ASSESSMENT OF GENETIC DIVERSITY IN *LIMNOPHILA AQUATICA* (ROXB.) ALSTON USING RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) AND INTER SIMPLE SEQUENCE REPEATS (ISSR) MARKERS

Dissertation submitted in partial fulfillment of the requirements

for the award of the degree of "**Masters of Science**" in

BOTANY

By

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ST. TERESA'S COLLEGE (AUTONOMOUS), ERNAKULAM

2021-23

CERTIFICATE

This is to certify that the dissertation entitled **"Assessment of genetic diversity in *Limnophila aquatica* (Roxb.) Alston using Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) Markers"** is an authentic record of work carried out by **Aishwarya Nair** under my supervision and guidance in the partial fulfillment of the requirement of the M.Sc. Degree of 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.

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I hereby declare that the work which is being presented in the dissertation, entitled **"Assessment of genetic diversity in *Limnophila aquatica* (Roxb.) Alston 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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ABSTRACT

Limnophila aquatica (Roxb.) Alston commonly known as giant ambulia, is an under-exploited plant of the Plantaginaceace family. Genetic diversity of *Limnophila aquatic* was evaluated using Random amplified polymorphic DNA (RAPD) and Inter simple sequence repeats (ISSR) markers. A total of 10 genotypes of *Limnophila aquatica* were collected from different regions of Kerala. Plant populations are named LA 1 (*Limnophila aquatica* 1) to LA 10 (*Limnophila aquatica* 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 (92.36 %) was higher than RAPD (83.94%). RAPD and ISSR marker system were found to be useful for the genetic diversity studies in *Limnophila aquatica* (Roxb.) Alston and to identify the variation.

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

I. INTRODUCTION

The macrophytes play a crucial role in healthy aquatic ecosystems. They serve as the primary producers of oxygen, through photosynthesis. These plants form habitats for a wide variety of aquatic organisms. Macrophytes form a diverse assemblage of taxonomic groups and are divided into four categories based on their growth habits namely free-floating, attached floating, submerged, and emergent (Kumar, 2015). Aquatic macro-vegetation includes macroalgae of the divisions Chlorophyta (green algae), Xanthophyta (yellow-green algae) and Rhodophyta (red algae), and the ‘‘blue-green algae’’ (more correctly known as Cyanobacteria), Bryophyta (mosses and liverworts), Pteridophyta (ferns) and Spermatophyta (seed-bearing plants), the vegetative parts of which actively grow either permanently or periodically (for at least several weeks each year) submerged below, floating on, or growing up through the water surface (Denny, 1985). The macroscopic flora plays a vital role in maintaining the ecological balance of an aquatic ecosystem. The eutrophic water bodies are characterized by the presence of aquatic plants (Bronmark and Hansson, 2017). The increased number of macrophytes indicates the eutrophic condition of the water bodies. Some 2,614 aquatic vascular macrophytes occur globally, representing only 1% of the total number of vascular plants (Ansari, *et al*., 2017). The total number of freshwater aquatic plants in India exceeds 1,200 species (Gopal, 1995). Many aquatic plant species are invasive (Oyedeji and Abowei, 2012).

1.1 GENUS *LIMNOPHILA*

Genus *Limnophila* is an aquatic flowering plants belonging to the family Plantaginaceace. The species are commonly known as marshweed. These are annual or perennial herbs. They grow in moist habitats such as marshes, and some are aquatic. Some species are glandular and aromatic. Plants of this genus vary in shape, from erect to deciduous, with branched or unbranched stems. Submerged leaves become whorled. Air leaves are arranged spirally or oppositely. The leaves are lanceolate or pinnate with smooth or serrated edges. Some species grow solitary in leaf axils, while others bear flowers on inflorescences. The sepals are organized in a tubular calyx, and the corolla is tubular or funnel-shaped. The crown has a three-lobed lower lip and an unlobed or double-lobed upper

lip. A separate family of plants, *Cabomba caroliniana* is known for its *Limnophila*-like leaves. *Limnophila* usually propagates through fragmentation of the stem or by seeds. After the rains, the *Limnophila* fruits ripen and the mats move away from the hydrosoil. The seeds spread over a wider area as the floating mats are displaced.

1.2 TRADITIONAL USES

Limnophila plants are widely distributed throughout the world, finding immense application in the traditional medical system in many countries. Several studies have already been conducted on the chemical and pharmacological aspects of these plants, but largely unexplored.

L. rugosa has shown numerous medicinal uses in the traditional system. Rub the juice of the plant over the body in pestilent fever. It is used for elephantiasis along with coconut oil. It is used for diarrhea and dysentery. Used as a carminative and tonic. Essential oils are used as food flavorings and hair oil fragrances. *L. aromatica* is used as spinach and can be eaten raw or steamed. It is acidic, slightly bitter, cooling, emollient, antiseptic, lactating, hypnotic, appetite stimulant, carminative, anthelmintic, anthelmintic, anti-inflammatory, diuretic, and antipyretic. It helps with disturbed conditions of pitta, septic ulcers and anthrax, galactic impurities, anorexia, indigestion, helminthiasis, constipation, inflammation, and suffocation.

L. indica has a fresh, pleasant scent reminiscent of camphor and lemon oil. *L. indica* is believed to have digestive and antiseptic properties. *L. conferta* has been used in traditional medicine to treat various types of skin diseases and inflammatory conditions. *L. gratissima* is a strongly aromatic herb that smells like turpentine and contains 0.13% essential oil.

1.3 PHARMACOLOGICAL ACTIVITIES OF LIMNOPHILA

A large number of biological or pharmacological works with different parts of *Limnophila* plant as crude extracts and also purified chemical components are isolated from these plants have been reported.

Limnophila plants are reported to have significant antibacterial activity. Extracts from *L. racemosa* and *L. indica* inhibited the growth of *Xanthomonas campestris* and *X. malvacearum* in vitro. It is reported that the essential oil of *L. gratissima* showed antibacterial

activity in an order similar to that shown by the reference standards, streptomycin, and chloramphenicol. Nevadensine and isothymusine (6,7-dimethoxy-5, 8, 4'- trihydroxyflavone), isolated from chloroform extracts of the terrestrial parts of *L. geoffrayi*, showed growth inhibitory activity against *Mycobacterium tuberculosis.*

Based on studies with *L. conferta* essential oil on a variety of worms, it appears that this essential oil can be used as a powerful and effective antidote against these parasites. The oil showed dose-dependent anthelmintic activity against the test organisms, and in each case, the oil was found to be more effective than the standards used. Suksamrarn, *et al.,* (2003) reported a significant amount of antioxidant activity in the chloroform extract of the aerial part of *Limnophila geoffrayi*. The Bioassay-guided fraction of the extract resulted in the isolation of two pentaoxygenated flavones.

1.4 *LIMNOPHILA AQUATICA* (ROXB.) ALSTON

Limnophila aquatica, commonly known as giant ambulia, is a plant of the Plantaginaceace family. Native to Asia, Sri Lanka, and India, *Limnophila aquatica* is characterized by fine leaves and a bushy pine-like appearance. It grows best in moderate to very high lighting, preferably in acidic soil. It can reach 25-50 cm in height and 9-15 cm in width.

The species of *Limnophila* are usually small aquatic or semi-aquatic herbs. *Limnophila aquatica* (Roxb.) Alston. is an aquatic dicotyledonous plant. *L.aquatica* is a semi-aquatic annual with a stout stem. The basal part of the stem is usually submerged, thick, and tumid at the nodes. The aerial part is erect, glabrous, or sessile glandular. Leaves on aerial stems are opposite or verticillate or linear-lanceolate to lanceolate elliptical. They are sessile, sub-amplexicaul, glabrous, densely but minutely and obscurely punctate. The submerged leaves are multifid, pinnately dissected, and glabrous. Flowers are either terminal or axillary racemose or occasionally solitary axillary. Peduncles of terminal racemose are finely glandular-hispid. Pedicels are slender, 2-12 mm. long, and glandular with minute sessile glands. The calyx tube is 2-3 mm long with lobes more or less equal, ovate-lanceolate, long acuminate, and scarious at margins. Corolla is infundibuliform, 8-13 mm long, and violet to white with purple markings. Stamens are all glabrous with posterior filaments 2 mm long and anterior filaments 5 mm long. The capsule is globose which is enclosed by longer calyx lobes

and tipped by a withered style. Seeds are oblong-cuneate to globular, 0.5-0.7 mm long, quadrangular, and muricate (Philcox, D.1970)

1.5 GENETIC DIVERSITY

Genetic diversity is a broad term encompassing all the variability occurring among different genotypes with respect to the total genetic makeup of single species or between species. Genetic diversity forms the basis of evolutionary change. The extent and distribution of genetic diversity in a plant species depend on many factors which include its evolution and breeding system, and, ecological and geographical factors. A better understanding of genetic diversity and its distribution is required for its efficient utilization and conservation (Ramanatha Rao, 2002).

For the effective conservation, management, and efficient utilization of plant genetic resources (PGR) it is important to understand the molecular basis of the essential biological phenomena in plants. The assessment of genetic diversity is usually performed at the molecular level using various laboratory-based techniques, such as allozyme or DNA analysis, which measure levels of variation directly (Mondini, L., *et al.* 2009). Molecular analyses of genetic diversity comprise a large variety of DNA molecular markers. A genomic locus can be identified by a probe or specialized starters (primer) and is referred to as a molecular marker if its presence clearly distinguishes the chromosomal feature it represents from the neighboring areas at the 3' and 5' extremities (Barcaccia, G., *et al.* 2000).

The importance of plant genetic diversity (PGD) is now being recognized since growing population pressure and urbanization of agricultural lands and rapid modernization are the critical factors contributing to food insecurity in the developing world. Genetic diversity is of the greatest interest among workers. Reduced genetic variability and diversity among crop plant species have raised serious concerns and further improvement in crop varieties will be an arduous task.

1.6 MOLECULAR MARKERS

Every organism has its own unique set of DNA. Mutations occurring in the DNA sequences of an organism are referred to as variations and this will result in the creation of new alleles in a population. The occurrence of two or more variant forms of a specific DNA sequence that can occur among different individuals or populations is called polymorphism. A population is said to be in a state of balanced polymorphism when non-identical alleles for a trait are maintained at frequencies greater than 1%. The survival of a species and its evolutionary potential is determined by the extent of polymorphism within the gene pool of a species. Genetic variation is very common in the natural populations of most plants and such variations that distinguish within species and among species can be called Molecular Markers (Sebastian, V. A., 2009)

A genetic marker is a gene or DNA sequence with a known location on a chromosome and it is always associated with a particular gene or trait. A genetic marker may be a short DNA sequence (single nucleotide polymorphism, SNP), or a long one, like mini & microsatellites. Molecular markers reveal polymorphism at the DNA level (Al-Samarai, F. R., 2015).

Genetic diversity within the gene pool of a species has traditionally been measured using morphological and biochemical traits like allozymes. However, allozymes are no longer sufficient for assessing genetic diversity within populations (Heun *et al*. 1994). A broad range of molecular-based technologies has made it possible for such studies to be made at the population level, including RAPD (Random Amplified Polymorphic DNA), ISSR (Inter Simple Sequence Repeat), and RFLP (Restricted Fragment Length Polymorphism).

Among molecular markers, RAPDs have been extensively used in genetic research due to their speed and simplicity (Penner, 1996). Each technique has got its own advantages and limitations. These methods differ in the extent to which they can resolve genetic differences, the type of data they generate, and the level to which they can be applied. Molecular markers are used in many different areas such as genetic mapping, paternal tests, detecting mutant genes which are connected to hereditary diseases, cultivars identification, marker-assisted breeding of crops, population history, etc.

1.7 RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) MARKERS

Over the past decade, polymerase chain reaction (PCR) technology has become a popular research technique and has led to the development of several new genetic tests based on selective DNA amplification. The creation of genetic markers has been made easier by the realization that random primer-based PCR may be used to amplify a collection of randomly distributed loci in any genome. RAPD Markers or Random Amplification of Polymorphic DNA is a type of PCR reaction, and the DNA segments are amplified at random. No knowledge of the DNA sequence for the target gene is necessary because the primers will bind somewhere in sequence, but it is not known exactly where. The RAPD technique is sensitive and specific because the organism's entire genome is used as the basis for DNA profiling. This technique allows the detection of multi-locus genetic variation using short primers of arbitrary sequences. A single oligodeoxyribonucleotide of 10 to 34 base pairs or more is sufficient for the RAPD-PCR which utilizes two or more primers (Mullis, K. B. 1987; Williams, *et al.1990*). Perhaps the main reason for the success is the gain of a large number of genetic markers that require small amounts of DNA without the need for cloning, sequencing, or other molecular characterization of the species' genome.

