide variety of loci. The RAPD (random amplified polymorphic DNA) technique is one such approach that has attracted significant interest. The evaluation of populace shape with RAPD statistics is hampered through the dearth of entire genotypic facts due to dominance when you consider that this complements the sampling variance related to unmarried loci in addition to inducing bias in parameter estimation. We gift estimators for numerous populace-genetic parameters (gene and genotype frequencies, within- and between-populace heterozygosities, diploma of inbreeding and populace subdivision, and diploma of man or woman relatedness) at the side of expressions for his or her sampling variances. Although absolutely independent estimators do now no longer appear viable with RAPDs, numerous steps are cautioned with a view to ensure that the unfairness in parameter estimates is negligible. To attain an equal degree of statistical power, at the order of two to ten instances extra people want to be sampled according to locus while dominant markers are relied upon, compared to codominant (RFLP, isozyme) markers. Moreover, to keep away from bias in parameter estimation, the marker alleles for the maximum of those loci have to be in notably low frequency. Due to the want for pruning loci with low-frequency null alleles, extra loci additionally want to be sampled with RAPDs than with extra traditional markers, and a few issues of bias cannot be absolutely eliminated. M. Lynch and B.G Milligan (1994).

Inter-simple sequence repeats (ISSRs) area unit regions within the ordination flanked by microsatellite sequences. PCR amplification of those regions employing single primer yields multiple amplification products which will be used as a dominant multi-locus marker system for the study of genetic variation in varied organisms. ISSR markers are a unit straightforward to use, low-cost, and methodologically less stern compared to alternative dominant markers, creating it a perfect factor for beginners and for organisms whose genetic data is lacking.

A major advantage of ISSR is that no sequence data is required for primer design. Since the analytical method involves PCR, only small amounts of template DNA are required. Moreover, ISSRs are randomly distributed throughout the genome. Since ISSR is a multilocus method, it has the disadvantage that similarly sized fragments may lack homology. Furthermore, like RAPD, ISSR can also have reproducibility issues.

In the present, we studied 10 populations of *Hygrophilum ringens* collected, and primarily DNA isolation was done using the suitable protocol. There are different protocols used by different workers. Good quality DNA is a prerequisite for all experiments of DNA manipulation. All plant DNA extraction protocols comprise the basic disruptions of the cell wall, cell membrane, and nuclear membrane to release the DNA into solution followed by precipitation of DNA while ensuring the removal of contaminant biomolecules such as the protein polysaccharides, and lipids. phenols, and other secondary metabolites. this is brought by disruptions of the tissue in mortar and pestle aided by liquid nitrogen and the various components of homogenizations or extraction buffer followed by the precipitating and purification method employed .since DNA can be extracted from various types of tissues such as seedlings, leaves, cotyledons, seeds, endosperm, tissue culture callus, roots, etc. the tissue type along with the concentration of DNA finally determine the methodology of DNA extraction to followed by the experimenter. the most commonly used basic plant DNA extraction protocols are those of Dellaporta *et.al.* 1983 and saghaiMaroof *et al.*1984 along with the many others that are modifications of the components of these protocols to suit a particular tissue type or downscaling them for miniprep

The protocol adopted in this work is ideal to isolate a considerable amount of DNA which was quantified in a spectrophotometer. The isolated DNA was treated with 5 ISSR primers ie ISSR1, ISSR2, ISSR3, ISSR4, ISSR5 and 5 RAPD primers ie, OPG-03, OPB-04, OPA-02, OPB 01, and OPA-5. In PCR thermocycler with appropriate conditions amplified fragments of DNA produced by the PCR were subjected to gel electrophoresis to separate the bands according to their size and charge. a clear banding pattern was obtained by viewing the gel under the gel imager. the genetic analyzed these scoring these bands. The data of scored bands from amplified products of PCR revealed the polymorphism exhibited by these populations.

The binary scored data used NTSYS software and it generated the genetic distance and genetic similarity. A dendrogram was generated from UPGMA (Unweighted Pair Group Method with

Arithmetic Mean) cluster analysis of RAPD and ISSR primers in which plant populations were grouped into certain clusters. The genetic parameters include polymorphism among the populations, EMR (Effective Multiplex Ratio), and MI (Marker Index). In RAPD maximum identity showed between population HR1 and HR4, HR1 and HR7 by showing a maximum identity value of 0.7692. The genetic identity ranges from 0.1842 shared by population HR8 and shared by population HR4 & HR7 to 0.7692. In ISSR, maximum genetic diversity is shown by population HR1 and HR8 by showing a maximum identity value of 0.8387. The genetic identity ranges from 0.3182 shared by population HR9 and shared by population HR8 to 0.8387.

