MOLECULAR TAXONOMICAL STUDIES ON SOUTH INDIAN SPECIES OF *SELAGINELLA*

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DECLARATION

I hereby declare that the thesis entitled **"MOLECULAR TAXONOMICAL STUDIES ON SOUTH INDIAN SPECIES OF *SELAGINELLA*"** submitted by me for the Degree of Doctor of Philosophy in Biotechnology is the result of my orginal and independent research work carried out under the guidance of **Dr. M. Johnson**, Assistant Professor, Department of Botany, St. Xavier's College (Autonomous), Palayamkottai and it has not been submitted for the award of any degree, diploma, associateship, fellowship of any University or Institution.

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M. NARAYANI

CONTENTS

ABBREVIATIONS

$^{\circ}C$	:	Degree Celsius
$(NH_4)_2SO_4$	:	Ammonium Sulphate
µL	:	Micro Litre
2-DE	:	2-Dimensional Electrophoresis
A	:	Adenine
AAT	:	Aspartate Amino Transferase
ABA	:	Abscissic acid
ACN	:	Acetonitrile
ACP	:	Acid Phosphatase
AFLP	:	Amplified Fragment Length Polymorphism
ALP	:	Alkaline Phosphatase
APS	:	Ammonium Per Sulphate
BLAST	:	Basic Local Alignment Search Tool
bp	:	basepairs
C	:	Cytosine
CBOL	:	Consortium for the Barcode Of Life
CLUSTAL W	:	CLUSTAL with a command line interface
cm	:	Centimetre
CO1	:	Cytochrome c oxidase
Cp DNA	:	Chloroplast DNA
CTAB	:	Cetyl Trimethyl Ammonium Bromide
DNA	:	Deoxy ribo Nucleic Acid
dNTP	:	Deoxy Nucleotide Tri Phosphate
EDTA	:	Ethylene Diamine Tetra Acetic acid
EST	:	Esterase

Fig	:	Figure
G	:	Guanine
GenBank	:	Comprehensive databank
gm	:	gram
HK	:	Hexo Kinase
IBOL	:	International Barcode Of Life
Ii	:	Identical pairs
ISSR	:	Inter-Simple Sequence Repeats
ITS	:	Internally Transcribed Spacer
KCl	:	Potassium Chloride
kDa	:	Kilo Daltons
L	:	Lane
LAP	:	Leucine Amino Peptidase
M	:	Molar
m/z	:	Mass spectral
MALDI-TOF MS	:	Matrix Associated Laser Desorption Ionization-Time Of Flight Mass Spectrometry
matK	:	Maturase K
MDH	:	Malate Dehydrogenase
ME tree	:	Minimum Evolutionary tree
MEGA (5.2)	:	Molecular Evolutionary Genetic Analysis (version 5.2 software)
$MgSO_4.7H_2O$	:	Magnesium Sulphate
Min	:	Minutes
MIP's	:	Major Intrinsic Proteins
mL	:	milli Litre
mM	:	milli Molar

Mol. Wt	:	Molecular weight
MULTALIN	:	Multiple Sequence Alignment
MW-Rf	:	Molecular Weight-Retardation Factor
N	:	Normality
NaOH	:	Sodium hydroxide
NCBI	:	National Centre for Biotechnology Information
NIP	:	NOD26- like intrinsic proteins
NJ tree	:	Neighbor Joining tree
NTSYSpc-2.0	:	Numerical Taxonomy System, Version 2.2 for Windows XP
PAUP	:	Phylogenetic Analysis Using Parsimony
PCR	:	Polymerase Chain Reaction
PGD	:	Phospho Gluconate Dehydrogenase
PGI	:	Phospho Gluco Isomerase
PGM	:	Phospho Gluco Mutase
pH	:	Power of Hydrogen Ion Concentration
Pi	:	Inorganic phosphate
PIP	:	Plasma membrane Intrinsic Proteins
PO	:	Peroxidase
PPO	:	Polyphenol oxidase
PVP	:	Polyvenyl Pyrrolidone
RAPD	:	Random Amplified Polymorphic DNA
rbcL	:	Ribulose,1-5,bisphosphate large subunit
rDNA	:	Ribosomal DNA
Rf	:	Retardation Factor
RFLP	:	Restriction Fragment Length Polymorphism
RNA	:	Ribo Nucleic acid

rpm	:	revolutions per minute
RT	:	Retention Time
SDH	:	Shikimate Dehydrogenase
SDS-PAGE	:	Sodium Do-decyl Sulphate Polyacrylamide Gel Electrophoresis
Sec	:	Seconds
si	:	Transitional pairs
SKD	:	Shikimate Dehydrogenase
SNP's	:	Single Nucleotide Polymorphisms
SlTPS1	:	Trehalose-6-Phosphate Synthase
SPR	:	Subtree-Prunting-Regrafting
SSR	:	Single Sequence Repeats
sv	:	Transversional pairs
T	:	Thymine
Taq DNA	:	*Thermus aquaticus* DNA polymerase
TEMED	:	N,N,N',N'-Tetramethyl ethylenediamine
TFA	:	Tri Fluoro Acetic acid
TPI	:	Triose Phosphate Isomerase
Tris HCl	:	Tris (Hydroxymethyl) aminomethane Hydrochloride
U	:	Uracil
UPGMA	:	Unweighed Pair Group Mean Average
V	:	Volts
v/v	:	volume per volume
w/v	:	weight per volume
β ME	:	β - Mercapto Ethanol

Introduction

''*All the organic beings which have ever lived on this earth have descended from someone primordial form*''

Charles Darwin

Each and every characteristic feature of a species on earth is an outcome of evolutionary developments (Futuyma, 2005). Studies from the morphology to molecular level in different genera of plants have shed light on the evolution of plants. This has led us to gain an improved insight into evolution and the processes that drive evolution. The phylogenetic relationships between different taxa have also been clarified due to these studies (Pryer *et al*., 2004). The geneticist Theodosius Dobzhansky (1973) famously argued that ''*Nothing in biology makes sense, except in the light of evolution*''. Evolution governs diversity on earth, and plants are the most diverse organisms in the whole history of life. Biological diversity refers to the variability of living organisms including their genetic variability of which they are a part; it is indeed the biological capital and the basis for human survival and civilization. The relevance of biodiversity lies encoded in the genes and the genetic diversity is the basis of all bio-industrial developments (Mannion, 1995). The study and evolution of biodiversity therefore, forms an important part of the ecosystem analysis, ecological economics and developmental planning (Costanza, 1997).

India is one among eighteen megadiverse countries with significant biodiversity. The major part of India lies within the Indo-Malaya and Palearctic ecozone. With 2.5 percent of the world's land area, India accounts for 7.8% of the recorded species of the world including 45,500 species of plants and more than 1200 species of vascular plants (MoEF, 2009; Chandra *et al*., 2008). India hosts three biodiversity hotspots viz., the Himalayas, Western Ghats and the Indo-Burma region. Among the three, the Western Ghats to a large extent

presides over the ecology of peninsular India. This rugged range of hills lie between 8°-20º40'N and 73°77'E covering a distance of about 1600 Km from Tapti valley in Gujarat to Kanyakumari in Tamil Nadu is situated in the equatorial belt. The South Western Ghats region falls within the states of Kerala and Tamil Nadu (Radhakrishna, 2001). The Western Ghats harbor about 4,000 species of flowering plants and 320 species of ferns and fern allies with more species diversity in the southern part (Sumesh *et al*., 2012).

Pteridophytes are non-flowering, vascular and spore-bearing plants which possess a simple organization and are characterized by independent gametophytic and sporophytic generations (Dixit, 1984). During the Jurassic period, pteridophytes formed the dominant part of earth's vegetation, but in present day flora, they have been largely replaced by the seed bearing plants. The pteridophytes form a connecting link between the lower group of plants (bryophytes) and the higher group of seed bearing plants (Dixit, 2000). Pteridophytes show promise for their economic values and many of them had been used in various ways such as folk remedies and medicine, plant protection, food and fodder, bio-fertilizer, environmental monitoring, insect repellents and ornamentals (Kaur, 1989).

Genetic diversity in germplasm collections is fundamental to broadening the genetic base of plant species. The data on agronomic, morphological and physiological plant traits are generally used to estimate the magnitude of genetic diversity present in germplasm. However, such data may not provide an accurate indication of genetic diversity because of environmental influences upon the expression of observed traits (Shahid *et al*., 2000). Considering these difficulties, several molecular and biochemical analysis has been applied to establish germplasm differences at various taxonomic levels (Rabbani *et al*., 2010; Mumtaz *et al*., 2010).

Molecular marker technology provides overall estimates of the genetic diversity of a species and act as an important biological tool for the identification of raw materials of

medicinal plants (Sozen *et al*., 2010). Molecular genetic markers include biochemical markers which detect variation at the gene product level (proteins and isozymes) and molecular markers which detect variation at the nucleotide (DNA) level (Pervaiz *et al*., 2010).

During the molecular era, a variety of tools have been developed for the analysis of genetic diversity (Kumar and Kumari, 2009). One of the biochemical methods extensively used in taxonomic and evaluation of genetic diversity studies is the electrophoretic analysis (SDS-PAGE) that separates proteins according to their electrophoretic mobility (Syed *et al*., 2011; Johnson *et al*., 2012; Warren *et al*., 2013; Sen *et al*., 2014). The mass spectrometric (MS) techniques were developed for the ionization of proteins and peptides which include matrix-associated laser desorption ionization (MALDI) combined with time of flight (TOF). MS offers high sensitivity and accuracy for determining the molecular weight of proteins according to the difference of m/z (mass/electric charge) ratio (Franc *et al*., 2013). MALDI-TOF MS has advantages over other spectroscopic methods because of good sensitivity and high tolerance towards contaminants (Hailat and Helleur, 2014).