The use of a single decamer oligonucleotide promotes the generation of multiple discrete DNA products, and these products are considered to be derived from different genetic loci. Polymorphisms result from mutations or rearrangements at or between primer binding sites and are detected as the presence or absence of a specific RAPD band. This means that RAPD is a dominant marker and therefore cannot be used to determine heterozygosity.

RAPD Markers have been widely used because of the following advantages:

- For the creation of particular primers, DNA probes, and sequence information are not needed.
- It involves no blotting or hybridization steps, hence, it is quick, simple, and efficient.
- It can be automated and just modest amounts of DNA (approximately 10 ng) are needed for each reaction.
- A high number of fragments.
- Arbitrary primers are easily purchased.
- Unit costs per assay are low compared to other marker technologies (Kumar, N. S., 2011)

1.8 INTER-SIMPLE SEQUENCE REPEATS (ISSRS) MARKERS

Inter-simple sequence repeats (ISSRs) are regions within the genome flanked by microsatellite sequences. PCR amplification of these regions utilizing a single primer yields multiple amplification products that can be utilized as a dominant multi-locus marker system for the study of genetic variation in sundry organisms. ISSR markers are facile to utilize, low-cost, and methodologically less demanding compared to other ascendant markers, making them an ideal genetic marker for tyros and for organisms whose genetic information is destitute.

The generation of ISSR markers uses microsatellite sequences that are highly diverse and ubiquitous throughout the genome. It is more reproducible than using RAPD and less time-consuming and expensive than using AFLP. All these make ISSR an ideal genetic marker for various studies, most eminently on genetic variation/diversity, DNA fingerprinting, and phylogenetics (Ng, W. L., & Tan, S. G., 2015). ISSR markers exhibit the Mendelian mode of inheritance and are segregated as dominant markers. This technique has been widely utilized in the studies of cultivar identification, genetic mapping, gene tagging, genetic diversity, evolution, and molecular ecology.

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. In addition, ISSR, like his RAPD, is subject to reproducibility problems. The multilocus fingerprinting profiles obtained make ISSR analysis applicable to studies dealing with genetic identity, phylogeny, clone and strain identification, and taxonomic studies of closely related species. In addition, ISSR is believed to be useful for genetic mapping studies.

This study aims at the genetic diversity study of *Limnophila aquatica* plants using RAPD and ISSR markers for a better understating of its genome characters for the effective conservation, management, and utilization of it in various areas

II. OBJECTIVES

1. Survey and collection of *Limnophila aquatica* from different parts of Kerala.

2. Isolation of genomic DNA from collected sample populations.

3. PCR amplification of isolated genomic DNA using Random Amplified Polymorphic DNA (RAPD) Markers and Inter Simple Sequence Repeats (ISSR) Markers.

4. Analysis of genetic diversity in *Limnophila aquatica.*

III. REVIEW OF LITERATURE

3.1 GENUS *LIMNOPHILA*

The word *Limnophila* comes from the Latin word meaning pond loving and is commonly known as “Ambulia” (Asian marsh weed). The genus *Limnophila* includes short-growing herbaceous plants that live in wetlands and are essentially semi-aquatic plants widely distributed in tropical and subtropical regions of the world. The genus *Limnophila* belonging to the family Plantaginaceae are aquatic dicotyledonous plants. *Limnophila aquatica* commonly called Giant Ambulia is a semi-aquatic annual with a stout stem. Khan and Halim (1987) in their study reported four species of Limnophila, viz. *L. cana* Griff, *L. heterophylla* (Roxb.) Benth, *L. indica* (L.) Druce, and *L. sessiliflora* Blume from regions of Bangladesh. Ahmed, *et al.* (2009) reported the distribution of *L.aquatica* in Bangladesh, Chinese, Indonesia, Japan, Sri Lanka, and Taiwan.

3.2 *LIMNOPHILA AQUATICA* (ROXB.) ALSTON

Alfasane, *et al.*, in 2021 studied the occurrence of *Limnophila Aquatica* (Roxb.) Alston of Bangladesh. Rooting occurs at the lower stem node. The base of the stem is usually submerged, thick, and swollen with nodes. Its leaves are fine, pine-like, and bushy. The upper leaves are usually crenulate, opposite or in verticals of 3, ovate-lanceolate to oblong-lanceolate, 2.7-6.8 cm long x 0.7-2.0 cm wide, rounded and semi-amplexicaul at the base, acuminate at apex, finely spinulose-serrate, strongly 3-5-nerved at the base, glabrous on both surfaces and punctate above. The lower leaves are whorled, in verticals of more or less 10-12, pinnately dissected, up to 6 cm long, frequently deflexed, and root-like. Inflorescence is terminal racemes with peduncles finely glandular-hispid. Bracts are usually ovate to broadly lanceolate, 5-7 x 3-4 mm, apex sub acuminate, finely glandular-pubescent on both surfaces and bracteoles are linear-lanceolate. Flowers are numerous with pedicels up to 5 mm long, finely glandular-pubescent. The calyx segments are deltoid-ovate, acute, tubes 2-3 mm long, lobes more or less equal, ovate-lanceolate, long acuminate, scarious at margins. The corolla tubes are pale greenish with finely pubescent petals and whitish pale blue limbs. Lobes of the upper lip are broadly orbicular with a pale purple blotch at the center whereas middle lobes are broader than lateral ones. Stamens are four in number with posterior filaments 1.5-2.5

mm long and anterior ones longer. Capsules are globose and enclosed by longer calyx lobes. Flowering and fruiting usually occur during the months of June-April. It grows well in very high to medium sunlight, preferably in mud soil and water prominent areas.

3.3 MEDICINAL USE

Limnophila plants are extensively used in the indigenous system of medicine. Brahmachari. G, (2008) has cited the medicinal uses of these plants on the basis of an extensive literature survey. According to Prajapati, N.D. *et al*., (2003), *L. aromatica* plant juice is used as a cooling remedy for fever and pharyngitis. It is given to lactating women when the milk is sour. The tree gives off a turpentine smell and produces essential oils. Ambasta, S. P., (1986) reported *L. aquatica* has a refreshing and agreeable odor resembling camphor or the oil of lemon. *L. indica* is considered to have carminative and antiseptic properties.

In Southeast Asia, *Limnophila aromatica* is commonly used as a spice and herbal medicin*e.* The effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of *Limnophila aromatica* was reported by Do, Q.D., *et al.* in 2014. Water and various concentrations (50%, 75%, and 100%) of methanol, ethanol, and acetone in water were used as solvents for *L. aromatica* extraction. The antioxidant activity, total phenolic content, and total flavonoid content of the lyophilized *L. aromatica* extract were investigated by various in vitro assays. The extract obtained with 100% ethanol showed the highest overall antioxidant activity, reducing power, and DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity. The same extract also had the highest phenol content and the highest flavonoid content. The highest extraction yield was obtained using 50% aqueous acetone. From these results, they concluded that *L. aromatica* can be used in dietary applications that may reduce oxidative stress.

Kukongviriyapan, *et al*. (2007) reported the antioxidant and vascular protective activities of *Cratoxylum formosum*, *Syzygium gratum,* and *Limnophila aromatica*. Phytochemicals contained in dietary plants offer many health benefits and may reduce the risk of cardiovascular disease. Aqueous extracts of three common Thai dietary and herbal plants, *Cratoxylum formosum*, *Syzygium gratum*, and *Limnophila aromatica*, were investigated for their antioxidant and vascular protective activities in in-vitro and in-vivo

models. It has been concluded that plant extracts have antioxidant properties and have a potential role in protection against vascular dysfunction.

In a study by Thongdon, A. J., & Inprakhon, P. (2009), essential oils from the dried aerial parts of the aquatic plant *Limnophila geoffrayi* Bonati were obtained by water distillation. Gas chromatography-mass spectrometry analysis was used to characterize the major constituents such as d-pulegone (27.14%), perillaldehyde (19.13%), and limonene (9.00%). The antimicrobial activities of essential oils and their main components were evaluated against microorganisms commonly found in contaminated cosmetic products, using agar and broth dilution methods. Their insecticidal activities against the oriental fruit fly *Bactrocera dorsalis* (Hendel) were reported using a bioassay with impregnated filter paper. The results showed that the essential oils had high antimicrobial activity. Strong insecticidal activity as a fumigant has also been observed.

Gorai, *et al.* in 2014 reviewed the chemical and pharmacological aspects of *Limnophila aromatic.* The review addressed *Limnophila aromatica* (Lamk.) Merr. as one of the most significant plant species in the traditional system of medicine and it is established as a source of flavonoids, terpenoids, etc. The isolated phytochemicals as well as the various extracts exhibited important biological activities such as antibacterial, antioxidant, and vascular protective activities. Extensive research involving the isolation of many phytochemicals and pharmacological studies of this medicinal plant is still needed to explore the plant for its medicinal significance. The purpose of this review is to inspire today's researchers to do further research on this plant in search of new drugs. The plant is used as water spinach, eaten raw or steamed. It is an acidic, slightly bitter softening antiseptic refrigerant, galactagogue, aperitif, aperitif, digestive, carminative, anthelmintic, anti-inflammatory, diuretic, and cooling agent.

3.4 GENETIC DIVERSITY

Hughes, A. R *et al.* conducted a study on ‘Ecological consequences of Genetic Diversity’ in 2008. Their study revealed the significant effect of genetic diversity on ecological processes such as primary productivity, community structure, population recovery from disturbance, interspecific competition, and fluxes of energy and nutrients. Genetic diversity can have important ecological consequences at the population, community, and

ecosystem levels. In some cases, the ecological consequences are comparable in magnitude to the effects of species diversity.

The work of Booy, G, *et al.* (2000) on Genetic Diversity and the Survival of a population, reports that genetic diversity is essential for the fitness of populations where quantitatively inherited fitness-related traits are important. They considered many factors that could influence the importance of genetic diversity for population survival. The level of genetic diversity in a population is highly dependent on the mating system, the species' evolutionary and population history, and the degree of environmental heterogeneity. Underlying the importance of species diversity for ecosystem functioning is the importance of genetic diversity at the population level, which is thought to determine species survival and thus species diversity. This is the subject of ongoing debate at the population level. In, this review, a survey of the importance at the population level is presented.

Natarajan, C., *et al.* 1988, conducted a study of genetic relatedness and diversity among forty-five genotypes of diverse green gram origins for eight characters. High genotypic coefficients of variation were shown for seed yield, pod number, and plant height. High heritability changes were observed for 100 seed weights, days to flowering, plant height, and pod length. Seed yield showed the highest genetic progress, followed by plant height. Correlation studies found seeds to be positively and significantly correlated with plant height, racemes and pods per plant, and seeds per pod. For the selected materials, seed weight contributed the most to genetic divergence.

Studies on genetic variability, association, and diversity in wheat (*Triticum aestivum* L.) germplasm were performed by Ali, Y., *et al.* (2008). They evaluated seventy local and exotic wheat genotypes for variation parameters, correlations, and path coefficients for eight metric traits, which include plant height, number of productive tillers per plant, number of spikelets per panicle, spike length, number of grains per earring, fertility percentage, 1000 seed weight and yield per plant. Substantial genotypic differences were observed for all traits studied, indicating a significant amount of variation between genotypes for each trait. The study showed that seed yield per plant was strongly and positively correlated between genotype and number of productive tillers per plant and number of grains per panicle with maximum direct effect. These two characteristics are the main contributors to yield per plant, suggesting a need to focus more on these components to increase wheat grain yield. The

number of spikelets per spike and the length of the spike, although strongly correlated with grain yield, were not taken into account in the selection criteria due to their high indirect effect through the number of productive tillers per plant and the number of grains per spike. The distribution patterns of all genotypes in different groups indicate the presence of significant genetic differences between genotypes for most traits.

Crawford, K. M, and Rudgers, J. A, (2012) discussed how plant species diversity and genetic diversity within a dominant species independently and interactively affect the biomass of plant communities in the Great Lakes sand dune ecosystem. To test the independent effects of diversity, they set up two experiments. In one, they manipulated genetic diversity in the dune-dominant *Ammophila breviligulata*. On the other hand, they manipulate the number of plant species, with the exception of *A. breviligulata*. Then, to test interaction effects, they constructed communities that altered the number of species and levels of genetic diversity in *A. breviligulata.* Although there was no independent effect on species diversity or genetic diversity in *A. breviligulata* on biomass production in this system, the interacting effects of species diversity and genetic diversity significantly influenced the overall aboveground biomass production of plant communities. The results suggest that the interaction between plant species diversity and genetic diversity within a dominant species can alter biomass production.