The effective marker index considers all the possible attributes such as information content, a fraction of polymorphic fragments, multiplex ratio as well as the qualitative issues of the given marker system (Mitra *et.al*. 2011). According to the calculation for the RAPD marker, EMR is highest for OPB 4 marker which is 117, and lower for OPB 1 marker which is 40. For Marker Index (MI), as per calculations, OPB 4 showed the highest MI at 49.14 and OPB 1 showed the least value which was 15.6. ISSR markers were found to be more efficient than the RAPD markers with regard to polymorphism detection). According to the calculations for ISSR markers, the EMR is highest for ISSR 1 marker which is 121, and lower for ISSR 4 marker which is 28. For Marker Index (MI), as per calculations, ISSR 1 showed the highest MI at 55.66 and ISSR 4 showed the least value which was 10.08. It was observed that the percentage of ISSR polymorphism bands (80.2%) was higher than RAPD (77.7%).

7. SUMMARY AND CONCLUSION

The present study was aimed at developing RAPD and ISSR-based molecular markers for studying genetic diversity among the different accessions of plant populations collected from various geographical regions of India. The importance of plant genetic diversity (PGD) is now being recognized as a specific area since exploding population with urbanization. Common marshy lands and other natural habitats of many plants are at the edge of destruction. Diversity helps in increasing the resistance of ecosystem productivity to climatic extremes. If ecosystems have value because they provide services to humans and ecosystems function better when they contain more species, then the loss of species could diminish the value of ecosystems. It is cruciate to know the existing variability among Hygrophila plant species in different habits for the effective utilization and conservation of these plants

The present work deal with the isolation of genomic DNA obtained from 10 populations and then the amplification of this DNA in PCR using Random Polymorphic DNA Markers. 5 primers of RAPD and ISSR were used in the study and they are 0PA 2, OPA5, OPB1, OPB4, OPG 3, ISSR1, ISSR2, ISSR3, ISSR4, and ISSR 5. The amplified products are then visualized using gel electrophoresis and a qualitative scoring band was done using gel photographs. The binary data obtained here from the marker system were analyzed using software called NTSYSpc version 2.2. NTSYSpc can be used to discover patterns and structures in multivariate data. The UPGMA method is used for constructing dendrograms. The genetic parameters viz, the polymorphism among the population (PIC), Effective multiplex ratio (EMR), and Marker Index (MI) were calculated using the same program. RAPD and ISSR markers could reveal the genetic characteristics of each population and the comparison of the genome diversity among different geographical locations was carried out in the study.

DNA technologies are reliable and powerful tools for the identification of taxa at various taxonomic levels as they provide consistent results irrespective of age, tissue, origin, physiological conditions, environmental factors, harvest, storage, and processing of samples. With the increasing demand for high-quality herbs, the need for DNA authentication will accelerate for ensuring therapeutic effectiveness, fair trade of drugs, and raise consumer confidence. However, for the modernization of traditional medicines, it is inevitable in the future to compile a comprehensive

database for all investigated medicinal taxa with reference information on nomenclature, phylogenetic relationships, macroscopic and microscopic features, chemical constituents, and profiling, toxicity and voucher specimens in herbaria or museums. Through this work, it is proved that RAPD and ISSR markers are a good choice among other molecular markers for the assessment of the genetic diversity of *Hygrophila ringens.*

Among them ISSR markers were found to be more efficient than others with regards to polymorphism detections. From the study, more genetic diversity was shown by the *Hygrophila ringens* population collected from Iringallur, Kasargod (HR1), and the population from Muthanga, Wayanad (HR 8) for both RAPD and ISSR markers. Populations collected from the Northern parts of Kerala are more genetically diverse than those collected from the Southern parts of Kerala. ISSR markers were found to be more efficient than the RAPD markers with regard to polymorphism detection. It was observed that the percentage of ISSR polymorphism band is higher than RAPD both RAPD and ISSR might detect noncoding and therefore more polymorphic DNA by exploiting the different regions of the genome. The difference in the dendrograms generated by RAPD and ISSR could be explained by the different number of PCR products analyzed or it may be attributed to marker sampling error.

8. FUTURE PROSPECTS

The results of the study can be seen as a starting point for many types of research aimed at defining the level of intra and interspecific genetic diversity and finding hybrids among these species. These studies have given important clues in understanding genotype relationships, which may further assist in planning and developing breeding strategies. *Hygrophila ringens* is one of the under-exploited plants in the Acanthacea family, containing great potential for medicinal purposes, this can be explored in future research. It is also useful for studying the diversity of different germplasm as possible sources of genes that can improve the performance of cultivars. It helps to access genetic diversity of accessions in order to set up good conservation programs. Thus this study helps to reveal different fields of research on plants that are not explored.

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