Isozymes are good estimators for elucidating the possible mechanisms leading to the formation of genetic variability in plant populations (Zeidler, 2000). It is widely used in the studies of inter and intra-specific variation (Johnson and Raja, 2007; Johnson *et al*., 2010; Nanthini *et al*., 2011; Johnson *et al*., 2012; Wang and Sheng, 2013). They also show lower levels of polymorphism in a population where the expression of various isozymes differs both temporally and spatially which can correctly identify several levels of taxa, accessions and individuals since the assumption of homology can be more accurate than for same genomic DNA markers (Smila *et al*., 2007).

In recent years there has been an explosion in the number of different types of genetic markers and statistical tools are available for the analyses of molecular data. In most cases,

the new DNA based markers provide the same type of information as allozymes, but allow for clear resolution of genetic differences (de Groot *et al*., 2012; Parveen, 2013; Wynns and Asmussen, 2014). The study of relationships among major lineages of organisms, phylogenetic analysis, has been closely associated with the classification and taxonomy in which both are branches of the science of systematics (Mauro and Agorreta, 2010). In particular, the use of molecular data and DNA sequences are appropriate for testing and improving classifications, especially for highly diverse groups of flora such as pteridophytes (Li *et al*., 2011).

Molecular methods used for detecting DNA sequence variation are generally based on the use of Restriction Fragment Length Polymorphism (RFLP), Polymerase Chain Reaction (PCR), Random Amplified Polymorphic DNAs (RAPD), Amplified Fragment Length Polymorphisms (AFLPs), Inter-Simple Sequence Repeats (ISSRs), microsatellites and Single Nucleotide Polymorphisms (SNPs) which proved especially important in diversity studies. Newer and more powerful molecular techniques that detect variation at specific gene loci are highly becoming available (Singh, 2013).

Barcoding has been equated with "DNA taxonomy" (Tautz *et al*., 2003) or "Molecular taxonomy" (Blaxter, 2004). Molecular taxonomy supports the discovery of new species with genetic measures using universal barcodes (Hebert *et al*., 2003). The non-coding chloroplast (cp) DNA regions, as well as the nuclear ITS and the chloroplast protein-coding sequences, have been commonly used as the barcoding markers for more species includes plants (Wang *et al*., 2010; Ma *et al*., 2010; Mahadani *et al*., 2013). In case of pteridophytes, *rbcL* would serve as a locus for species identification because it is the most characterized plastid coding region in Genbank and provide a good baseline for comparison with other plastid genes (Korall *et al*., 2007; Pryer *et al*., 2010; Li *et al*., 2011).

Selaginella Pal. Beauv., a perennial herbaceous plant (spike moss) is the only surviving genus within the Selaginellaceae family. *Selaginella* is a unique terrestrial heterosporous pteridophyte and is represented by about 700-750 species widely distributed around the globe (Little *et al*., 2007). They grow in damp shady habitats; others are xerophytic and grow on dry rocky cliffs. These xerophytic species are able to withstand desiccation for months and expands when it is moistened. Such species are called resurrection plants. Due to the drought-tolerance capacities, these species have different bioactive chemical compounds which are medicinally important (Irudayaraj and Johnson, 2013). *Selaginella* has been used as an alternative medicine to cure wound after childbirth, menstrual disorder, skin disease, headache, fever, infection of exhalation channel and urethra, cirrhosis, cancer, rheumatism, bone fracture etc (Setyawan and Darusman, 2008; Setyawan, 2009; Setyawan, 2011).

Many methods have been carried out to characterize *Selaginella* species which includes cytology (Manickam and Irudayaraj, 1988; Morbelli *et al*., 2001; Mukhopadhyay, 2002), histochemistry (Manickam and Irudayaraj, 1992), chemodiversity (Cheng *et al*., 2008; Setyawan, 2011; Weng and Noel, 2013), phytochemistry and pharmacological studies (Ma *et al*., 2002; Gayathri *et al*., 2005; Janaki *et al*., 2009; Duraiswamy *et al*., 2010; Irudayaraj *et al*., 2010; Suganya *et al*., 2011; Sivaraman *et al*., 2013). Measurement of morphological, anatomical and cytological traits alone may not serve as a useful criterion to identify the fern ally *Selaginella*. Earlier work has revealed a great deal about concealed genetic variability caused due to deleterious genes and cytological variations in *Selaginella* (Manickam and Irudayaraj, 1988; Manickam and Irudayaraj, 1992; Morbelli *et al*., 2001; Mukhopadhyay, 2002).

Globally, only very few reports are available on the molecular characterization and systematic studies of *Selaginella* (Rodolfo *et al*., 1999; Korall and Kenrick, 2002; Korall and

Kenrick, 2004; Li *et al*., 2007; Banks *et al*., 2011; Hanna *et al*., 2012; Yobi *et al*., 2012; Das *et al*., 2012; Harholt *et al*., 2012; Lydia *et al*., 2012; Weng and Noel, 2013; Gu *et al*., 2013). But there is no report on the Indian *Selaginella* species. Hence, the present investigation is aimed to determine the genetic similarities and variation of selected *Selaginella* species from the Western Ghats of India using the biochemical and molecular analysis.

The specific objectives of the present investigation are as follows:

- To reveal the protein profile of selected *Selaginella* species using SDS-PAGE.
- To identify the protein similarities and variations among the *Selaginella* species by applying MALDI-TOF MS analysis.
- To determine the biochemical variation by means of isozymic profile.
- To estimate the phylogenetical and evolutionary relationship among the selected species of *Selaginella* using *rbcL* gene.

Plants have always been the source of energy for survival and evolution of the animal kingdom, thus forming the base for every ecological pyramid. Over the last few decades, plant genomics has been studied extensively revolutionizing this area of research. During the early period of research, classical strategies including comparative morphology, anatomy, physiology and embryology were employed in genetic analysis to determine inter and intra-species variability (Weising *et al*., 1995). In recent times, molecular markers have very rapidly complemented the traditional strategies. Molecular taxonomy is an aspect of molecular systematics, a broader term that includes the use of molecular data in phylogenetics and biogeography (Schneider and Schuettpelz, 2006). The impact of molecular data on the field of taxonomy can hardly be overstated. In combinations with explicit methods for phylogenetic analysis, molecular data have reshaped concepts of relationships and circumscriptions at all levels of the taxonomical hierarchy (Qui *et al*., 1999; Soltis *et al*., 1999). As molecular phylogenetic studies have accumulated, it has become apparent that different molecular tools are required for different questions because of varying rates of sequence evolution among genomes, genes and gene regions. The choice of molecular tool is very important to ensure that an appropriate level of variation is recovered to answer the phylogenetic questions at hand. Nonetheless, the plant systematic community is using only a small fraction of the available molecular tools (Small *et al*., 2004).

Every living organism contains DNA, RNA and proteins. In general, closely related organisms which look morphologically similar have a high degree of agreement in the molecular structure of these substances, while the molecules of distantly related organisms usually show a pattern of dissimilarity. Conserved sequences are expected to accumulate mutations over time and assuming a constant rate of mutation provides a molecular regulator

for dating divergence (Crawford, 2000). Such molecular composition can be studied by genetic markers that represent genetic differences between individual organisms or species. Such markers themselves do not affect the trait of interest because they are located only near or linked to genes controlling the trait. Earlier, morphological markers were applied to characterize phenotypic characters such as flower colour, seed shape, growth habits or pigmentation (Sumarani *et al*., 2004). The main disadvantage of using morphological markers is that they may be limited in number and are influenced by environmental factors or the developmental stage of the plant and so measurement of morphological traits alone may not serve as a useful criterion to evaluate plant germplasm (Winter and Kahl, 1995).

The theoretical frameworks for molecular systematic were laid in the 1960s. Early attempts at molecular systematics or chemotaxonomy made use of proteins, enzymes, carbohydrates and other molecules that were separated and characterized using biochemical techniques such as chromatography and electrophoresis (Diaz *et al*., 2008). In addition, biochemical techniques offered a powerful means of detecting introgression and distinguishing it from other phenomena, such as joint retention of the ancestral condition and convergence (Soltis, 1992). The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) technique is a powerful tool for estimating the molecular weights of proteins (Weber *et al*., 1971). A major advantage of electrophoresis over morphological evaluation is the speed with which a large number of test samples can be analyzed. It simultaneously exploits differences in molecular size to resolve proteins differing by as little as 1% in their electrophoretic mobility through the gel matrix (Scopes, 1994). Electrophoretic banding pattern of polypeptides can be an efficient approach for assessment of different plant samples (Felsentein, 2004).

Isozymes analysis has been used for over 60 years for various research purposes in biology viz., to delineate phylogenetic relationships, to estimate genetic variability, to study

population genetics and developmental biology, to characterize the plant genetic resources management and plant breeding. Isozymes originate through amino acid alterations which cause changes in net charge or the spatial structure of the enzyme molecules and their electrophoretic mobility (Soltis and Soltis, 1989). The strength of using isozymes is simplicity; it does not require DNA extraction or the availability of sequence information, primers or probes; they are quick and easy to use. Some plant species, however, can require considerable optimization of techniques for certain enzymes. Isozymes have been applied in many population genetic studies, including measurements of crossing rates, population structure and population divergence (Freville *et al*., 2001). Isozymes are particularly useful at the level of specific populations and closely related species and are therefore useful to study diversity in crops and their relatives (Hamrick and Godt, 1997).