3.5 MOLECULAR MARKERS

A study by Kumar, P., *et al.* (2009) "Potential molecular markers in plant biotechnology" describe biochemical and molecular markers, their advantages, disadvantages, and applications compared to other types of markers. The use of molecular markers, which reveal polymorphisms at the DNA level, plays an increasing role in studies of plant biotechnology and genetics. There are different types of markers, such as morphological, biochemical, and DNA-based molecular markers. These DNA-based markers are differentiated into two categories: non-PCR-based markers (RFLP) and PCR-based markers (RAPD, AFLP, SSR, SNP, etc.). Microsatellite DNA markers are the most widely used, due to their ease of use by simple PCR, followed by denaturing gel electrophoresis for allele size determination and the high level of information provided by a large number of alleles per locus. Even so, a new type of marker, named SNP, for Single Nucleotide Polymorphism, has gained popularity, even though it is only a type of tandem marker. The

daily evolution of these new and specific types of markers makes them important for understanding genome variability and diversity between similar and different plants.

A study by Wang, J. B., *et al*. (2006) revealed a lack of genetic variation of an invasive clonal plant *Eichhornia crassipes* in China by RAPD and ISSR markers. Random amplified polymorphic DNA (RAPD) and Inter simple sequence repeat (ISSR) markers were used to analyze the genetic structure of six populations of invasive *Eichhornia crassipes* plants collected from an introduced area in Southern China. Using 25 RAPD and 18 ISSR primers, 172 RAPD, and 145 ISSR bands were generated, respectively. However, no polymorphic bands were detected within or between populations by either marker. This suggests that the genetic diversity of *E. crassipes* in southern China is very low, and all populations are mostly composed of the same genotype. In addition to genetic diversity, this study suggested several other factors related to the expansion of *E. crassipes* in China.

Gupta, P.K., *et al.* in 1999 conducted research on molecular markers and their application in wheat breeding. They reported on the different molecular markers currently available for gene mapping and marking different traits in wheat. The Molecular markers studied include restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPDs), sequence-tagged sites (STS), DNA amplification fingerprinting (DAF), amplified fragment length polymorphisms (AFLPs), and microsatellites (STMS). Other markers including microsatellite-initiated polymerase chain reaction (MP-PCR), expressed sequence tag (EST), and single nucleotide polymorphism (SNP) are also discussed. They also reviewed recent information on synteny in cereal genomes, marker-assisted selection, marker validation, and their relevance to cereal breeding in general and wheat breeding.

Tanya, C. and Kumar, R., reviewed molecular markers and their applications in fishing and aquaculture in 2010. Molecular markers and their statistical analysis have revolutionized the analytical capabilities required to explore genetic diversity. Various protein, DNA, or molecular markers are used today in fisheries and aquaculture. These markers provide many important scientific observations in recent aquaculture practice, such as 1) Species identification, 2) Studies of genetic variation and population structure in natural populations, 3) Comparison between wild and hatchery populations,4) Assessment of

demographic bottlenecks in wild populations, 5) Propagation assisted rehabilitation programs. The study presented the basics of molecular genetics, an overview of commonly used markers and their applications, and their limitations in fisheries and aquaculture research.

Cloutier, S. and Landry, B. S., (1994) studied the application of molecular markers in plant tissue culture. Plant tissue culture techniques have been successfully applied to a number of crops. The study of plant molecular genetics is growing exponentially. Molecular markers are currently used to study many of the current limitations of tissue culture. They have been used to study the mechanisms underlying somatic lineage variation in nuclear, mitochondrial, and chloroplast genomes. Molecular markers represent powerful tools for the precise identification of plant origins from microspore or anther culture, protoplast fusion, and other tissue culture studies, where this information is very important. With improved tissue culture techniques, populations of doubled haploid lines have been generated in several major plant species. Doubled haploid populations have been shown to be useful in generating molecular maps and marking important agronomic traits. This review describes the use of molecular markers in plant tissue culture and also discusses the potential for improving molecular techniques and novel molecular markers such as SCAR and STS as well as High-resolution map sequencing strategies.

Pourmohammad, A., (2013) studied the application of molecular markers in medicinal plants. DNA-based techniques are widely used for the authentication of medicinal important plant species. Geographical conditions influence the active ingredients of medicinal plants and their efficacy profile. DNA-based molecular markers help improve medicinal plant species. DNA markers are more reliable because the genetic information is unique to each species and independent of age, physiological condition, and environmental factors. RAPD markers have been reported to help distinguish different specimens of his *Codonopsis pilosula*, *Allium schoenoprasum* L., and *Andrographis paniculata* collected from different geographic regions. Similarly, different specimens of *Arabidopsis thaliana* are distinguished by ISSR markers. RFLP technology was used to assess the intraspecific genetic diversity of the genus Capsicum and the DNA fingerprinting of pepper cultivars. Markers differ in their ability to discriminate, mechanism of polymorphism, and genomic coverage. Therefore, they can complement each other, depending on the technology available. Molecular techniques offer an independent method for describing medicinal plant materials.

3.6 RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) MARKERS

Bardakci, F., (2001) reported in a study that advances in molecular biology techniques have led to the development of a large number of highly informative DNA markers for the identification of genetic polymorphisms. Randomly amplified polymorphic DNA (RAPD) technology, based on the polymerase chain reaction (PCR), is one of the most commonly used molecular techniques for the development of DNA markers. The RAPD marker is the amplification product of an anonymous DNA sequence using single, short, and random oligonucleotide primers, so no prior knowledge of the DNA sequence is required. Although the reproducibility of RAPD profiles remains a focus of debate, the low cost, the efficiency of developing large numbers of DNA markers in a short time, and the need for less sophisticated equipment make the RAPD technique worthwhile.

Xu, C. Y., *et al*. in 2003 examined the Genetic diversity of Chinese alligator weed by RAPD analysis. Applying random amplified polymorphic DNA (RAPD), they analyzed the genetic diversity of an invasive weed species, *Alternanthera philoxeroides*, collected from eight different locations in southern China. 391 bands were amplified by 108 RAPD primers at fairly large spatial intervals from samples collected from different sites, but no genetic variation was found between samples. Molecular data have shown that alligator weeds have very low genetic diversity. Alligator weed is a seriously invasive weed and is widespread in China. They felt that the low genetic diversity of alligator weed does not affect its successful dissemination in China and that low genetic diversity does not necessarily lead to endangered plant species. Furthermore, molecular evidence from this study suggests that alligator weeds in southern China may have originated from very few clones or from a single clone. The expansion is most likely the result of extensive vegetative propagation since its introduction in China.

An assessment of genetic variation and clonality in the seagrass *Posidonia australis* using RAPD and allozyme analysis was performed by Waycott, M. in 1995. The genetic structure of seagrasses endemic to the coast of South Australia was studied at Warnbro Sound, Western Australia. High levels of genetic variation were detected within small regions

of the meadow, as determined by Allozyme and his RAPD (randomly amplified polymorphic DNA) analysis. The level of genetic variation in *P. australis* is comparable to or higher than that observed in other hydrophilic species. The genetic variation within the population demonstrated in this study indicates that *P. australis* meadows need not be monoclonal in all cases. Genetic diversity exists in *Posidonia australis* and the meadows studied are multiclonal. This is important because polyclonal populations have a high potential for successful outcrossing and sexual reproduction. Therefore, it is of great interest to determine the degree of outbreeding in populations of this species and to assess sexual reproductive success in polyclonal and monoclonal populations.

The two most widely recognized subspecies of rice (*Oryza sativa* L.) are the *indica* and *japonica* varieties. Mackill, D.J. 1995 classified *Japonica* rice cultivars with RAPD markers. The study included 134 predominantly *japonica* cultivars and two wild varieties (*O. nivara* Sharma *et* Shastry and *O. rufipogon* Griffith). Ten oligonucleotide primers produced 30 bands showing distinct polymorphisms. *Indica* and *japonica* varieties were separated into separate groups by cluster analysis. Clustering was reduced within the japonica group. Although tropical japonica cultivars usually cluster together, no clear boundaries were found between tropical and temperate species. Canonical discriminant analysis showed that the distances between the *indica* group and each *japonica* subgroup were approximately equal and were much larger than the distance between the two *japonica* subgroups. Additional primers resulted in better resolution of closely related strains. It was concluded that the RAPD marker is useful for the taxonomy of *japonica* rice cultivars, but many primers are required to resolve closely related *japonica* cultivars. Maximum genetic diversity for genetic mapping, or exploitation of F1 hybrid vigor within *japonica* cultivars, can be achieved by crosses between selected temperate and tropical parents.

Wilkie, S.E., *et al.* (1993) performed a genetic analysis study on Allium using randomly amplified polymorphic DNA (RAPD) markers. RAPD analysis has been applied to onion (*Allium cepa*) and other Allium species to assess the degree of polymorphisms within the genus and to determine if the approach is suitable for onion genetic studies. Seven varieties of *A. cepa*, including shallots, and varieties of Japanese onion (*A. fistulosum*), chives (*A. schoenoprasum*), leeks (*A. ampeloprasum*) and wild relatives of onions (*A. royalei*), was evaluated for variability using a set of 20 random primers. Seven of the twenty primers

revealed remarkable polymorphisms among *A. cepa* varieties and they were further evaluated for use in gene mapping. Large variations in band profile between species were observed with almost all primers tested. Ninety-one band positions were recorded (+/-) for all breeds studied. The genetic distance between each cultivar was calculated and cluster analysis was used to generate a dendrogram showing the phylogenetic relationships between them. The resulting analysis largely agrees with the earlier classifications of the species studied, confirming the validity of the method.

Klein-Lankhorst, R. M. *et al.* in 1991 conducted a study using RAPD assay on tomatoes. Isolation of molecular markers for tomato (*L. esculentum*) using random amplified polymorphic DNA (RAPD) was reported. A set of 11 oligonucleotide decamer primers were used, and each primer directed the amplification of a genome-specific "fingerprint" of DNA fragments. The potential of the original RAPD assay to generate polymorphic DNA markers with a given set of primers was further increased by combining two primers in a single PCR reaction. By comparing the "fingerprints" of *L. esculentum*, and *L. pennellii*, 6-specific RAPD markers were directly identified in the set of amplified DNA fragments. Their chromosomal positions on the classical genetic map of tomatoes were then established by restriction fragment length polymorphism (RFLP) association analysis. It was found that one of the RAPD markers was closely associated with the nematode resistance gene Mi.

3.7 INTER-SIMPLE SEQUENCE REPEATS (ISSRS) MARKERS

Wang, J. B. 2002 reviewed ISSR markers and their applications in plant genetics. Intersimple Sequence Repeat (ISSR) markers have emerged as an alternative system with the reliability and advantages of microsatellites (SSR). This technique involves the amplification of genomic segments flanked by microsatellite sequences that are contiguous in reverse orientation with a single primer or pair of primers based on SSRs anchored 5' or 3' by 1-4 purine or pyramidine residues. The sequences of repeats and anchor nucleates are chosen arbitrarily. Combined with the separation of amplification products on polyacrylamide or agarose gels, ISSR amplification shows a much higher number of fragments per primer than RAPD. It was concluded that ISSR technology provides a fast, reliable and informative DNA fingerprinting system. ISSR markers are inherited in Mendelian mode and segregated as

dominant markers. This technique can be widely used in breed identification, genetic mapping, genetic marking, genetic diversity, evolution, and molecular ecology studies.

Han, Y. C.*et al*, in 2007 conducted a study on the Genetic variation and clonal diversity in populations of *Nelumbo nucifera* (Nelumbonaceae) in central China detected by ISSR markers. In order to obtain accurate population structure estimates for conservation planning purposes of wild lotus (*Nelumbo nucifera* Gaertn.) in central China, genetic diversity between and within six populations and other populations of this species were analyzed. They analyzed clonal diversity within two populations of genetic diversity was high at the species level but low within each study population. The total mean estimated number of gene flows indicated limited gene flow among the populations studied. The level of clonal diversity found within the population was rather high, suggesting that clonal diversity contributes significantly to the overall genetic variation in the *N. nucifera* gene structure. Based on this study, It was recommended that future conservation plans for this species should be specifically designed to include representative populations with the highest genetic variation, both in situ conservation and germplasm collection expeditions.

Khodaee, *et al.,* in 2021 studied the genetic diversity among Iranian *Aegilops triuncialis* accessions using ISSR, SCoT, and CBDP markers. Crop Wild Relatives (CWRs) are commonly used as genetic reservoirs suitable for plant breeding. They can be used to increase the tolerance of plant cultivars to biotic and abiotic stresses. Since Iran is one of the most important centers of genetic diversity and distribution of her Aegilops species, studying the genetic diversity of related wheat species in Iran could improve various traits of bread wheat. Therefore, the aim of this study was to identify the relationship between 48 *Aegilops triuncialis* accessions using three DNA marker systems, including Start Codon Targeted (SCoT), CAAT box-derived polymorphism (CBDP), and Inter-simple Sequence Repeat (ISSR). The distinguishability of the three markers was assessed using Polymorphic Information Content (PIC), Marker Index (MI), and Resolution (Rp). The average PIC values for the ISSR, SCOT, and CBDP markers were 0.3, 0.26, and 0.34, respectively, indicating the efficiency of the three markers in detecting polymorphisms among the accessions investigated. ISSR markers had the highest MI, and percentage polymorphism values compared to SCOT and CBDP markers. Based on the Shannon index and heterozygosity scores, genetic diversity was greater in the Alborz population than in the other populations.

Using the UPGMA method, we classified enrollments into 6, 5, and 5 groups based on ISSR, SCOT, and CBDP. According to the results of cluster and PCoA analyses, the variation patterns corresponded to the geographical distribution of *Aegilops triuncialis*. Three markers provided a comprehensive pattern of genetic diversity among *Aegilops triuncialis* accession. This information may provide future insight into wheat breeding programs.

Prakashkumar, *et al.* (2015) analysed the genetic diversity of Lagenandra species. (Araceae) of Kerala (South India) using ISSR Markers. This study revealed molecular variation in different species of Lagenandra, an aquatic plant collected from different geographical regions in Kerala, India. Molecular analysis was performed using ISSR markers. A total of 66 evaluable polymorphic markers were generated from the 18 primers screened. Genetic distances between populations ranged from 0.0016 to 0.0271, and genetic identities ranged from 0.9732 to 0.9984. It is evident that there is significant genetic diversity among the Lagenandra species found in Kerala. This study demonstrated that ISSR-PCR is a suitable method for detecting genetic variation in Lagenandra species.

Bahmani, *et al.* in 2012 used ISSR markers to examine the genetic diversity of Iranian fennel. Fennel (*Feniculum vulgare* Mill.) is an important medicinal plant used for various purposes in various industries. In this study, 25 different ecotypes of fennel were collected from across Iran and their genetic diversity was examined by seven ISSR primers. The seven ISSR primers generated 52 amplified fragments, 49 of which were polymorphic. This study found that ISSR markers could adequately segregate these ecotypes based on their geographical distribution and climatic similarities, demonstrating high genetic diversity in Iranian fennel. In this study, ISSR markers provided comprehensive insight into the genetic diversity of Iranian fennel ecotypes. Furthermore, the results showed that the ISSR markers efficiently discriminate 25 ecotypes of fennel, enabling the characterization of the ecotype under study.

IV. MATERIALS AND METHODS

4.1 PLANT MATERIALS

A total of 10 genotypes of *Limnophila aquatica* were collected from different regions of Kerala (Table 1). These plants were then successfully maintained in the aquatic plant conservatory at KSCSTE- Malabar Botanical Garden and Institute for Plant Science, Calicut, Kerala, India. Plant populations are named LA 1 (*Limnophila aquatica* 1) to LA 10 (*Limnophila aquatica* 10) (Table 10). 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.

Limnophila aquatica (Roxb.) Alston. is an aquatic dicotyledonous plant belonging to the family Plantaginaceace. *L.aquatica* is a semi-aquatic annual with a stout stem. The basal part of the stem is usually submerged, thick, and tumid at the nodes. The aerial part is erect, glabrous, or sessile glandular. Leaves on aerial stems are opposite or verticillate or linear-lanceolate to lanceolate elliptical. They are sessile, sub-amplexicaul, glabrous, densely but minutely and obscurely punctate. The submerged leaves are multifid, pinnately dissected, and glabrous. Flowers are either terminal or axillary racemose or occasionally solitary axillary (Plate 1 and 2).

Plate 1: *Limnophila aquatica* habit

Plate 2: *Limnophila aquatica* single plant

Table 1: Details of *Limnophila aquatica* accession collected from different regions of Kerala for genetic diversity analysis

Sl.no	Location	Latitude	Longitude	Altitude (M)
LA1	Manjeshwar, Kasaragod	12.7221248	74.89042002	1
LA2	Nilambur, Malappuram	11.29645274	76.306536	62
LA3	Paravur, Ernakulam	10.1173345	76.2239894	1
LA5	Kadavathur , Kannur	11.7834654	75.65565216	107
LA4	Thirunelli, Wayanadu	11.89699077	76.00800128	872
LA6	Kutyadi, Kozhikode	11.63361109	75.74477837	49
LA7	Chalakkudi, Trissur	10.28805559	76.33438394	36
LA8	Cherthala, Alappuzha	9.674473503	76.34926892	4
LA9	Agasthyamala , Thiruvanathapuram	8.631637889	77.1362981	176
LA10	Elathur , Kozhikode	11.3286813	75.74957142	1

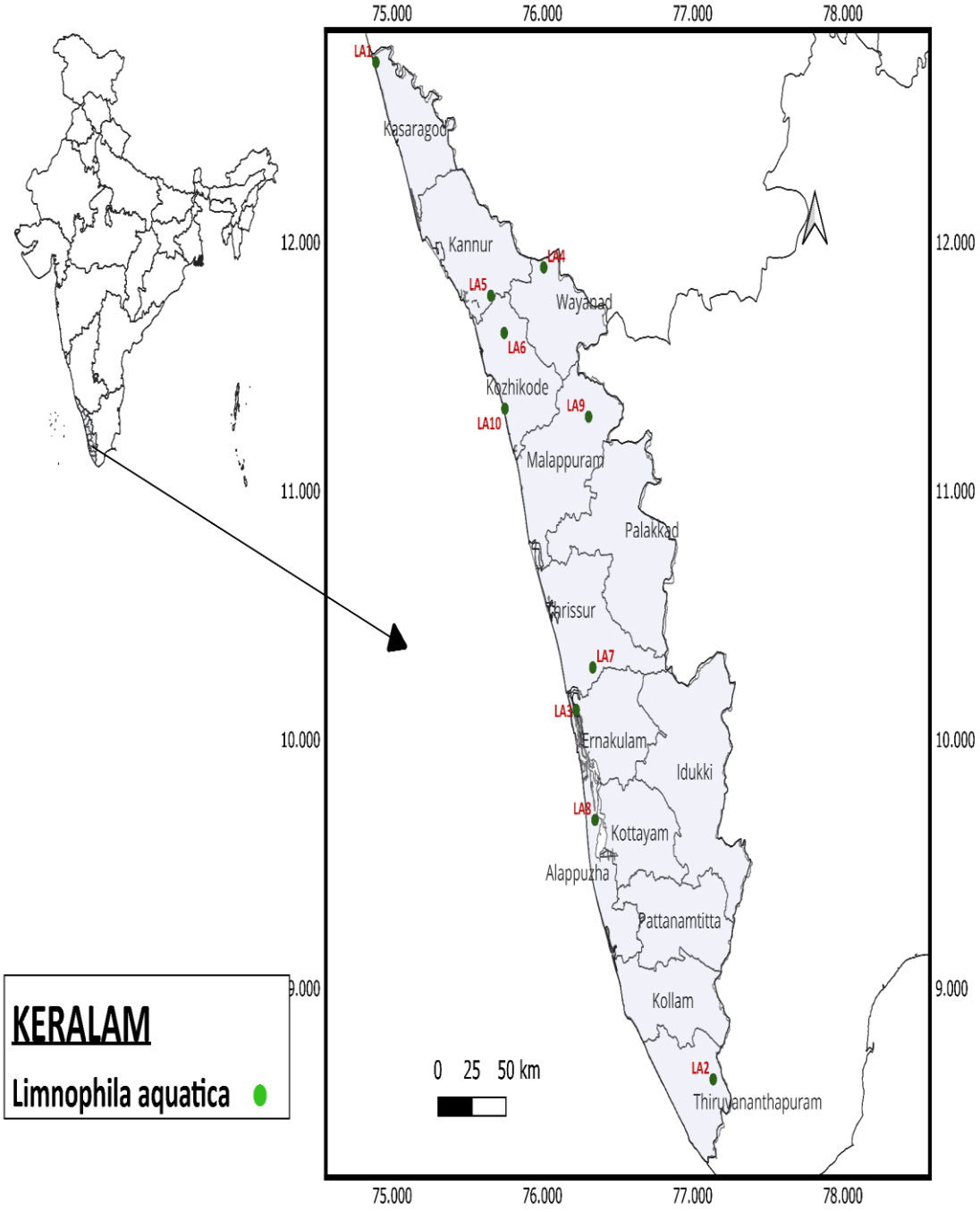

Figure 1: *Limnophila aquatica* geographical distribution in Kerala

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. Rinse thoroughly and dry with sterile filter paper. Weigh 1 g of leaf sample and cut it into pieces in a pre-cooled mortar. Pre-warm the CTAB Extraction Buffer and add polyvinylpyrrolidone (PVP) before extraction. Grind the tissue to a powder with a mortar and pestle, add 500-1000 µl of extraction buffer (Table 2), mix it into a slurry, and transfer it to a 2 ml centrifuge tube Incubate the tubes at 65°C for 45 min in the water bath, stirring occasionally.

Cool the mixture to room temperature and add 70 µl of the chloroform: isoamyl alcohol (24:1) mixture, mix well to form an emulsion, and centrifuge at 12,000 rpm for 15 min at 25℃. Collect the upper aqueous phase into a new tube and add 1/10 volume of CTAB/NaCl solution. Gently swirl the mixture and add an equal amount of chloroform and centrifuge at 12000 rpm for 10 minutes at 4°C. Collect the upper aqueous phase into a new eppendorf tube and add an equal amount of chloroform and centrifuge at 12,000 rpm for 10 minutes at 4℃. Transfer the supernatant to a fresh eppendorf tube and add chilled 100% isopropanol through the walls, mixing gently by inverting the tubes. Keep the mixture at -20℃ for 1 hour. Centrifuge at 7850 rpm for 15 min at 4℃. Collect the pellets twice with cold 70% ethanol at 1000 rpm for 5 min and dry the pellets in the air. Suspend the pellets in 200 µl of TE buffer. Add 4 µl of RNase and incubate at 37℃ for 1-2 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 500 µl 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 50 µl TE buffer (Table 2). The resuspended pellets were collected and stored at -20 ℃.

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 mM 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. DNA samples were prepared by mixing, 6X loading dye up to a final concentration of 1X. 1µl loading dye (Table 3) and 5µl isolated genomic DNA were loaded on 0.8% agarose gel stained with ethidium bromide and subjected to electrophoresis in 1x TAE buffer (Table 3) 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 acetic acid Na_2EDTA 0.5Mm H_2O	21.6 gm 11 gm 8 ml 100 ml
Gel loading buffer	Bromo phenol blue 0.25% Xylene cyanole 0.25 % Sucrose 40%(w/v) H_2O	250 gm 250 gm 40 gm 100 ml
Ethidium bromide	Ethidium bromide H_2O	1gm 100 ml

4.4 PCR AMPLIFICATION

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

The ISSR amplification 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 (Table 6).

Table 4: 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
ISSR3	AGAGAGAGAGAGAGAGG	52.2
ISSR4	GAGAGAGAGAGAGAGAT	47.05
ISSR5	GAGAGAGAGAGAGAGAC	52.2

RAPD amplification was performed using 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 was held at 12℃ (Table 7).

Table 5: List of RAPD primers used in the study

Primer name	Primer sequence (5 ' -3 ')	G C content (%)
OPA 2	TGCCGAGCTG	70
OPA 5	AGGGGTCTTG	60
OPB 1	GTTTCGCTCC	60
OPB 4	GGACTGGAGT	60
OPG 3	GAGCCCTCCA	80

Table 6: Conditions for 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 data 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 the Numerical Taxonomy and Multivariate Analysis System (NTSYSpc) (version 2.02) software package.

The coefficient selected in NTSYS is the 'J' coefficient, and UPGMA (Unweighted Pair Group Method with Arithmetic Mean) was used for generating a dendrogram for data interpretation and diversity patterns.

4.6 PIC (POLYMORPHISM INFORMATION CONTENT)

In dominant markers, a binary matrix can be elaborated for the presence and absence of bands as a result of the polymorphism revealed by the marker. The frequency of bands present is considered as *p,* and the frequency of bands absent is *q.* The general equation to estimate the Polymorphism Information Content (PIC) is,

$$PIC = 1- (p^2+q^2)$$ (Nunes dos Santos, K., *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 informativeness of a genetic marker for linkage studies. 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 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.

4.7 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.

$E=n\beta$

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

4.8 MARKER INDEX

For determining the overall utility of a given marker system, the marker index MI was calculated for all ten markers. The high MI is the reflection of the efficiency of the marker to simultaneously analyse the larger number of bands rather than the level of polymorphism detected. MI is the product of PIC and EMR (Varshney *et.al* .2005).

MI=PIC *EMR

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

V. RESULT

In this study 10 samples of *Limnophila aquatica* was subjected to amplification using RAPD and ISSR markers in PCR thermal cycler. The banding patterns 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 by using RAPD and ISSR markers. In the present study, 5 RAPD primers and 5 ISSR primers were screened for polymorphism survey in pooled DNA accessions of *Limnophila aquatica.*

5.1 ISSR BAND ANALYSIS

The PCR analysis of these 10 samples taken in the study, with 5 Inter Simple Sequence Repeats markers generated 55 scorable bands. An average of 11 bands per primer was generated. Among the 5 ISSR markers, ISSR 2 produced the maximum number of bands (12 bands in all the samples) followed by ISSR 1, ISSR 3, and ISSR 4 producing 11 bands in all the samples (Plate 3). ISSR 5, both produced a minimum number of bands (10 bands) in all the genomic pools.