Isozymes are considered as molecular markers since they represent enzyme variants. However, isozymes are in fact phenotypic markers since they may be affected by environmental conditions (Krieger and Ross, 2002). They have been used often in concert with other markers for fingerprinting purposes and diversity studies (Manjunatha *et al*., 2003), to study inter-specific relationships (Garvin and Weeden, 1994), the mode of genetic inheritance (Warnke *et al*., 1998), allelic frequencies in germplasm collections (Reedy *et al*., 1995) and also to identify the parents of hybrids (Parani *et al*., 1997). The main disadvantage of isozymes is their relatively low abundance and low level of polymorphism. Moreover, proteins with identical electrophoretic mobility may not be homologous for distantly related germplasm (Hudson *et al*., 1994).

With the advent of molecular era, a new generation of markers has been introduced over the last two decades, which has revolutionized the entire scenario of biological sciences. DNA-based molecular markers have acted as versatile tools and have found their own position in various fields like taxonomy, physiology, embryology and genetic engineering.

They are no longer looked upon as simple DNA fingerprinting markers in variability studies or as mere forensic tools. Ever since their development, they are constantly being modified to enhance their utility and to bring about automation in the process of genome analysis (Miwa *et al*., 2009). The discovery of PCR (Polymerase Chain Reaction) was a landmark in this effort and proved to be an unique process that brought about a new class of DNA profiling markers. This facilitated the development of marker-based gene tags, map-based cloning of agronomically important genes, variability studies, phylogenetic analysis, synteny mapping and marker-assisted selection of desirable genotypes. The DNA marker based approach gives new dimensions to intensive efforts of breeding and marker-aided selection which can reduce the time span of developing new and better varieties. These DNA markers offer several advantages over traditional phenotypic markers, as they provide data that can be analyzed objectively (Kumar *et al*., 2009). DNA markers may be broadly divided into three classes based on the method of their detection viz., hybridization-based, PCR based and DNA sequence based (Joshi *et al*., 2004).

DNA barcoding is an innovative molecular technique, which uses short and agreed upon DNA sequences from either nuclear or cytoplasmic genome for rapid identification of biological specimens at species level. Based on the study on 200 allied Lepidopteran species, Hebert *et al*. (2003) were the first to propose the use of short DNA sequences as “Taxon Barcodes” for species level identification. The DNA sequence which was found to be applicable for barcoding animal species in their pioneering and subsequent studies was the "Folmer region" at the 5' end of cytochrome c oxidase 1 (CO1), present in mitochondrial genome (Hebert *et al*., 2003; 2004). Based on this initial success with animals, this was projected as the locus that could provide recognition tags to all organisms including plants. Realizing the importance of this technology, an international “Consortium for the Barcode of Life” (CBOL) was established in 2004 (Lahaye *et al*., 2008). CBOL’s mission is to promote

the exploration and development of DNA barcoding as global standard for species identification, through rapid compilation of high quality DNA barcodes in a public library of DNA sequences. Another organization, International Barcode of Life (IBOL), based at Guelph, Ontario in fact, is involved in barcoding of different group of plants and animals. It aimed to generate DNA barcodes for 5 million specimens and 500,000 estimated species existing on earth by 2015 (Muellner *et al*., 2011). DNA barcoding besides helping taxonomists in rapid identification of new, cryptic, polymorphic species, is also a powerful diagnostic tool in hands of enforcement agencies for checking illegal trade of endangered species of both animals and plants especially bio-piracy (Jeanson *et al*., 2011). As opposed to the current taxonomic methods which require whole plant preferably in flowering stage for its authentic identification, DNA barcodes once standardized can identify the species even if a minute amount of tissue/fragment is available (Yesson *et al*., 2011).

Diversity of pteridophytes

"*Biodiversity is the degree of variety in nature and not nature itself*". The number of species of plants, animals and microorganisms, the enormous diversity of genes in these species; the different ecosystems on the planet such as deserts, rainforests and coral reefs are all part of a biologically diverse earth (IIRS, 2002). India possess only 2% land mass of world and harbors more than 7% of the global vascular plant species. Pteridophytes are primitive vascular plants, which can adapt well in terrestrial habitat and form a conspicuous element of the earth's vegetation. Moreover they are important from evolutionary point of view and reflect the emergence of seed habitat in the plants (Sumesh *et al*., 2012).

The global diversity of pteridophytes is yet not very clear; however the estimated number of species in the world is about 9000-15000 (Haufler 1996; Smith *et al*., 2008). On the other hand, according to Moran (2008) the exact number of pteridophytes is about 13,600 species. With reference to various enumerations, checklists and recent publications, the

number of Indian pteridophyte species is about 950-1000 species (Fraser-Jenkins, 2008; Chandra and Fraser Jenkins, 2008; Chandra *et al*., 2008). Fraser-Jenkins (2009) enumerated 1050-1100 species of pteridophytes from India. Recently Kholia (2010) listed out 1200 species of pteridophytes. In India, the pteridophytes diversity is observed in the Himalayas, Eastern and Western Ghats (Dixit, 2000) where as Western Ghats are a natural reservoir of large number of important medicinal pteridophytes (Benjamin and Manickam, 2007).

After Beddome (1883) much work has not been done on Indian pteridophytes. However an upswing was seen after 1950 in pteridological research and in almost all the fields. Caius (1935) is supposed to be the first man who has described the medicinal uses of some ferns of India. The most important studies on medicinal values of ferns and fern allies were conducted by Nayar (1957), Hodge (1973), Dixit (1974), Dixit and Vohra (1984) and Ghosh (1984). Jain and Sastry (1980) reported 17 rare and endangered species of pteridophytes from India, followed by Dixit (1984) who included twenty five rare and interesting pteridophytes from India. Bir (1987 and 1988) enumerated 156 rare and endangered species of pteridophytes from various regions of India. Nayar and Sastry (1987; 1988; 1990) included 31 threatened pteridophytes in the volumes of the Botanical Survey of India's Red Data Book of Indian Plants. Bharadwaja *et al*. (1987) enumerated 36 endangered species belonging to 21 genera of ferns and fern-allies from Rajasthan. Pangtey and Samant (1988) listed some rare and endangered pteridophytes of the Kumaun Himalaya, Uttarakhand. Pande and Bir (1994); Pande and Pande (2003) identified 30 rare, threatened and endemic taxa of the Kumaun Himalaya and later, Fraser-Jenkins *et al*. (2009) listed 123 rare and endangered taxa of pteridophytes. Singh and Panigrahi (2005) reported 48 new and rare taxa from Eastern Arunachal Pradesh and listed 16 endemics to North East India.

Manickam (1984) worked on the pteridophytes of Western Ghats in detail. Some of the earlier prominent publications are ecological studies on the pteridophytes of Palni hills

(Manickam, 1984; Manickam, 1986), Cytology of the pteridophyte flora of Western Ghats, South India (Manickam and Irudayaraj 1988; 1992). Madhusoodanan (1991) listed rare and endangered ferns of the Western Ghats of Kerala, South India and later on, Manickam (1995) reported 44 rare and endangered ferns and fern allies in the Western Ghats region of South India. Benniamin *et al*. (2008) developed a key for identification of rare and endangered ferns and fern allies of the Western Ghats.

Numerous works have been carried out in several pteridophytic species which includes ethnobotanical (Rao *et al*., 2007; Sharief and Rao, 2007; Shil *et al*., 2009; Rout *et al*., 2009; Sen and Ghose, 2011), taxonomical, phytochemical (Raja *et al*., 1995; Okwu, 2004; Shokeen *et al*., 2005; Hassan *et al*., 2007; Irudayaraj *et al*., 2010; Paulraj *et al*., 2011; Britto *et al*., 2012; Mithraja *et al*., 2012; Johnson *et al*., 2013), cytological (Bir, 1962; 1965; Ghatak, 1963; Bir and Vasudeva, 1979; Mahabale and Kamble, 1981; Manickam and Irudayaraj, 1988; 1992; Irudayaraj *et al*., 1993; Irudayaraj and Bir, 1994; Bir *et al*., 1996), pharmacognostical (Irudayaraj *et al*., 2005; Irudayaraj and Johnson, 2013), anti-bacterial (Manickam *et al*., 2005; Khan *et al*., 2007; Maridass and Ghantikumar, 2008; Lai *et al*., 2010; Panda *et al*., 2014), antifungal (Singh *et al*., 2008; Dolly *et al*., 2010), antidiabetic (Nair *et al*., 2006), antioxidant (Beknal *et al*., 2010; Lai *et al*., 2010), anti-inflammatory (Zakaria *et al*., 2006; Anuja *et al*., 2010), antihyperglycaemic activity (Ahmed *et al*., 2009; Sujith *et al*., 2011; Paul *et al*., 2012), antidermatophytic activity (Nejad and Deokule, 2009) and other pharmacological studies (Haripriya *et al*., 2010; Paul *et al*., 2012). In addition to the above phytochemical and pharmacological examinations, the biochemical and molecular systematic studies were also carried out by a number of pteridologists; Wolf *et al*., (1994); Pryer *et al*., (1995); Ebihara *et al*., (2002); Pinter *et al*., (2002); Hennequin *et al*., (2003); Skog *et al*., (2004); Zhang *et al*., (2005); Reid *et al*., (2006); Korall *et al*., (2007); Kirkpatrick (2007);

Johnson *et al*., (2007); Grusz *et al*., (2009); Johnson *et al*., (2010); Beck *et al*., (2011); de Groot *et al*., (2012) and Johnson *et al*., (2012).