Out of the 55 bands, all bands were found to be polymorphic. They showed 92.36 % polymorphism and the average number of the polymorphic band per primer was 10. The primers ISSR 1, ISSR 2, ISSR 3, ISSR 4, and ISSR 5 produced 92.36 % polymorphism.

PIC values obtained for the primers ranged from 0.45 for ISSR 1 and ISSR 4 to 0.47 for ISSR 2 and ISSR 5. Intermediate PIC values are shown in table 7. The PIC value of ISSR 2 and ISSR 5 is found to be more convenient for studies as the value 0.47 is closest to 0.5.

The effective multiplexing ratio takes into account all possible attributes such as information content, percentage of polymorphic fragments, multiplexing ratio, and qualitative aspects of a particular marker system (Mitra *et al*., 2011). According to the calculations, the EMR is the highest for the ISSR 2 marker and the lowest for ISSR 5 marker (Table 7 and 8). The Marker Index is highest for ISSR 2, with a value of 56.4 and it is lowest for ISSR 5, with a value of 47.

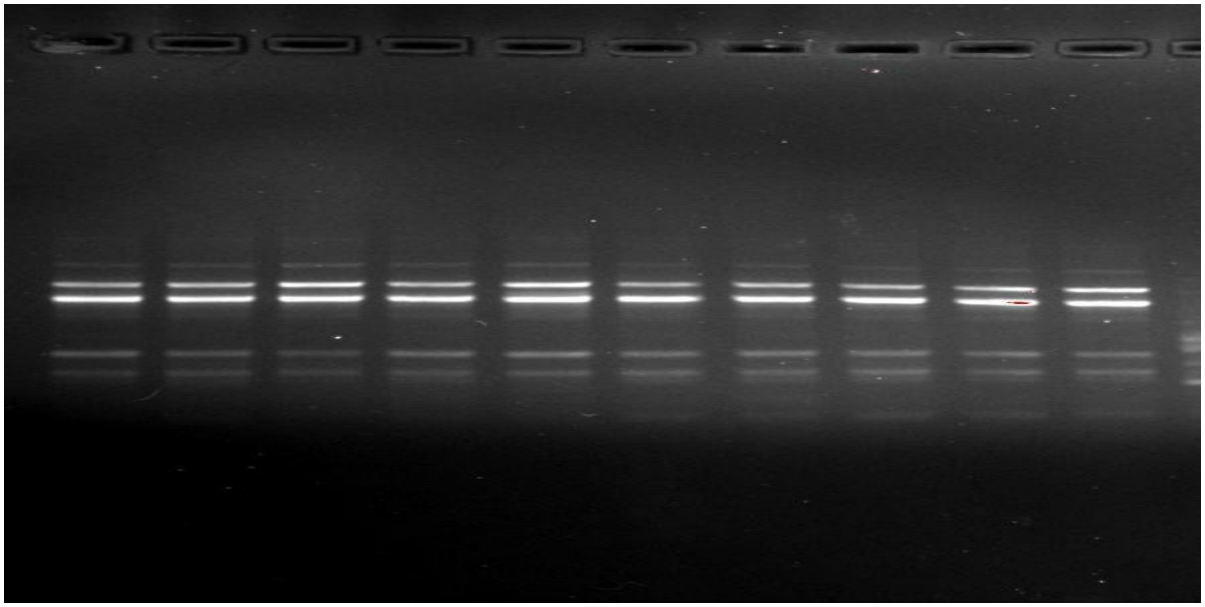

Plate 3: ISSR fingerprint obtained with ISSR primer in different accessions of *Limnophila aquatica*

Table 7: Data for ISSR primers used for analyzing 10 accessions of *Lymnophila aquatica*

ISSR Primer	Number of bands	No. of Polymorphic bands	Percentage of Polymorphism	PIC	EMR	MI
ISSR 1	11	10	90 %	0.45	110	49.5
ISSR 2	12	12	100 %	0.47	120	56.4
ISSR 3	11	9	81.81 %	0.46	110	50
ISSR 4	11	11	100 %	0.45	110	49.5
ISSR 5	10	9	90 %	0.47	100	47
Total	55	51				
Average	11	10	92.36 %			

Table 8: List showing ISSR markers details obtained from 10 *Limnophila aquatica* accessions

Primer	**ISSR**
Number of Primers used	5
Total number of polymorphic bands	55
Total number of monomorphic bands	0
Total number of bands	55
Percentage polymorphism	92.36 %
Average number of bands	11
Average number of polymorphic band	11

5.2 GENETIC IDENTITY AND GENETIC DISTANCE USING ISSR

Numerical Taxonomy and Multivariate Analysis System (NTSYS) was used to estimate phylogenetic tree using the UPGMA methods for constructing dendrograms. The genetic distance and genetic identity between 10 samples were able to relate. Genetic identity is shown above the diagonal of the table and genetic distance is shown below the diagonal of it.

According to the table (Table 9), least genetic diverse is shown by population LA1 and LA5 with a minimum identity value of 0.3953. Genetic identity value exhibited between population LA 4 and LA 9 is 0.7917. This shows that these two population are highly diverse.

Table 9: NTSYSpc ISSR Genetic distance

	LA1	LA2	LA3	LA4	LA5	LA6	LA7	LA8	LA9	LA10
LA1	1.0000									
LA2	0.2609	1.0000								
LA3	0.2609	0.3171	1.0000							
LA4	0.4286	0.3333	0.2444	1.0000						
LA5	**0.6047**	0.3830	0.3000	0.4255	1.0000					
LA6	0.5227	0.3404	0.3125	0.4773	0.5417	1.0000				
LA7	0.3404	0.4048	0.3721	0.2979	0.4894	0.3878	1.0000			
LA8	0.3333	0.3333	0.5385	0.3778	0.3396	0.4681	0.3542	1.0000		
LA9	0.3636	0.3659	0.3333	**0.2083**	0.4565	0.3265	0.5641	0.2653	1.0000	
LA10	0.3333	0.3333	0.3659	0.4146	0.3673	0.4130	0.3864	0.3478	0.2889	1.0000

5.3 CLUSTER ANALYSIS OF ISSR

Cluster analysis is a data analysis technique that examines naturally occurring groupings within a data set, called clusters. Cluster analysis does not require grouping data points into predefined groups. So this is an unsupervised learning method. A good clustering algorithm will result in high within-cluster similarity and low inter-cluster similarity. The purpose of cluster analysis is to assign observations to groups such that the observations within each group are similar. The groups themselves are separate from each other in terms of the variable or attribute of interest. Cluster analysis is also used to group variables into homogeneous and distinct groups.

A dendrogram is a diagram that shows the hierarchical relationships between objects. Most commonly produced as the output of hierarchical clustering. The main use of dendrograms is to find the best way to assign objects to clusters. A dendrogram is made up of stacked branches (called clades) that are further broken down into smaller branches. At the lowest level are individual elements, grouped into clusters by attributes, with fewer clusters at higher levels. At the end of each group (called a leaf) is the data. The method of unweighted average binding among clusters, better known as UPGMA, is most commonly used in ecology and phylogeny, and numerical taxonomy. As a clustering technique, UPGMA avoids characterizing dissimilarity by extreme values (minimum and maximum) between considered genotypes and uses the (unweighted) arithmetic mean of dissimilarity measures.

A dendrogram generated from UPGMA (Unweighted Paired Group Method using Arithmetic Averages) cluster analysis of ISSR primer is shown in figure 2. The coefficient value of dendrogram ranges from 0.33 to 0.60. The dendrogram consist of two major clusters, Cluster 1 and Cluster 2. Cluster 1 is composed of 8 populations, LA1, LA5 LA6, LA4, LA10, LA2, LA7 and LA 9. Cluster 1 is divided into 2 sub-clusters.

In sub-cluster 1 there are 5 populations, LA1, LA5, LA6, LA4, and LA 10. In this, LA1 and LA 5 are closely related with a similarity value 0.6047. Population LA 6 shares a common ancestor even though it is genetically diverse from populations of sub-cluster 1. The

major cluster 2 divides into LA3 and LA 8. They are closely related with a similarity index 0.5385. The populations LA 4 and La 9 are highly diverse with a similarity index 0.208

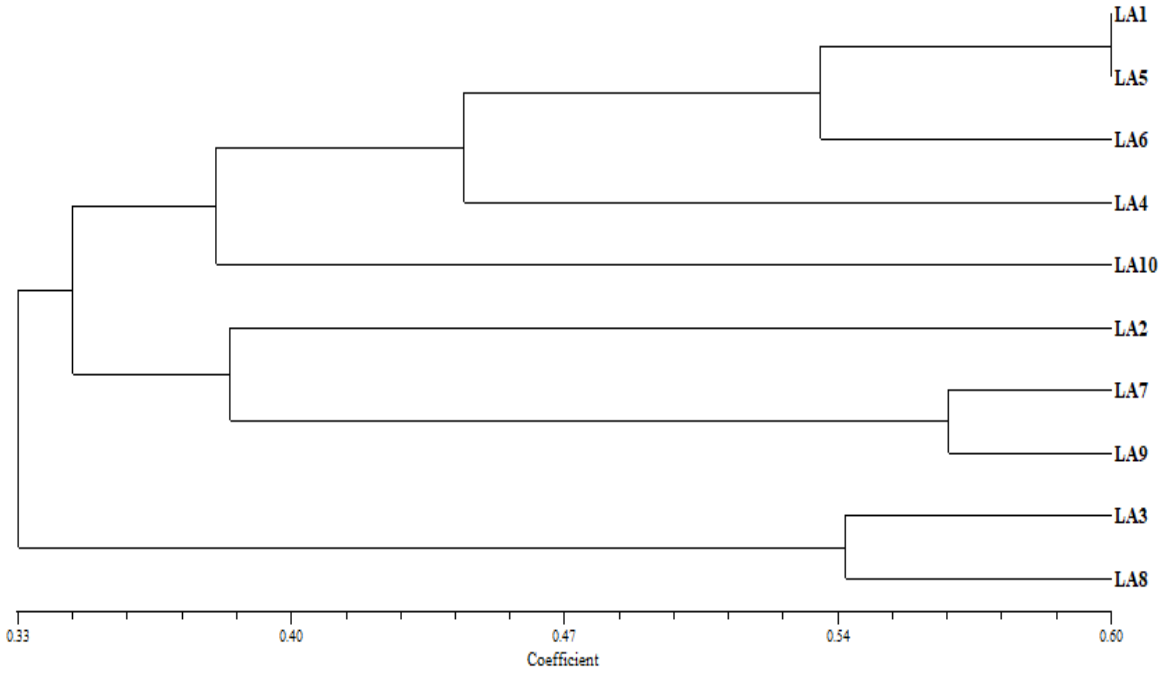

Figure 2: Dendrogram based on ISSR analysis for the estimation of genetic diversity in different accessions of *Limnophila aquatica* collected from different locations

5.4 RAPD BAND ANALYSIS

The PCR analysis of these 10 samples taken in the study, with 5 Random Amplified Polymorphic DNA markers generated 50 scorable bands. An average of 10 bands per primer was generated. Among the 5 RAPD markers, OPA 5 produced the maximum number of bands (11 bands in all the samples) followed by OPA 2, OPB4, and OPG3 producing 10 bands in all the samples (Plate 4). OPB1 produced a minimum number of bands (9 bands) in all the genomic pools.

Out of the 50 bands, 42 bands were found polymorphic. They show 83.94% polymorphism and the average number of the polymorphic band per primer was 8 (Table 11 and 12). All the primers produced the same levels of polymorphism among the different genotypes. The primers exhibited 83.94% polymorphism.

PIC values obtained for the primers ranged from 0.44 to 0.45. The PIC value for OPB 4 and OPB 1 is 0.44 and that of OPA 2, OPG 3, and OPA 5 is 0.45. The PIC value 0.45 is found to be more convenient for studies as the value is closest to 0.5.

The effective multiplexing ratio takes into account all possible attributes such as information content, percentage of polymorphic fragments, multiplexing ratio, and qualitative aspects of a particular marker system (Mitra *et al*., 2011). According to the calculations, the EMR is the highest for the OPA 5 marker and the lowest for OPB 1 marker. The Marker Index is highest for OPA 5, with a value of 44.55 and it is lowest for OPB 1, with a value of 35.64.