Molecular systematic studies on plants

The long-term survival and evolution of a species depends on the maintenance of sufficient genetic variation within and among populations to adapt to new selection pressures and those exerted by environmental changes (Barrett and Kohn, 1991). Genetic diversity maintained in a plant species would be influenced by many processes, such as long-term evolutionary history and characteristics of the species including genetic drift, gene flow and mode of reproduction (Hamrick and Godt, 1990). Understanding the genetic variation within and between populations is therefore required for the establishment of effective and efficient conservation strategies for endemic and endangered plant species (Hamrick and Godt, 1996). Characterization of genetic diversity in a plant species offers an opportunity to have a better knowledge of the genetic affinity of germplasm (Syed *et al*., 2011).

The extent of genetic diversity in germplasm can be assessed through morphological characterization and genetic markers. Morphometry could not provide solution of complex taxonomic problems, modern biological techniques are now available that can resolve these issues (Zubaida *et al*., 2006). Molecular marker technology provides a valuable tool to obtain overall estimates of the genetic diversity of a species. In recent years, there has been explosion in the availability of different types of molecular genetic markers which includes proteins (Terry *et al*., 2007; Malik *et al*., 2009; Shah *et al*., 2011; Johnson *et al.*, 2012), isozymes (Smila *et al*., 2007; Johnson *et al*., 2010; Johnson *et al*., 2012), DNA hybridizations (Chen *et al*., 2012), restriction enzymes (Tzfira *et al*., 2012), random PCR amplifications, species-specific PCR primers (Periera, 2008; Bellstedt *et al*., 2010), RAPD (Pervaiz *et al*., 2010; Elham *et al*., 2010), microsatellite markers (Rabbani *et al*., 2010), SNPs (Clarke *et al*.,

2013) and DNA sequence variation (Ren *et al*., 2010; Korall *et al*., 2010; Beck *et al*., 2011; Alvarez *et al*., 2012; Pomponio *et al*., 2013).

Biochemical studies on plants

Proteomic analysis using biochemical techniques constructed a pathway to study the genetic differentiation in plant populations. The word "proteome" was combined by the front-half of protein and the back-half of genome (Wasinger *et al*., 1995) which indicates the presence of all the proteins and their action modes in a cell, tissue or an organism. Analyses of the variants occurring in plants concerning biochemical phenotype have been undertaken to a lesser extent (Johnson and Manickam, 2003). Like other biological macromolecules such as polysaccharides and nucleic acids, proteins are essential parts of organisms participate in virtually every process within cells. Proteins have structural or mechanical functions and are involved in biochemical reactions which are vital to metabolism (Omnia, 2010). Gene allele codes for the production of amino acids that string together to form proteins. Thus, differences in the nucleotide sequence of alleles result in the production of slightly different strings of amino acids or variant forms of the proteins. These proteins code for the development of the anatomical and physiological characteristic of the organism, which are also responsible for determining aspects of the organism's behavior (Bhat and Kudesia, 2011).

Proteins (SDS-PAGE) are simple to use in a variety of applications in plant taxonomical research. The information on polymorphism using protein profiles in a set of genotype is useful in tagging genes of interest and genetic mapping in extensive run to facilitate marker assisted selection (Johnson *et al*., 2012) where as the banding positions and regions of activity are variable based on the development of plants (Johnson and Patric Raja, 2007). Protein profiles are still powerful tools for determining genetic homology at the molecular level and solving problems in systematic methodology (Johnson *et al*., 2012). The

methods available to isolate and distinguish proteins are electrophoresis, chromatography, gel filtration, catalysis, immuno-techniques and sedimentation. Among these, electrophoresis is often treated as the best investigative tool that has been applied successfully in many botanical disciplines. It is especially employed for characterization and comparison of germplasm as well as evaluation of protein expression at different developmental stages of plants (Smila *et al*., 2007). Originating in the 1930s and till now electrophoresis technique has been used as a tool for the study of heritable and developmental variations by geneticists, systematists and population biologists because of their relative efficiency and cost effectiveness (Siva and Krishnamurthy, 2005; Johnson, 2007; Smila *et al*., 2007; Johnson *et al*., 2010).

Seigneurin-Berny *et al*. (1999) developed a novel approach based on the selective extraction of hydrophobic proteins using organic solvents, followed by classical polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). This procedure allowed the characterization of well known envelope membrane proteins that displayed several transmembrane segments. Interestingly, most of these proteins were found to be basic proteins which are detected by SDS-PAGE and shown to contain putative transmembrane domains. SDS-PAGE also provided valid evidence for assessing inter-specific relationships and intra-specific variation (Kamel *et al*., 2003; Javaid *et al*., 2004; Celebi *et al*., 2009; Hameed *et al*., 2009). Many plant genotypes have been characterized on the basis of electrophoresis profiles by SDS-PAGE (Zhan and Lin (1991); Montalavan *et al*., (1995); Santhy *et al*., (1998); Habib *et al*., (2000); Sengupta and Chattopadhayy (2000); Ghafoor *et al*., (2002); Asghar *et al*., (2004); Vivekananthan *et al*., (2005); Weber *et al*., (2005); Zubaida *et al*., (2006); Johnson *et al*., (2007); Babu *et al*., (2010); Malik *et al*., (2009); Muhammed *et al*., (2010); Joelri *et al*., (2011); Johnson *et al*., (2012); Johnson and Narayani (2013), Neilson *et al*., (2014). Revathy *et al.* (2011) studied

the protein variation of *Adiantum raddianum, Arachniodes tripinnata, Dryopteris sparsa* and *Odontosoria chinensis* using SDS-PAGE. Sivaraman *et al*. (2011) determined the proteomic expression of *Tectaria paradoxa, Araiostegia hymenophylloides* and *Deparia petersonii* using electrophoresis.

MALDI-TOF MS analysis on plants

Knowledge of plant development and function can be obtained by determining the distribution of proteins and metabolic processes within plant tissues. Development of improved high-throughput proteomics techniques has shifted attention to 'protein profiling', while attempts were made to identify all proteins that are present in a cell (Yates, 2004). Analysis of a complete proteome remains a challenge, despite significant advances in mass spectrometry technology and peptide fractionation tools. Such a challenge can best be tackled by community effort of MALDI-TOF MS analysis because ionization method is generally quite selective for different peptides. MALDI was first introduced in 1988 by Karas and Hillenkamp as a revolutionary method for ionizing and mass-analyzing large biomolecules where MALDI-TOF MS was first reported by Spengler (1994) and which has been applied to visualize peptides and proteins by Caprioli (1997). MALDI-TOF MS analysis has the potential to provide new insights into the molecular analysis of plants by providing high spatial resolution information about proteins and potentially quantitative changes during plant development or those induced by environmental variation (Grassl *et al*., 2011). Genome annotation based on peptide identification in particular requires an open source platform to collect and integrate MS data (Desire *et al*., 2007). The ionization principles will improve the coverage of proteins and therefore MS data will increase the success of identification of proteins in plants. Researchers have isolated and identified more than 200 protein complexes using MALDI spectroscopic profile, providing clues as to how an entire proteome is organized into functional units (Ohkmae, 2004).

Proteomic research using mass spectrometry is advanced in animals and yeast, plant proteomics and its subcellular proteins are still at the initial phase (Zivy and de Vienne, 2000; van Wijk, 2001; Kersten *et al*., 2002). Rice is the first crop genome to be decoded where the rice proteome has been systematically analyzed using MS methods (Komatsu *et al*., 2003; Rakwal and Agarwal, 2003). Cellular proteins are actively and passively transported across cellular and organelle membranes during normal homeostasis and in response to stress and external stimuli (Agarwal *et al*., 2005). Accurate identification and quantification of a sub-cellular proteome is very useful and can provide insight into cellular/organelle function and dynamics. In addition to the field of functional proteomics, sub-cellular proteomics can provide insight into the molecular mechanisms of plant cell modulation of protein accumulation in intracellular compartments in response to various perturbations and thus provide refined knowledge about signal transduction in organelles (Hossain *et al*., 2012). Some of the major sub-cellular organelles are plasma membranes, chloroplasts, mitochondria and nucleus in which specific proteins from such organelles are studied by several researchers (Rouquie *et al*., 1997; Peltier *et al*., 2000; Prime *et al*., 2000; Kruft *et al*., 2001; Bae *et al*., 2003). By using proteomic tool of mass spectrometry analysis, over 400 proteins, including 30 proteins from the tricarboxylic acid cycle, 78 proteins from the ETC and more than 20 proteins from amino acid metabolism pathways have been identified in mitochondria from the model plant *Arabidopsis thaliana* (Ito *et al*., 2007).

Peltier *et al*. (2000) investigated luminal and peripheral thylakoid proteins by a combination of 2-DE and MALDI-TOF MS, and allowed a unique comparison of the two sub proteomes in the plant chloroplast. The results estimated 200-230 different luminal and peripheral proteins. Sixty-one proteins were identified, of which 33 are identified as a functional domain, whereas 10 proteins had no annotated function. Peltier (2001) identified 350 kDa chloroplast protein protease complexes with 10 different subunits in the chloroplast

of *Arabidopsis thaliana* using MALDI-TOF MS analysis. A new chloroplast protein was thus discovered that does not belong to any of the known chloroplast gene families. Yamaguchi and Subramanian (2000) identified the proteins of 30S and 50S ribosomal subunits in spinach chloroplasts by a combination of 2-DE, chromatography and mass spectrometry. The results estimated that the *Spinach* plastid ribosome comprises 59 proteins, of which 53 are *Escherichia coli* orthologues and six were non-ribosomal plastid specific proteins. Koller *et al*. (2002) studied a systematic proteomic analysis of rice leaves, root and seed tissue using independent technology of mass spectroscopic methods. The results allowed the identification of 2,258 unique proteins. Among them, 189 proteins were detected in all of the tissues which found to be involved in the central metabolic pathways, transcription control, mRNA biosynthesis and protein biosynthesis.

Castillejo *et al*. (2008) analyzed the drought stress proteins in sunflower (*Helianthus annus*) leaves by MALDI-TOF mass spectrometry. Out of the 23 identified proteins, *Helianthus annus* matched 6 proteins followed by *Arabidopsis thaliana* (10), *Nicotiana* spp. (3), *Solanum tuberosum* (1), *Pisum sativum* (1), *Flaveria bidentis* (1) and *Selaginella utahensis* (1). Natarajan *et al*. (2009) elucidated a total of 107 low abundant proteins from soybean seed using combined action of MALDI-TOF mass spectrometry and liquid chromatography-mass spectrometry (LC-MS).

Kumar and Kumari (2009) described MALDI-TOF MS as an efficient method to identify the variation of protein expression in *in vitro* and *in vivo* plants of *Artemisia vulgaris*. The results showed that 59 µg of protein is present in *in vitro* leaf samples (50 µl), where as *in vivo* plants have 58 µg of proteins in 50 µl of leaf sample. In addition proteins with molecular weight 64, 48 and 37 kDa were significantly increased in *in vivo* where as 79, 53 and 28 kDa proteins were significantly increased in *in vitro* plant samples. Tian *et al*. (2009) applied proteomic approaches of MALDI-TOF MS to identify genes and proteins involved in

antimicrobial defence, heavy metal uptake and translocation. A total of 74 proteins which encodes metal binding and transport proteins, transcription factors, ABC transporters and phenylpropanol biosynthetic enzymes were identified.

Kamal *et al*. (2010) identified the abiotic stress responsive proteins from wheat using MALDI-TOF mass spectrometry. Of the total number of 575 identified proteins, 345 proteins were recognized as abiotic stress responses unique proteins with isoforms. Of which 34% are induced by heat, 27% are induced by drought, 15% by salt, 13% by cold and 11% by other environmental stress.

Zhao *et al*. (2012) analyzed the differential expression of proteins in the rice leaves and roots using MALDI-TOF MS. A total of 16 proteins were expressed in the rice leaves and 25 proteins were expressed in the rice roots. The identified proteins were involved in photosynthesis, carbohydrate metabolism, nitrogen metabolism, oxidative phosphorylation, oxidative stress responses, signal transductions and cell division respectively. Hong *et al*. (2012) used two-dimensional electrophoresis (2-DE), coupled with MALDI-TOF MS to identify the mitochondrial protein expression between two plants *Ananas comosus* and *Kalanchoe pinnata*. Peptide mass spectrometry permitted identification of 12 and 15 proteins in mitochondrial 2-DE gels of *A. cosmosus* and *K. pinnata* respectively. Mitochondria-specific proteins of *A. cosmosus* included three isoforms of ATP synthase sub unit beta, an ATP synthase subunit alpha, phenylalanine ammonia-lyse 2, a mitochondrial ribosome protein S3, a glucose-6-phosphate isomerase and a 14-3-3-like protein GF 14-F. Major proteins specific to *K. pinnata* mitochondria included ADP-ATP carrier protein, an ATP catalytic subunit A, a malate dehydrogenase and an outer mitochondrial membrane protein (porin 2).

Datta *et al*. (2013) studied the changes in leaves proteome profile of *Arabidopsis thaliana* in response to salicylic acid using MALDI-TOF MS analysis to identify

differentially expressed proteins. This proteomic profiling of salicylic acid treated leaves versus control leaves demonstrated the changes of many defense related proteins like pathogenesis related protein, disease-resistance protein, putative late blight-resistance protein, WRKY4 and MYB4 along with gross increase in the rate of energy production. Lee *et al*. (2013) characterized the sub-cellular organelle proteomes such as nuclei and mitochondria as well as proteins distributed in plasma or microsomal membranes from various tissues in *Medicago truncatula* by applying MALDI-TOF MS. The results enhanced the detection of low abundance proteins and increased the overall detectable proportion of the sub-cellular proteome.

Isozyme analysis on pteridophytes

Multiple forms of enzymes generally called as isozymes which catalyze the same chemical reaction if their polypeptide constituents are coded by more than one gene locus and allozymes coded by different alleles of the same locus (Hamrick and Gadt, 1997). The existence of isozymes permits the fine-tuning of metabolism to meet the particular needs of a given tissue or developmental state. Isozymes are useful as genetic and biochemical markers and also as good estimators of genetic variability in plant populations. Isozymes were first described by Hunter and Markert (1957). The expression of various isozymes differs both temporally and spatially. Isozymes are usually the result of gene duplication, but can also arise from nucleic acid hybridization. Isozyme markers can correctly identify several levels of taxa, accessions and individuals since the assumption of homology can be more accurate than for same genomic DNA markers (Karp *et al*., 1997).

Numerous enzymes were tested as markers for developmental processes in plants, but there are few reports related to pteridophytes. The most interesting group of enzymes called esterases (EST, E.C.3.1.1.6), which hydrolyze ester bonds, generally have a broad spectrum of substrates and act on a wide variety of natural and xenobiotic compounds. 1-and 2-

naphthylacetate are often used for visualization of these enzymes in plant tissues (Peskan *et al*., 2000). A few reports have been made on the presence of esterases in fungi, bacteria (Lee *et al*., 1987; Oakeshott *et al*., 1993) and in plants (Krsnik-Rasol *et al*., 2001; Cummings and Krafsur, 2005). Characterization of purified enzyme is critically important to elucidate physiological functions and mechanisms of plant peroxidase. Peroxidase (PO, E.C.1.11.1.7) is an enzyme found in all aerobic cells, which functions to decompose toxic hydrogen peroxide (Petersen and Anderson, 2005). Knowledge of catabolic processes and the properties of enzymes involved enables manipulation of peroxidase catabolism using molecular approaches has been investigated by several researchers (Sebela *et al*., 2001; Yoda *et al*., 2003; Cona *et al*., 2003).

Acid phosphatases (ACP, EC 3.1.3.2) are widely distributed in plants and animals which catalytically break down a wide variety of phosphate esters and exhibit pH optima below 6.0 (Vincent *et al.*, 1992). Acid phosphatase is believed to be important for many physiological processes, including regulation of phosphorus efficiency (Asaduzzaman *et al*., 2011). Acid phosphatases have been purified and characterized from many of the plant species (Ullah and Gibson, 1988; Panara *et al.*, 1990; Staswick *et al.*, 1994; Olczak *et al*., 1997; Guo and Pesacreta, 1997; Kusudo *et al.*, 2003; Kouadio, 2004; Gonnety *et al*., 2006). The metalloenzyme known as alkaline phosphatase (ALP, EC 3.1.3.1) exists as several tissue-specific isozymes encoded by separate genes. ALP is expressed in many species which includes plants, animals and bacteria which catalyze the hydrolysis of phosphomonoesters (Holtz and Kantrowitz, 1999). More than 80 years ago, the high level of ALP expression was first noted in humans (Golub and Boesze-Battaglia, 2007).

Polyphenol oxidase (PPO, E.C.1.10.3.2) is a ubiquitous copper-containing protein widely distributed in plant kingdom which uses molecular oxygen to oxidize common *ortho*-diphenolic compounds such as caffeic acid and catechol to *ortho*-quinones. The *o*-quinones

are highly reactive substances that can react with aminoacids, peptides and proteins and thus altering structural and functional properties of the cell (Zivkovic, 2009). PPO has been characterized in a wide variety of plants (Mayer, 2006; Yoruk and Marshall, 2003). Recent studies have indicated that phenol oxidizing enzymes may participate in response to various abiotic stresses including drought (Zivkovic *et al*., 2009).

Isozymic studies on several fern taxa were carried out electrophoretically viz., *Polypodium* (Gastony, 1982; Bryan, 1987), *Asplenium* (Werth, 1985), *Dryopteris* (Werth, 1991), *Bommeria* (Haufler, 1985), *Cystopteris* (Haufler, 1987) and *Pellaca* (Gastony and Gottlieb, 1985). Woodin *et al*. (1978) isolated two isozymic bands of chorismate mutase and such bands are applied as diagnostic characters of *Selaginella*. Almost 30 years ago, isozyme analyses of a small group of ferns in the genus *Asplenium* provided the first solid evidence that a single polyploid species could have multiple, independent origins (Werth *et al*., 1985). Haufler and Soltis (1986) elucidated the genetic constitution of the complex polypodiaceous pattern in homosporous ferns by the expression of phosphoglucomutase and triose-phosphate isomerase using isozymic analysis comparing with diploid angiosperms. Phosphoglucomutase has a duplicated locus in *Bommeria chrenbergiana*, *Asplenium montanum* and *Polypodium virginianum* where such locus is also observed in diploid angiosperms. The complex banding patterns of triose-phosphate isomerase detected in homosporous ferns and in diploid seed plants typically display two isozymes, one in the chloroplast and the other in the cytosol.