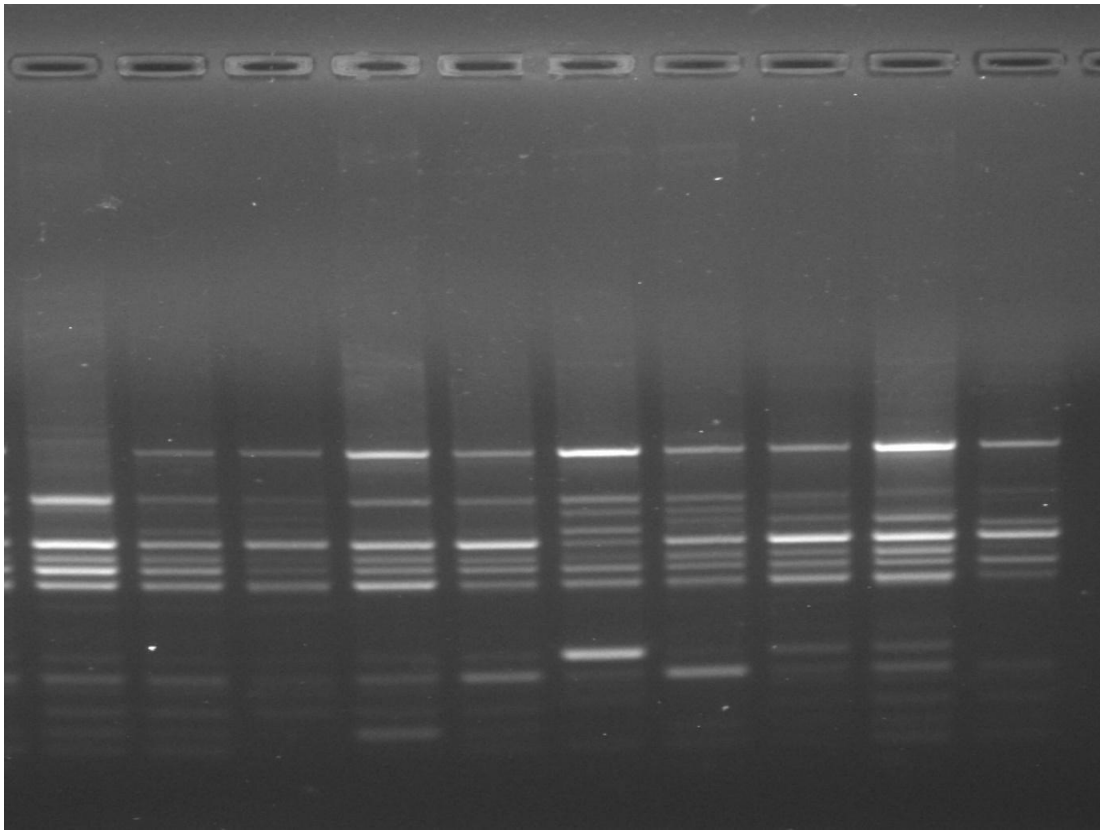

Plate 4: RAPD fingerprint obtained with RAPD primer in different accessions of *Limnophila aquatica*

Table 10: Data for RAPD primers used for analyzing 10 accessions of *Lymnophila aquatica*

RAPD Primer	Number of bands	No. of Monomorphic bands	No. of Polymorphic bands	Percentage of Polymorphism	PIC	EMR	MI
OPA 2	10	2	8	80%	0.45	90	40.5
OPB 4	10	1	9	90%	0.44	90	39.6
OPG 3	10	3	7	70%	0.45	90	40.5
OPB 1	9	1	8	88.8%	0.44	81	35.6
OPA 5	11	1	10	90.9%	0.45	99	44.5
Total	50	8	42				
Average	10		8	83.94%			

Table 11: List showing RAPD markers details obtained from 10 *Limnophila aquatica* accessions

PRIMERS	RAPD
Number of primers used	5
Total number of polymorphic bands	42
Total number of monomorphic bands	8
Total number of bands	50
Percentage polymorphism	83.94%
Average number of bands	10
Average number of polymorphic bands	9

5.5 GENETIC IDENTITY AND GENETIC DISTANCE USING RAPD

Numerical Taxonomy and Multivariate Analysis System (NTSYS) were used to estimate phylogenetic tree using the UPGMA methods for constructing dendrograms. The genetic distance and genetic identity between 10 samples were able to relate. Genetic identity is shown above the diagonal of the table and genetic distance is shown below the diagonal of it.

According to the table, genetic diversity ranges from 0 to 1.The population LA 5 shows close genetic relation with LA6 with an identity value of 0.4545.The similarity index of LA1 and LA8 is only 0.2439. Therefore they shows maximum genetic diversity (Table 12).

Table 12: NTSYSpc RAPD Genetic distance

	LA1	LA2	LA3	LA4	LA5	LA6	LA7	LA8	LA9	LA10
LA1	1.0000									
LA2	0.2632	1.0000								
LA3	0.2683	0.3500	1.0000							
LA4	0.2632	0.3158	0.3846	1.0000						
LA5	0.5128	0.3864	0.5116	0.4524	1.0000					
LA6	0.4474	0.3256	0.4524	0.4615	**0.5455**	1.0000				
LA7	0.3415	0.4250	0.4524	0.3902	0.4468	0.4222	1.0000			
LA8	**0.2439**	0.3250	0.4250	0.3250	0.4545	0.4634	0.3953	1.0000		
LA9	0.2632	0.2500	0.3846	0.4286	0.3864	0.3902	0.3571	0.3250	1.0000	
LA10	0.3250	0.4474	0.4048	0.4865	0.5000	0.4419	0.4091	0.3810	0.3415	1.0000

5.6 CLUSTER ANALYSIS FOR RAPD

Cluster analysis is a data analysis technique that examines naturally occurring groupings within a data set, called clusters. Cluster analysis does not require grouping data points into predefined groups. So this is an unsupervised learning method. A good clustering algorithm will result in high within-cluster similarity and low inter-cluster similarity. The purpose of cluster analysis is to assign observations to groups such that the observations within each group are similar. The groups themselves are separate from each other in terms of the variable or attribute of interest. Cluster analysis is also used to group variables into homogeneous and distinct groups.

A dendrogram is a diagram that shows the hierarchical relationships between objects. Most commonly produced as the output of hierarchical clustering. The main use of dendrograms is to find the best way to assign objects to clusters. A dendrogram is made up of stacked branches (called clades) that are further broken down into smaller branches. At the lowest level are individual elements, grouped into clusters by attributes, with fewer clusters at higher levels. At the end of each group (called a leaf) is the data. The method of unweighted average binding among clusters, better known as UPGMA, is most commonly used in ecology and phylogeny, and numerical taxonomy. As a clustering technique, UPGMA avoids characterizing dissimilarity by extreme values (minimum and maximum) between considered genotypes and uses the (unweighted) arithmetic mean of dissimilarity measures.

A dendrogram generated from UPGMA (Unweighted Paired Group Method using Arithmetic Averages) cluster analysis of RAPD primer is shown in the figure 3. The coefficient value of dendrogram ranges from 0.33 to 0.55. This dendrogram clearly separates the whole genotype into a major clusters. Populations LA 1 and LA 2 are separated from the cluster as they are highly diverse. The cluster is divided into two sub-clusters, sub-cluster 1 and subcluster 2. Sub cluster 1 is composed of 7 populations which include LA 3, LA 5, LA6, LA8, LA 7, LA 4 and LA 10. LA 5 and LA 6 are highly similar with a similarity value 0.5455. LA 4 and La 10 has a similarity value of 0.4865, therefore they are less genetically diverse. Sub-cluster 2 consists of population LA 9 which share a common ancestor even though it is genetically diverse from populations of sub-cluster 1.

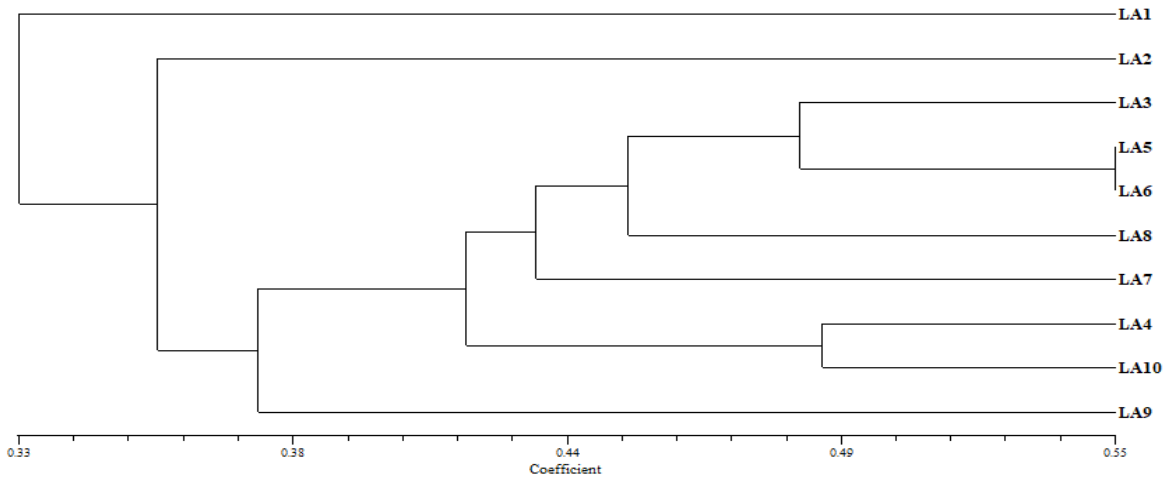

Figure 3: Dendrogram based on RAPD analysis for the estimation of genetic diversity in different accessions of *Limnophila aquatica* collected from different locations.

VI. DISCUSSION

The genus *Limnophila* belonging to the family Plantaginaceae are aquatic dicotyledonous plants. *Limnophila aquatica* commonly called “Giant Ambulia” is a semi-aquatic annual with the stout stem. Khan and Halim (1987) in their study reported four species of *Limnophila*, viz. *L. cana* Griff, *L. heterophylla* (Roxb.) Benth, *L. indica* (L.) Druce, and *L. sessiliflora* Blume., from regions of Bangladesh. *L. australis*, is an Australian endemic related to *L. heterophylla* and *L. aquatica*.

Limnophila aquatica (Roxb.) Alston is an aquatic dicotyledonous plant belonging to the family Plantaginaceae. *L.aquatica* is a semi-aquatic annual with a stout stem. Rooting occurs from the lower nodes of stems. The basal part of the stem is usually submerged and it is thick, and tumid at the nodes. Leaves are usually fine, pine-like, and bushy. Upper leaves are crenulate, opposite or tristichous, ovate-lanceolate to oblong-lanceolate, rounded and semiamplexicaul at the base, and acuminate at the apex. Both surfaces of the upper leaves are glabrous. Lower leaves are whorled, pinnately dissected, frequently deflexed, and root-like. The inflorescence is terminal racemes and peduncles finely glandular-hispid. Bracts ovate to broadly lanceolate, and the apex is sub-acuminate. Bracteoles are linear-lanceolate. Flowers are numerous. Calyx segments are deltoid-ovate, acute, tubes 2-3 mm long, lobes more or less equal, ovate-lanceolate, acuminate, and scarious at margins. Corolla tubes are pale greenish, white, 8-11 mm long, finely pubescent, limb whitish pale blue. Lobes of upper lip broadly orbicular with a pale purple blotch at the center. Lower lips are 8-16 mm across and middle lobes are broader than the lateral ones. The stamens are 4in number with posterior filaments 1.5-2.5 mm long, and anterior ones longer. Capsules are globose which are enclosed by longer calyx lobes. *Limnophila aquatica* grows well in very high to medium sunlight preferably in mud soil and water prominent areas (Alfasane *et al.* 2021)

Limnophila plants are extensively used in the indigenous system of medicine. Goutam Brahmachari, (2008) has cited the medicinal uses of these plants on the basis of an extensive literature survey. Prajapati, N.D *et al.* (2003) has reported that juice of the plant *L. aromatica* is used as a cooling medicine in fever and pharyngitis. It is given to nursing women when breast milk is sour. The plant emits a turpentine-like odor and yields an essential oil.

Ambasta, S. P (1986) reported *L. aquatica* has a refreshing and agreeable odor resembling camphor or the oil of lemon. *L. indica* is believed to have digestive and antiseptic properties.

Conventionally, genetic diversity study is based on phenotypic traits as it is easy, cheap, and does not require sophisticated tools and techniques. However, phenotypic measures of genetic diversity may not be trustworthy and accurate because of the influence of the environment on gene expression. Hence, further assessment of the genetic diversity of germplasm accessions is required at the molecular level (Fonseca R. M, 2008).

Population genetic studies are essential for conservation programmes and restoring the threatened population. The development in molecular genetics has laid the groundwork for genomics**.** Population ecology and plant demography are integrated research fields that provide insights into the performance of plant populations in the environment. In the context of conservation biology, it is important to understand how plant populations function. This knowledge is relevant to the potential for developing comprehensive and effective conservation plans for endangered plant species. On the other hand, because evolutionary change begins at the population level, studies of population ecology shed light on the forces that influence the survival and fertility of individual plants, and assess the fitness of all different variants of a population.