Shigeo and Yasuyuki (1994) conducted an electrophoretic analysis of enzymes for the Japanese fern *Cratopteris thalictroides* and confirmed the hybrid sterility between two isozymic types of *C. thalictroides*. Korpelainen (1995) carried out enzyme genetic variation in bracken fern *Pteridium aquilinum* by means of electrophoretic analysis. The mean value of the differentiation among populations was high and the level of genetic divergence was

considerable. Hooper and Haufler (1997) explored the genetic diversity and breeding system of six members of the epiphytic fern genus *Pleopeltis*, using electrophoretic analysis of isozymes such as phosphogluco isomerase (PGI), triosephosphate isomerase (TPI), aspartate aminotransferase (AAT), leucine aminopeptidase (LAP), hexokinase (HK), aldolase, malate dehydrogenase (MDH), isocitrate dehydrogenase and shikimic dehydrogenase (SKDH). A total of nine isozymes encoded by 11 putative gene loci were consistently resolved in all *Pleopeltis* species and varieties showed that *Pleopeltis* species are diploid in nature.

Huang *et al*. (1997) determined the genetic diversity in *Asmina triloba* from nine states using electro-focusing of a 24 enzyme system viz., acid phosphatase, alcohol dehydrogenase, aconitate hydratase, adenylate kinase, formate dehydrogenase, fructose-bisphosphate aldolase, fructose-bisphosphatase, fumarate hydratase, glucose-6-phosphate dehydrogenase, glutamate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, isocitrate dehydrogenase, dihydrolipoamide dehydrogenase, malic enzyme, phosphogluco isomerase, peroxidase, malate dehydrogenase, isocitrate dehydrogenase, mannose phosphate isomerase, peroxidase, phosphoglucomutase, phosphogluconate dehydrogenase, triosephosphate isomerase and shikimate dehydrogenase (SKD). Of the 24 enzyme systems 23 presumptive loci were observed and 49 alleles were detected across populations. The results also explained mean number of alleles per locus (1.54), polymorphic loci (43.5%), effective number of alleles per loci (1.209) and heterozygosity (0.172). Partitioning of genetic diversity was observed as 88.2% within populations. Santiago (1999) studied the genetic diversity in populations of *Cryptogramma crispa* (Pteridaceae) by means of isozymic analysis. The percentage of polymorphic loci and the similarity levels obtained from the isozyme analyses indicate a level of genetic variability of *C. crispa* are usually associated with diploid fern species rather than polyploid species.

Herrero (2001) tested the isozyme variation and genetic relationships among diploid and tetraploid taxa in the *Asplenium obovatum* group (Aspleniaceae) encoded by electrophoretic analyses of eight enzyme system which includes triose phosphate isomerase (TPI), phosphogluco isomerase (PGI), malate dehydrogenase (MDH), 6-phosphogluconate dehydrogenase (6PGD), shikimate dehydrogenase (SDH), phosphoglucomutase (PGM), aminotransferase (AAT) and leucine aminopeptidase (LAP). Gel stained with each enzyme system showed two regions of band activity corresponding to both diploids and tetraploids in homozygous and heterozygous pattern. Pajaron *et al.* (2005) evaluated seventeen populations of *Asplenium seelosii* using isozyme electrophoresis. There was no genetic identity within and between populations. It also clearly differentiated two groups viz., *Asplenium seelosii* and *Asplenium celtibericum* and do not support the recognition of subspecies in this complex. He *et al*. (2005) purified and characterized polyamine oxidase from the aquatic nitrogen-fixing fern *Azolla imbricata* using electrophoretic analysis. SDS-PAGE gave a single protein band corresponding to a molecular mass of 65.5 kDa. The overall purification yield of polyamine oxidase was 7% of total activity which was purified by a factor of 82-fold mg protein.

Various studies have been carried out on the isoperoxidase profiling on the south Indian ferns and fern allies (Soltis and Soltis, 1993, 1999). Irudayaraj *et al*. (2009) illustrated the isozymic evidence for the common origin of *Diplazium* species confined to South India and Sri Lanka. A total of 30 bands in twenty five different positions with seven activity regions were observed in the isoperoxidase enzyme system of *Diplazium*. Maximum degree of diversity has been observed in *D. travancoricum* with the presence of fourteen bands, followed by *D. polypoides* with nine bands, *D. cognatum* with four bands and *D. dilatum* with two bands respectively. Johnson *et al*. (2010) identified the genetic distinction of three filmy ferns viz., *Trichomonas obscurum*, *Trichomonas proliferum* and *Trichomonas plicatum*

using isoperoxidase analysis. A total of sixteen bands were obtained in *Trichomonas* enzyme system. Bands were observed between zone four to nine and other zones were failed to express in *Trichomonas* isoperoxidase enzyme system. Johnson *et al*. (2010) illustrated the isoperoxidase banding profile for some selected species of *Pteris* from India. A total of thirty eight bands were scored in thirty one different positions with eight active zones. The study revealed the biochemical positions of the *Pteris* complex in which the similarity and variation index between the five species of *Pteris* were reported with reference to isoperoxidase profiles.

Johnson *et al*. (2010) studied the biochemical difference between the different developmental stages of four rare and endangered ferns of Western Ghats of South India viz., *Cheilanthes viridis*, *Phlebodium aureum* and *Pronephrium triphyllum* and *Sphaerostephanos unitus* using isozymic analysis. Four zones of activity were observed in *C. viridis*, multiple zones of activity were obtained for *P. aureum* and *P. triphyllum*. Five zones of activity were obtained in *S. unitus*. The isozyme profiles revealed the biochemical changes between the different developmental stages of selected ferns. Johnson *et al*. (2010) studied the isozymic variation of *Tectaria coadunata*, *Tectaria wightii* and *Tectaria paradoxa* using PAGE. A total of seventeen bands with active regions were expressed in isoperoxidase enzyme system. The study revealed hundred percentage genetic differentiations between the three *Tectaria* species. Johnson *et al*. (2010) revealed inter-specific variation in the crude drugs of Maiden hair ferns (*Adiantum*) from the Western Ghats, South India by means of isoperoxidase analysis. *Adiantum raddiannum* and *Adiantum lunulatum*; *Adiantum caudatum* and *Adiantum zolingeri* banding profile showed high percentage of similarity indices compared to other species. The band PRX 1^3 and PRX 1^4, PRX 2^4, PRX 4^4 and PRX 7^1 showed their unique presence in *A. raddianum*, PRX 5^1 for *A. caudatum*, PRX 6^4 and PRX 7^2 for *A. zolingeri*, PRX 3^1, PRX 9^1, PRX 9^3 and PRX 10^1 for *A. lunulatum*, PRX 2^3 and PRX 6^1 for *A.*

hispidillum and PRX 4^3 for *A. incisum*. These unique bands can be used to distinguish and characterize the species and differentiate the original crude drug from the adulterant.

Irudayaraj and Johnson (2011) identified the phylogenetic relationships among three medicinally important species of *Sphaerostephanos* viz., *S. arbuscula, S. unitus and S. subtruncatus* from South India using isozymic profile. A total of six different bands in five different positions with different molecular weight were obtained. One band with MW/R_f 0.399 is common to *S. arbuscula* and *S. unitus*. Two bands (R_f 0.23 and 0.47) are present in *S. subtruncatus* and one distinct band has been observed individually in *S. arbuscula* (R_f 0.507) and *S. unitus* (R_f 0.56). Johnson *et al*. (2012) revealed inter-specific variation on three South Indian species of tree fern *Cyathea* viz., *C*. *gigantea*, *C*. *nilgirensis* and *C*. *crinita* by applying isoperoxidase analysis. A total of seven bands with three active regions were expressed in the *Cyathea* spp. enzyme system. The banding pattern revealed hundred percentage genetic differentiations between the three *Cyathea* species which belong to three different intra generic taxonomical groups. Johnson *et al*. (2012) assessed the genetic variation between different populations of *Thelypteris ciliata* collected from different localities on Tirunelveli hills, using isoperoxidase profiling. A total of six bands in six different positions with five active regions were observed in the isoperoxidase system of *T. ciliata*. The isoperoxidase enzyme systems confirm the intra and inter population's genetical difference of *T. ciliata.*

DNA barcoding on pteridophytes

DNA barcoding is an emerging global standard for recognition and identification of eukaryotic species through comparison of sequences of a short DNA fragment called DNA barcode from an unknown specimen to a library of reference sequences from known species (Schori and Showalter, 2011). This allows identifying an organism at any stage of development from a very small tissue sample, fresh or conserved many years ago. In addition, to assigning specimens to known species, DNA barcoding will accelerate the ace of

species discovery by allowing taxonomists to rapidly sort specimens and by highlighting divergent taxa that may represent new species. By augmenting their capabilities in these ways, DNA barcoding offers taxonomists the opportunity to greatly expand and eventually complete a global inventory of life's diversity (Hebert and Gregory, 2005). An optimal DNA barcode region is a small fragment present in all species of a major taxonomic group, having invariable nucleotide sequence in all members of the same species, but with sufficient variation to discriminate among the species. This fragment should be flanked by low-variable regions for use of primers in PCR amplification and sequencing (Schneer, 2009).

A segment of the mitochondrial cytochrome *c* oxidase I gene is widely accepted as the standard barcode region in animals (Hebert *et al*., 2003). In plants, however, no equivalently accepted DNA barcode has been identified, despite the fact that many resolutions have been proposed in the leading literature (Lahaye *et al*., 2008; Kress and Erickson, 2010; Asahina *et al*., 2010; CBOL, 2010). Plastid genome (plastome) sequence information is of central importance to trace the evolutionary history of plastids and their hosts. The small genome size and high copy number per cell have made the plastid genomes sequencing much more amenable than nuclear genomes. Currently, there are at least 190 completely sequenced plastid genomes available, of them 163 are from various green plants, with land plants having the best representation (Lai *et al*., 2010). Analysis of over 10,000 plastid genome (*rbcL*) sequences from GenBank demonstrate that this locus could serve as the core region, with sufficient variation to discriminate among species in approximately 85% of congeneric pair-wise comparisions (Newmaster *et al*., 2006). Although efforts to identify a DNA barcode for discriminating among recognized species are less successful in plants than animals, in recent years, researchers have reported many efficient cases for plant DNA barcoding (Kress *et al*., 2010; Li *et al*., 2011; de Groot *et al*., 2012).

Green (1971) isolated DNA from three genera of ferns (*Angiopteris*, *Ophioglossum*, *Ciboteum*) and three genera of fern-allies (*Psilotum*, *Equisetum*, *Selaginella*). Pryer *et al*. (1995) recorded a broad sampling of 50 existing Pteridophyte taxa, with representatives of all major fern groups, and one seed plant (*Cycas*). Results from the morphological analysis are compared with an independent analysis of *rbcL* data carried out for the same set of pteridophyte taxa. Wolf *et al*. (1994) clarified the phylogenetic relationships among genera and families of dennstaedtioid ferns by sequencing 1320 bp of the chloroplast gene *rbcL* from 45 species representing 13 families. Sequence divergence for *rbcL* averaged 0.9% among species within genera, 10.3% among genera within families and 14.8% among families and suggested that the data are appropriate for phylogenetic analysis at the generic and familial levels in ferns. Maximum parsimony analysis resulted in four shortest trees of equal length. Tree ferns form a single clade and Hymenophyllaceae appear as sister groups to the dennstaedtioid ferns on all shortest trees. Polypodiaceae and adiantoid ferns groups considered separate from the dennstaedtioid families emerged within the dennstaedtioid clade.

Dubuisson *et al*. (2003) estimated the evolutionary relationships within the filmy fern genus *Trichomanas* (Hymenophyllaceae) using *rbcL* nucleotide data from 46 species of *Trichomanas* belonging to four subgenera: *Achomanes*, *Didymoglossum*, *Pachychaetum* and *Trichomanas.* Out groups included four species of *Hymenophyllum* and the monotypic genus *Cardiomanes* from New Zealand. The phylogenetic optimization includes two species belonging to Morton's Asiatic sections *Callistopteris* and *Cephalomanes* which are in unresolved basal positions within *Trichomanas* and suggested that *rbcL* data alone are inadequate for estimating the earliest cladogenetic events. Out of the four subgenera *Didymoglossum* appears monophyletic. *Lacostea* is more closely related to *Davalliopsis* (*Pachychaetum*) than to other members of subgenera *Achomanes*. *Davalliopsis* and *Lacostea*,

together with species of the morphologically different subgenera *Achomanes*, make up a strongly supported Neotropical clade. Hemiepiphytes and strictly epiphytic or epipetric species are grouped together in an ecologically definable clade that also includes *Nesopteris*. *Lacosteopsis* (*sensu* Morton) is polyphyletic and comprises two distantly related clades: large hemiepiphytic climbers and small strictly epiphytic/ epipetric taxa. Based on their work, each of these associations is also supported by cytological, geographical, and ecological evidence taxa.

Ranker *et al*. (2003) conducted a molecular phylogenetic analysis of the endemic Hqwaiian fern genus *Adenophorous* (Grammitididaceae) by employing DNA sequence variation from three cp DNA fragments viz., *rbcL*, *atpB* and the *trnL-trnF* intergenic spacer (IGS). Phylogenetic analyses of individual DNA fragments resulted in single strong supported phylogenetic hypothesis. The primary features of hypothesis are *Adenophorus* is monophyletic, subgenus *Oligadenus* is paraphyletic, the endemic Hawaiin species *Grammitis tenella* is strongly supported as the sister taxon to *Adenophorous*. Highly divided leaf blades are evolutionary derived in the group and simple leaves are the ancestral and the common ancestor of *Adenophorus tenella* clade remains unresolved.

Hauk *et al*. (2003) represented the phylogenetic studies of 36 species of Ophioglossaceae using *rbcL* and *trnL-trnF* plastid DNA sequences. Individual and combined analyses of the three data sets revealed two main clades of ophioglossoid and botrychioid within the family. In the botrychioid clade, *Helminthostachys* was sister to a broadly defined *Botrychium* and in some case *Sceptridium* was sister to *Botrychium*. *Botrypus* was paraphyletic with *Botrypus virginianus* sister to *Botrychium* and *Sceptridium*. *Botrypus strictus* was sister to all other botrychioid species except *Helminthostachys*. In the ophioglossoid clade, *Ophioglossum* was sister to *Cheiroglossa* and *Ophioderma* but relationships within *Ophioglossum* were not well supported.

Schneider *et al*. (2004) explored the phylogeny of the polygrammoid ferns using nucleotide sequences derived from three plastid loci for each of 98 selected species. The analyses recovered four major monophyletic lineages: the loxogrammoids, two clades and a largely neotropical clade includes the pantropical Grammitidaceae. The loxogrammoid lineage diverges first and is sister to a large clade comprising the three remaining species-rich lineages. One paleotropical clade includes the drynarioid and selligueoid ferns whereas the second paleotropical clade includes the platycerioids, lepisoroids, microsoroids and their relatives. The grammitids nest within the neotropical clade in which epiphytic group remains ambiguous. *Microsorum* and *Polypodium* were recovered as polyphyletic. In addition, the analysis revealed that the rate of substitution in the grammitids is remarkably higher relative to other polygrammoids.

Pryer *et al*. (2004) studied the phylogeny and evolution of ferns (monilophytes) with a focus on the early leptosporangiate divergences. More than 5000 bp from the plastid (*rbcL*, *atpB*, *rps4*) and the nuclear (18S rDNA) genomes were sequenced for 62 taxa. Phylogenetic analyses confirmed that Osmundaceae are sister to the leptosporangiates, Dipteridaceae, Matoniaceae, Gleicheniaceae, Hymenophyllaceae are monophyletic and schizaeoid ferns as sister to a large clade of "core leptosporangiates" included heterosporous ferns, tree ferns and polypods.

Korall *et al*. (2006) investigated phylogenetic relationships within tree ferns Cyatheaceae, Plagiogyriaceae and Hymenophyllopsidaceae based on analyses of four protein-coding, plastid loci (*atpA*, *atpB*, *rbcL* and *rps4*). The results revealed four well-supported clades with genera of Dicksoniaceae interspersed among Loxomataceae, *Culcita*, Plagiogyriaceae, *Calochlaena*, *Dicksonia*, Lophosoriaceae where *Cibotium* and Cyatheaceae nested within Hymenophyllopsidaceae. These four groups are related to one another in which

Thyrsopteris and Metaxyaceae are weakly supported. In addition the results showed Dicksoniaceae and Cyatheaceae are not monophyletic.

Schuettpelz and Pryer (2007) analyzed the most inclusive molecular dataset for leptosporangiate ferns using three plastid genomes (*rbcL*, *atpB* and *atpA*). More than 4,000 bp, were sequenced for each of 400 leptosporangiate fern species and five outgroups. Maximum likelihood analysis yielded strong phylogeny (80%) and the nodes were supported by a maximum likelihood bootstrap percentage ≥ 70. The analysis provided a record approaching overall fern relationships, not only delivering additional support for the deepest leptosporangiate divergences, but also uncovering the composition of more recently emerging clades and their relationships to one another. Schuttpelz *et al*. (2007) presented a molecular phylogeny of the fern family Pteridaceae by assessing overall relationships and the affinities of previously unsampled genera. The analyses resulted in two newly assembled data sets including 169 newly obtained sequences resolved five major clades within the Pteridaceae viz., cryptogrammoids, ceratopteridoids, pteridoids, adiantoids and cheilonthoids. Of the previously unsampled genera, *Neurocallis* and *Ochropteris* are nested within the genus *Pteris*, *Monogramma* and *Rheopteris* are early diverging vittarioid ferns, *Monogramma* resolved as polyphyletic and *Adiantopsis* occupied among cheilanthoids.

Korall *et al*. (2007) investigated the phylogenetic relationships of scaly tree ferns based on DNA sequence data from five plastid regions (*rbcL, rbcL-accD IGS, rbcL-atpB IGS, trnG-trnR* and *trnL-trnF*). The results resolved *Sphaeropteris* as sister to all other taxa and scale features support two clades. *Sphaeropteris* has conformed scales, whereas all other taxa have marginate scales. The marginate-scaled clade consists of three groups namely *Cyathea* including *Cnemidaria*, *Hymenophyllopsis*, *Trichipteris*, *Alsophila* includes *Alsophila capensis* and *Gymnosphaera*. Nagalingum *et al*. (2008) conducted a broad species level relationship for *Piluria* and *Salvinia* using coding (*atpB*, *rbcL* and *rpsA*) and non coding

plastid regions (*trnL*, *trnGR* and *rps4-trnS*). Their analyses resolved that Marsileaceae, Salviniaceae and all of the component genera as monophyletic. Salviniaceae incorporate *Salvinia* and *Azolla*; in Marsileaceae, *Marsilea* is sister to the clade of *Regnellidium* and *Pilularia*. Individual species level investigations for *Pilularia* and *Salvinia* provide phylogenies within all genera of heterosporous ferns. Nitta (2008) explored the utility of three plastid loci (*rbcL*, *trnSGG* and *trnH-psbA*) by sequencing the filmy ferns (Hymenophyllaceae) known from Morea.