The introduction of new types of molecular markers has increased the reliability of the genetic studies of plants and their improvement. These markers provide more information that is highly accurate and help in the better understanding of plant genetic resources. Markers generated by PCR have been in use since the 1990s which give access to information about intraspecific genetic variation at the nuclear level. The review work of Muhammad Idrees and Muhammad Irshad in 2004 on "Molecular markers in plants for analysis of genetic diversity" provides a detailed description of the major molecular markers. A molecular marker is a sequence of DNA, which are located with a known position on the chromosome (Pearce S. R, 1999), or a gene whose phenotypic expression is frequently easily discerned and used to detect an individual, or as a probe to mark chromosomes, nucleus, or locus (Schulmann, 2007). Markers exhibit polymorphisms resulting from nucleotide changes or mutations in genomic loci, allowing genetic differences between individual organisms or species to be identified (Collard, *et al*. 2005).

Arumugan, T., *et al.* (2019) reported that, among the polymerase chain reaction (PCR) based molecular techniques, random amplified polymorphic DNA markers have convenient performance and do not require information about the DNA sequence being amplified. RAPD markers are small oligonucleotide primers, typically 10bp in length, that are randomly sequenced to generate a banding profile. These primers bind to complementary sequences along the genome, and PCR amplification occurs when the region between opposing primer sites is within amplifiable distance.

Recently, Inter-Simple Sequence Repeat (ISSR) markers have emerged as an alternative system with the reliability and advantages of microsatellites (SSR). This technique involves the amplification of genomic segments flanked by microsatellite sequences that are contiguous in reverse orientation with a single primer or pair of primers based on SSRs anchored 5' or 3' by 1- 4 purine or pyrimidine residues. ISSR technology offers a fast, reliable, and informative system for DNA fingerprinting. ISSR markers exhibit the Mendelian mode of inheritance and are segregated as dominant markers. This technique has been widely utilized in the studies of cultivar identification, genetic mapping, gene tagging, genetic diversity, evolution, and molecular ecology (Wang, J. B., 2002). 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 study, 10 populations of *Limnophila aquatica* was 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. 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. The most commonly used basic plant DNA extraction protocols are those of Murray, M. G., & Thompson, W. (1980) 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, i.e., ISSR1 ISSR2 ISSR3 ISSR4 ISSR5 and 5 RAPD primers i.e., OPA 2, OPB 4, OPG 3, OPB 1, OPA 5. In a 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 PCR analysis of these 10 samples taken in the study, with 5 Inter Simple Sequence Repeats markers generated 55 scorable bands. An average of 11 bands per primer was generated. Among the 5 ISSR markers, ISSR 2 produced the maximum number of bands (12 bands in all the samples) followed by ISSR 1, ISSR 3, and ISSR 4 producing 11 bands in all the samples. ISSR 5, both produced a minimum number of bands (10 bands) in all the genomic pools. They showed 92.36 % polymorphism and the average number of the polymorphic band per primer was 10. The primers ISSR 2 and ISSR 4 produced 100 % polymorphism. PIC values obtained for the primers ranged from 0.45 for ISSR 1 and ISSR 4 to 0.47 for ISSR 2 and ISSR 5. Intermediate PIC values are shown in table 7. The PIC value of ISSR 2 and ISSR 5 is found to be more convenient for studies as the value 0.47 is closest to 0.5.The effective multiplexing ratio takes into account all possible attributes such as information content, percentage of polymorphic fragments, multiplexing ratio, and qualitative aspects of a particular marker system(Mitra *et al*., 2011). According to the calculations, the EMR is the highest for the ISSR 2 marker and the lowest for ISSR 5 marker (Table 7). The Marker Index is highest for ISSR 2, with a value of 56.4 and it is lowest for ISSR 5, with a value of 47.

The five Random Amplified Polymorphic DNA markers used in the study generated 50 scorable bands. An average of 10 bands per primer was generated. Among the 5

RAPD markers, OPA 5 produced the maximum number of bands (11 bands in all the samples) followed by OPA 2, OPB4, and OPG3 producing 10 bands in all the samples.OPB1 produced a minimum number of bands (9 bands) in all the genomic pools. Out of the 50 bands, 42 bands were found polymorphic. They show 83.94% polymorphism and the average number of the polymorphic band per primer was 8. All the primers produced the same levels of polymorphism among the different genotypes. The primers exhibited 83.94% polymorphism. The PIC value for OPB 4 and OPB 1 is 0.44 and that of OPA 2, OPG 3, and OPA 5 is 0.45. The PIC value 0.45 is found to be more convenient for studies as the value is closest to 0.5. The EMR value is the highest for the OPA 5 marker and the lowest for OPB 1 marker. The Marker Index is highest for OPA 5, with a value of 44.55 and it is lowest for OPB 1, with a value of 35.64.

Numerical Taxonomy and Multivariate Analysis System (NTSYS) were used to estimate phylogenetic tree using the UPGMA methods for constructing dendrograms. The genetic distance and genetic identity between 10 samples were able to relate. Genetic identity is shown above the diagonal of the table and genetic distance is shown below the diagonal of it.

From the dendrogram based on ISSR analysis, population LA 4 is highly distant from population LA9 with maximum identity value of 0.7917. The least genetic diversity is shown by population LA1 and LA5 with a minimum identity value of 0.3953. From the dendrogram based on RAPD analysis, population LA 1 is highly distant from population LA8 maximum identity value of 0.7561. The least genetic diversity is shown by population LA6 and LA5 with a minimum identity value of 0.4545. Thus least genetic diversity was shown by *Limnophila aquatica* population collected from Manjeshwar, Kasargod (LA 1) and the population from Kadavathur, Kannur (LA 5) for ISSR markers. And least genetic diversity for RAPD marker was shown by *Limnophila aquatica* population collected from Kutyadi, Kozhikode (LA 6) and the population from Kadavathur, Kannur (LA 5).

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 (92.36 %) was higher than RAPD (83.94%).

VII. 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 *Limnophila aquatica* plant populations collected from various geographical regions of Kerala.

Limnophila aquatica (Roxb.) Alston is an aquatic dicotyledonous. It is an aquatic, or semi-aquatic, perennial herb. It is commonly known as 'Ambulia' (Asian marsh weed). The genus belongs to the tribe Gratioleae of the family Scrophulariaceae. The basal part of the stem is usually submerged, thick and tumid at the nodes. The aerial part is erect, glabrous, or sessile glandular. Leaves on aerial stems are opposite or verticillate or linear-lanceolate to lanceolate elliptical. They are sessile, sub-amplexicaul, glabrous, densely but minutely and obscurely punctate. The submerged leaves are multifid, pinnately dissected, and glabrous. The inflorescence is a solitary- axillary or terminal or axillary spike or raceme.

We are living in an era of environmental degradation, marked by all around the loss of habitats. This is dangerous not only for human lives but also for all other forms of life on earth. 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 functions better when they contain more species, then the loss of species could diminish the value of ecosystems. It is crucial to know the existing variability among *Limnophila* plant species in different habits for the effective utilization and conservation of these plants.

The present work deal with the isolation of genomic DNA from *Limnophila aquatica* obtained from 10 population 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 *Limnophila aquatica* of different geographical location was carried out in the study.

From the study least genetic diversity was shown by *Limnophila aquatica* population collected from Manjeshwar, Kasargod (LA 1) and the population from Kadavathur, Kannur (LA 5) for ISSR markers. And least genetic diversity for RAPD marker was shown by *Limnophila aquatica* population collected from Kutyadi, Kozhikode (LA 6) and the population from Kadavathur, Kannur (LA 5). 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 (92.36 %) was higher than RAPD (83.94%). Both RAPD and ISSR might detect noncoding and therefore more polymorphic DNA by exploiting the different regions of genome. The difference in the dendrograms generated by RAPD and ISSR could be explained by the different number of PCR products analysed or it may be attributed to marker sampling error.

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 *Limnophila aquatica.*

VIII. SCOPE OF THE STUDY

Understanding the molecular basis of plants is crucial for the efficient conservation, management and use of plant genetic resources (PGR). In particular, adequate knowledge of the genetic diversity available, where it is found in plant populations and how to best use it, is of fundamental interest to basic science and application aspect.

Genetic diversity is the product of the recombination of genetic material during heredity. It changes over time and space. Sexual reproduction is important in maintaining genetic diversity because it produces unique offspring by combining genes from parents. Genetic diversity gives rise to different physical attributes of individuals and their ability to adapt to stress, disease, and adverse environmental conditions. Genetic diversity is important for a healthy population by maintaining different types of genes that can resist pests, diseases or other conditions.

The macrophytes play a crucial role in healthy aquatic ecosystems. The macroscopic flora plays a vital role in maintaining the ecological balance of an aquatic ecosystem. Limnophila is a genus of aquatic flowering plants in the family Scrophulariaceae. Limnophila plants are widely distributed throughout the world, finding immense application in the traditional medical system in many countries. Several studies have already been conducted on the chemical and pharmacological aspects of these plants. *Limnophila aquatica* (Roxb.) Alston is a largely unexplored plant species.

The *Limnophila* plant is widely used in indigenous systems of medicine. Medicinal plants are a valuable source of medicinal herbs. A good assessment of medicinal plant genetic resources is useful information for the development of conservation plans to protect genetic diversity. Regardless of resource management and conservation strategies, the sustainable use of medicinal plant resources should be fully considered. The application of biotechnological methods can help improve yield and change the effectiveness of medicinal plants. A large number of biological or pharmacological works with different parts of *Limnophila* plant as crude extracts and also purified chemical components are isolated from these plants have been reported.

Limnophila aquatica is one of the least explored plant species of the genus with very few available literature. Genetic diversity study of different populations of *L. aquatica* using RAPD and ISSR molecular markers, thus lead the way for further exploration. This study aimed at providing better understating of genomic characters of *L. aquatica* for the effective conservation, management, and utilization of it in various areas. The taxonomical importance of the plant can be studied. Medicinal and Phytochemical relevance can be explored in further studies. The results of the study can be seen as a starting point for future researchers aimed at defining the level of intra- and inter- specific genetic diversity. The two marker system, RAPD and ISSR used in the study are effective tools to evaluate genetic diversity and they also throw light on the phylogenetic relationships in different plants. Thus this study helps to reveals the unlimited possibilities of this plant in the field of research.

IX. REFERENCES

- Ahmed ZU, Hassan MA, Begum ZNT, Khondker M, Kabir SMH, Ahmad M & Ahmed ATA (2009) Encyclopaedia of flora and fauna of Bangladesh. Angiosperms: Dicotyledons (Ranunculaceae – Zygophyllaceae). Asiatic Society of Bangladesh, Dhaka.

- Alfasane, M. A., Jolly, J. A., & Hassan, M. A. (2021). Occurrence of *Limnophila Aquatica* (Roxb.) Alston in Bangladesh. *Bangladesh Journal of Plant Taxonomy*, *28*(2), 455-457.

- Ali, Y., Atta, B. M., Akhter, J., Monneveux, P., & Lateef, Z. (2008). Genetic variability, association and diversity studies in wheat (Triticum aestivum L.) germplasm. *Pak. J. Bot*, *40*(5), 2087-2097.

- Al-Samarai, F. R., & Al-Kazaz, A. A. (2015). Molecular markers: An introduction and applications. *European journal of molecular biotechnology*, *9*(3), 118-130.

- Ambasta, S. P. (1986). The Useful Plants of India, (Editor-in –chief), Publications & Information Directorate, CSIR: New Delhi-110012.

- Ansari AA, Saggu S, Al-Ghanim SM, Abbas ZK, Gill SS, Khan FA, Dar MI, Naikoo MI, Khan AA. (2017). Aquatic plant biodiversity: A biological indicator for the monitoring and assessment of water quality. In: Ansari AA, Gill SS, Abbas ZK, Naeem M (eds) Plant Biodiversity: Monitoring, Assessment, and Conservation. CAB International, Wallingford.

- Arumugam, T., Jayapriya, G., & Sekar, T. (2019). Molecular fingerprinting of the Indian Medicinal Plant Strychnos minor dennst. *Biotechnology Reports*, *21*.

- Bahmani, K., Izadi-Darbandi, A., Jafari, A. A., Noori, S. A. S., & Farajpour, M. (2012). Assessment of genetic diversity in Iranian fennels using ISSR markers. *Journal of Agricultural Science*, *4*(9), 79.

- Barcaccia, G., Mazzucato, A., & Falcinelli, M. (2000). Inheritance of apomictic seed production in Kentucky bluegrass (Poa pratensis L.). *Journal of New Seeds*, *2*(1), 43-58.

- Bardakci, F. (2001). Random amplified polymorphic DNA (RAPD) markers. *Turkish Journal of Biology*, *25*(2), 185-196.

- Bhagyaleena, P. and Gopalan, R. (2012). Aquatic plant diversity of ponds in Nenmara panchayath, Palakkad district, Kerala, India. International Journal of Scientific and Research Publications 2(8): 1-3.

- Booy, G., Hendriks, R. J. J., Smulders, M. J. M., Van Groenendael, J. M., & Vosman, B. (2000). Genetic diversity and the survival of populations. *Plant biology*, *2*(04), 379-395.

- Brahmachari, G. (2008). Limnophila (Scrophulariaceae): Chemical and pharmaceutical aspects. *The Open Natural Products Journal*, *1*(1).

- Bronmark, C. and Hansson, L.A. (2017). The biology of lakes and ponds Oxford University Press, UK, p 368.

- Chambers, P. A., Lacoul, P., Murphy, K. J., & Thomaz, S. M. (2007). Global diversity of aquatic macrophytes in freshwater. In *Freshwater animal diversity assessment* (pp. 9-26). Springer, Dordrecht.

- Chen, F. Q., Prehn, D., Hayes, P. M., Mulrooney, D., Corey, A., & Vivar, H. (1994). Mapping genes for resistance to barley stripe rust (Puccinia striiformis f. sp. hordei). *Theoretical and Applied Genetics*, *88*(2

- Cloutier, S., & Landry, B. S. (1994). Molecular markers applied to plant tissue culture. *In Vitro Cellular & Developmental Biology-Plant*, *30*(1), 32-39.

- Collard, B. C., Jahufer, M. Z., Brouwer, J. B., & Pang, E. C. (2005). An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for Crop Improvement: The basic concepts. *Euphytica*, *142*(1-2), 169–196.

- Crawford, K. M., & Rudgers, J. A. (2012). Plant species diversity and genetic diversity within a dominant species interactively affect plant community biomass. *Journal of Ecology*, *100*(6), 1512-1521.

- Dellaporta, S. L., Wood, J., & Hicks, J. B. (1983). A plant DNA minipreparation: version II. *Plant molecular biology reporter*, *1*(4), 19-21.

- Denny, P. (1985). Submerged and floating-leaved aquatic macrophytes (euhydrophytes). In *The ecology and management of African wetland vegetation* (pp. 19-42). Springer, Dordrecht.

- Do, Q. D., Angkawijaya, A. E., Tran-Nguyen, P. L., Huynh, L. H., Soetaredjo, F. E., Ismadji, S., & Ju, Y. H. (2014). Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of Limnophila aromatica. *Journal of food and drug analysis*, *22*(3), 296-302.

- Fonseca, R. M., Lopes, R., Barros, W. S., Lopes, M. T. G., & Ferreira, F. M. (2008). Morphologic characterization and genetic diversity of Capsicum chinense Jacq. accesions along the upper Rio Negro-Amazonas. *Embrapa Amazônia Ocidental-Artigo em periódico indexado (ALICE)*.

- Gopal B. (1995). Biodiversity in Freshwater Ecosystems Including Wetlands, Biodiversity and Conservation in India, A Status Report. Zoological Survey of India, Calcutta, India.

- Gorai, D., Jash, S. K., Singh, R. K., & Gangopadhyay, A. (2014). Chemical and pharmacological aspects of *Limnophila aromatica* (Scrophulariaceae): an overview. *AJPCT*, *2*(3), 348-356.

- Gupta, P. K., Varshney, R. K., Sharma, P. C., & Ramesh, B. (1999). Molecular markers and their applications in wheat breeding. *Plant breeding*, *118*(5), 369-390.

- Han, Y. C., Teng, C. Z., Zhong, S., Zhou, M. Q., Hu, Z. L., & Song, Y. C. (2007). Genetic variation and clonal diversity in populations of Nelumbo nucifera (Nelumbonaceae) in central China detected by ISSR markers. *Aquatic Botany*, *86*(1), 69-75.

- Heun, M., Murphy, J. P., & Phillips, T. D. (1994). A comparison of RAPD and isozyme analyses for determining the genetic relationships among Avena sterilis L. accessions. *Theoretical and Applied Genetics*, *87*(6), 689-696.

- Hughes, A. R., Inouye, B. D., Johnson, M. T., Underwood, N., & Vellend, M. (2008). Ecological consequences of genetic diversity. *Ecology letters*, *11*(6), 609-623.

- Huson, D. H., Mitra, S., Ruscheweyh, H. J., Weber, N., & Schuster, S. C. (2011). Integrative analysis of environmental sequences using MEGAN4. *Genome research*, *21*(9), 1552-1560.

- James, F. C., & McCulloch, C. E. (1990). Multivariate analysis in ecology and systematics: panacea or Pandora's box. *Annual review of Ecology and Systematics*, 129-166.

- Khan, M.S. and Halim, M. (1987). Aquatic angiosperms of Bangladesh. Bangladesh National Herbarium, BARC, Dhaka. 120 pg.

- Khodaee, L., Azizinezhad, R., Etminan, A. R., & Khosroshahi, M. (2021). Assessment of genetic diversity among Iranian Aegilops triuncialis accessions using ISSR, SCoT, and CBDP markers. *Journal of Genetic Engineering and Biotechnology*, *19*(1), 1-9.

- Klein-Lankhorst, R. M., Vermunt, A., Weide, R., Liharska, T., & Zabel, P. (1991). Isolation of molecular markers for tomato (L. esculentum) using random amplified polymorphic DNA (RAPD). *Theoretical and Applied Genetics*, *83*(1), 108-114.

- Kukongviriyapan, U., Luangaram, S., Leekhaosoong, K., Kukongviriyapan, V., & Preeprame, S. (2007). Antioxidant and vascular protective activities of Cratoxylum formosum, Syzygium gratum and Limnophila aromatica. *Biological and Pharmaceutical Bulletin*, *30*(4), 661-666.

- Kumar, A. (2015). Daya Freshwater plankton and macrophytes of India Publishing House, Delhi, p 362.

- Kumar, N. S., & Gurusubramanian, G. (2011). Random amplified polymorphic DNA (RAPD) markers and its applications. *Sci Vis*, *11*(3), 116-124.

- Kumar, P., Gupta, V. K., Misra, A. K., Modi, D. R., & Pandey, B. K. (2009). Potential of molecular markers in plant biotechnology. *Plant omics*, *2*(4), 141-162.

- Li, W., Wang, B., & Wang, J. (2006). Lack of genetic variation of an invasive clonal plant Eichhornia crassipes in China revealed by RAPD and ISSR markers. *Aquatic Botany*, *84*(2), 176-180.

- Mackill, D. J. (1995). Classifying japonica rice cultivars with RAPD markers. *Crop Science*, *35*(3), 889-894.

- Milbourne, D., Meyer, R., Bradshaw, J. E., Baird, E., Bonar, N., Provan, J., ... & Waugh, R. (1997). Comparison of PCR-based marker systems for the analysis of genetic relationships in cultivated potato. *Molecular breeding*, *3*, 127-136.

- Mitra, C., Kharb, P., Uppal, S., & Jain, S. (2011). Genetic diversity analysis in date palm (Phoenix dactylifera L.): a comparative assessment using ISSR and RAPD marker assays. *The Journal of Horticultural Science and Biotechnology*, *86*(4), 398-402.

- Mondini, L., Noorani, A., & Pagnotta, M. A. (2009). Assessing plant genetic diversity by molecular tools. *Diversity*, *1*(1), 19-35.

- Muhammad, I., Muhammad, I., Anwar, S., & Rehan, N. (2014). Genetic diversity among Asparagus species and cultivars of *Asparagus officinalis* L. using random amplified polymorphic DNA (RAPD) markers. *International Journal of Biodiversity and Conservation*, *6*(5), 392-399.

- Mullis, K. B., & Faloona, F. A. (1987). Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. In *Methods in enzymology* (Vol. 155, pp. 335-350). Academic Press.

- Murray, M. G., & Thompson, W. (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic acids research*, *8*(19), 4321-4326.

- Natarajan, C., Thiyagarajan, K., & Rathnaswamy, R. (1988). *Association and genetic diversity studies in greengram*.

- Ng, W. L., & Tan, S. G. (2015). Inter-simple sequence repeat (ISSR) markers: are we doing it right. *ASM Science Journal*, *9*(1), 30-39.

- Nunes dos Santos, K., Florentino, R. M., França, A., Lima Filho, A. C. M., Santos, M. L. D., Missiaggia, D., & Leite, M. F. (2019). Polymorphism in the promoter region of NFE2L2 gene is a genetic marker of susceptibility to cirrhosis associated with alcohol abuse. *International Journal of Molecular Sciences*, *20*(14), 3589.

- Oyedeji AA, Abowei JFN. 2012. The classification, distribution, control, and economic importance of aquatic plants. Intl J Fish Aquat Sci 1 (2): 118-128.

- Pearce, S. R., Stuart-Rogers, C., Knox, M. R., Kumar, A., Ellis, T. N., & Flavell, A. J. (1999). Rapid isolation of plant Ty1-copia group retrotransposon LTR sequences for molecular marker studies. *The Plant Journal*, *19*(6), 711-717.

- Penner, G. A. (1996). RAPD analysis of plant genome. In “Methods of genome analysis in plants”. (P. P. Jaguar, Ed.0, pp. 251-268, CRC Press, New York.

- Philcox, D. (1970). A taxonomic revision of the genus Limnophila R. Br. (Scrophulariaceae). *Kew Bulletin*, *24*(1), 101-iii.

- Pourmohammad, A. (2013). Application of molecular markers in medicinal plant studies. *Acta Univ. Sapientiae, Agric Environ*, *5*(1), 80-90.

- Prajapati, N.D.; Purohit, S.S.; Sharma, A.K.; Kumar, T.(2003). A Handbook of Medicinal Plants; Agrobios: India,; pp. 316-317.

- Prakashkumar, R., Anoop, K. P., Ansari, R., Sivu, A. R., Pradeep, N. S., & Madhusoodanan, P. V. (2015). Analysis of genetic diversity of Lagenandra spp.(Araceae) of Kerala (South India) using ISSR Markers. *Int J Sci Res*, *4*(6), 775-777.

- Ramanatha Rao, V., & Hodgkin, T. (2002). Genetic diversity and conservation and utilization of plant genetic resources. *Plant cell, tissue and organ culture*, *68*, 1-19.

- Ravi, V., Samimalaimurugan, K., Kalpana, P., Vijayakanth, P. and Ramamoorthy, R. (2020). Wetland and aquatic Angiosperm flora of Denkanikottai, Krishnagiri, Tamil Nadu. Indian Journal of Ecology (4): 1038-1043.

- Saghai-Maroof, M. A., Soliman, K. M., Jorgensen, R. A., & Allard, R. (1984). Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. *Proceedings of the National Academy of Sciences*, *81*(24), 8014-8018.

- Sebastian, V. A., (2009). Development and analysis of molecular markers for certain endangered species of Tylophora.

- Schulman, A. H. (2006). Molecular markers to assess genetic diversity. *Euphytica*, *158*(3), 313–321.

- Suksamrarn, A.; Poomsing, P.; Aroonrerk, N.; Punjanon, T.; Suksamrarn, S.; Kongkun, S. (2003). Arch. Pharm. Res., 26, 816-82

- Tanya, C., & Kumar, R. (2010). Molecular markers and their applications in fisheries and aquaculture. *Advances in Bioscience and Biotechnology*, *2010*.

- Thongdon, J., & Inprakhon, P. (2009). Composition and biological activities of essential oils from *Limnophila geoffrayi* Bonati. *World Journal of Microbiology and Biotechnology*, *25*(8), 1313-1320.

- Varshney, R. K., Graner, A., & Sorrells, M. E. (2005). Genic microsatellite markers in plants: features and applications. *TRENDS in Biotechnology*, *23*(1), 48-55.

- Wang, J. B. (2002). ISSR markers and their applications in plant genetics. *Yi Chuan Hereditas*, *24*(5), 613-616.

- Waycott, M. (1995). Assessment of genetic variation and clonality in the seagrass Posidonia australis using RAPD and allozyme analysis. *Marine ecology progress series. Oldendorf*, *116*(1), 289-295.

- Wilkie, S. E., Isaac, P. G., & Slater, R. J. (1993). Random amplified polymorphic DNA (RAPD) markers for genetic analysis in Allium. *Theoretical and Applied Genetics*, *86*(4), 497-504.

- Williams, J. G., Kubelik, A. R., Livak, K. J., Rafalski, J. A., & Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic acids research*, *18*(22), 6531-6535.

● Xu, C. Y., Zhang, W. J., Fu, C. Z., & Lu, B. R. (2003). Genetic diversity of alligator weed in China by RAPD analysis. *Biodiversity & Conservation*, *12*(4), 637-645.