Grusz *et al*. (2009) studied the origin of apomictic polyploids in the *Cheilanthes yavapensis* complex (Pteridaceae) by plastid and nuclear DNA sequencing. Plastid and nuclear DNA sequencing revealed *Cheilanthes lindheimeri* is an autopolyploid derived from a rare and undetected sexual diploid. The apomictic triploid *Cheilanthes wootonii* is an inter-specific hybrid between *Cheilanthes fendleri* and *Cheilenthes lindheimeri*, whereas the apomictic tetraploid *Cheilanthes yavapansis* comprised of two cryptic and geographically distinct lineages. Their work suggested that deciphering species relationship and hybrid origins in polyploidy agamic species complexes is difficult due to the cryptic nature of the morphological characters that distinguish species but it is possible to use plastid phylogenies to identify the maternal parents of hybrid ferns.

Ebihara *et al*. (2010) identified the pteridophytic flora of Japan (733 taxa including subspecies and varieties) in molecular level to test the utility of two plastid DNA barcode regions (*rbcL* and *trnH-psbA*) with the intention of developing an identification system for native gametophytes. DNA sequences were obtained from each of 689 taxa for *rbcL* and 617 taxa for *trnH-psbA*. Mean inter-specific divergence values across all taxon pairs did not reveal a significant difference in rate between *trnH-psbA* and *rbcL* but mean genetic distances of each genus showed significant heterogeneity according to systematic position. Their results suggested that DNA barcoding is expected to be an effective identification tool for

organisms with heteromorphic generations includes pteridophytes, which possess a morphologically simple gametophyte generation.

Pryer *et al*. (2010) exposed DNA barcoding approaches to a case of mistaken identity in the fern horticultural trade. Plastid *rbcL*, *atpA* and *trnG-R* sequence data demonstrated that a fern marketed as *Cheilanthes wrightii* in the horticultural trade is infact *Cheilanthes distans*. The results strongly advocated that the barcoding approach is a valuable technology to correct plant identification errors in the international trade of horticulture industry. Labiak *et al*. (2010) resolved molecular phylogeny, character evolution and biogeography of the grammitid fern genus *Lellingeria* (Polypodiaceae) among 61 species and sequences were obtained for two genes (*atpB* and *rbcL*) and four intergenic spacers (*atpB-rbcL*, *rps4-trnS*, *trnG-trnR* and *trnL-trnF*). *Lellingeria* is composed of two main clades, *L. myosuroides* and *Lellingeria* clades together are sister to *Melpomene*. Sister to all three of these is a clade with two species of the polyphyletic genus *Terpsichore*. In *L. myosuroides* clade several dispersal events occurred between the neotropics, Africa and Pacific Islands, whereas *Lellingeria* is restricted to the neotropics, with about 60% of its diversity in the Andes.

Gao *et al*. (2010) sequenced the complete chloroplast genome of a scaly tree fern *Alsophila spinulosa* (Cyatheaceae). The *Alsophila* cp genome is 156,661 base pairs (bp) in size, and has a typical quadripartite structure with the large (LSC, 86, 308 bp) and small single copy (SSC, 21, 623 bp) regions separated by two copies of an inverted repeats (IRs, 24,365 bp each). This genome contains 117 different genes encoding 85 proteins, 4 rRNAs and 28 tRNAs. Pseudogenes of ycf66 and *trnT*-UGU are also detected in this genome. A unique *trnR*-UCG gene (derived from *trnR*-CCG) is found between *rbcL* and *accD*. The *Alsophila* cp genome shares some unusual characteristics with the previously sequenced cp genome of the polypod fern *Adiantum capillus-veneris*, including the absence of 5 tRNA genes that exist in most other cp genomes. The genome shows a high degree of synteny with

that of *Adiantum*, but differs considerably from two basal ferns (*Angiopteris evecta* and *Psilotum nudum*).

Beck *et al*. (2011) estimated the ages of sexual and asexual diploids in the fern genus *Astrolepis* using a well-supported plastid phylogeny *trnGR*. The 50 asexual polyploidy samples were estimated to comprise 19 distinct lineages, including a variety of auto and allopolyploid genomic combinations. The confounding association between asexuality and polyploidy precludes regarding the effect of asexuality and suggested that asexuality limits evolutionary potential in *Astrolepis*. Li *et al*. (2011) assessed the discriminatory power of the core plant DNA barcode (*rbcL* and *matK*) as well as an alternative proposed fern barcodes (*trnH-psbA* and *trnL-F*) among two fern groups viz., *Deparia* (Woodsiaceae) and the *Cheilanthes marginata* group (Pteridaceae). With its high sequence variation, *matK* complements *rbcL* provided a strong resolving power of the loci. With sequence variation *matK* and *trnL-F* appears to be a suitable alternative barcode regions in ferns if universal primer development of *matK* fails. *trnH-psbA* showed reduced sequence variation for the majority of ferns. Their study suggested that DNA barcoding will revolutionized the understanding of fern ecology and identification of the cryptic gametophyte phase of the fern's life history.

Wolf *et al*. (2011) described evolutionary patterns and processes in fern plastid genomes (plastomes) and compared plastid organization and patterns of evolution in ferns to those in seed plants. A large clade of ferns is characterized by a plastome reorganized with respect to the ancestral gene order similar to seed plants and also observed high levels of RNA in fern plastomes.

Hao *et al*. (2012) evaluated the chloroplast barcoding markers in more than 9100 plant sequences by mean and smallest inter-specific distances. The results showed that the smallest inter-specific distances decrease with the number of species sampled in 6 out of 10

chloroplast markers. The differences in the size of barcode gaps based on mean versus smallest inter-specific distances have major implications for plant DNA barcoding. Johnson *et al*. (2012) studied the phylogenetic relationships among 49 taxa (30 notholaenids and 19 outgroup taxa) analyzed for three plastid loci *atpA*, *trnG-R* and *rbcL*. The phylogenetic analysis of the plastid DNA set showed that outgroup taxa never displayed gametophytic farina (with a single exception) and observed the unique expression of a sporophytic character on the gametophytes of Notholaenid ferns (Pteridaceae).

Chen *et al*. (2013) performed the discriminating power of three barcodes (*matK*, *rbcL* and *trnL-F*) on 16 vittarioid sporophytes. *trnL-F* showed highest primer universality and discriminatory ability scores whereas PCR success rates were very low for *matK* and *rbcL* regions (10.8% and 41.3%, respectively). BLAST analyses showed that all the sampled field gametophytes could be successfully identified to species level. Blanca *et al*. (2013) made an attempt to identify a cryptic fern (*Pityrogramma trifoliata*) distribution in the Western Andes of Peru through DNA sequencing. They identified that the collected sporophytes pointed to four different genera, two in Pteridaceae (*Anogramma* and *Pityrogramma*) and the others in Aspleniaceae (*Asplenium*) and Cystopteridaceae (*Cystopteris*). The results resolved that sequences of DNA (*rbcL* and *trnG-R*) pointed to *Pityogramma trifoliata* of Pteridaceae.

Studies on *Selaginella*

Selaginella, also known as spikemoss, is the only surviving genus within the Selaginellaceae family. *Selaginella* includes more than 700 species widely distributed around the world (Little *et al*., 2007). Selaginellaceae, together with the other two extant families Lycopodiaceae (clubmosses) and Isotaceae (quillworts) within the division Lycopodiophyta comprise the oldest lineage of vascular plants surviving on earth (Banks, 2009). Fossil records suggest that lycopodiophytes often referred to as lycophytes diverged from all other vascular plants including ferns and seed plants (euphyllophytes) more than 400 million year

ago (Pryer *et al*., 2004). As one of the few lycophyte genera that survived the Permian-Triassic extinction event, *Selaginella* has been a long standing subject of investigation for botanists and paleontologists.

Sah (2008) has explained the role and use of medicinal pteridophyte, particularly the fern allies *Selaginella* even in the famous ancient literature 'Ramayana'. Tulsidas, a famous epic of Hindi poet described *Selaginella* as a wonder herb called as *Sanjeevani booti* (Life giving herb), which according to the popular belief had given life to the dying Laxmana, the younger brother of Lord Shree Rama. It is believed that medicines prepared from this herb can even revive a dead person also.

Selaginella is traditionally used to cure several disease depending on species, such as cancer or tumor, wound, after childbirth, menstrual disorder, female reproduction disease, expulsion of the placenta, tonic (for after childbirth, increase body endurance, anti ageing, etc), pneumonia, respiratory infection, exhalation channel infection, inflamed lung, cough, tonsil inflammation, asthma, urethra infection, bladder infection, kidney stone, cirrhosis, hepatitis, cystisis, bone fracture, rheumatism, headache, fever, skin diseases, eczema, depurative, vertigo, toothache, backache, blood purify, blood coagulation, amenorrhea, hemorrhage, diarrhea, stomach-ache, sedative, gastric ulcers, gastro-intestinal disorder, rectocele, itches, ringworm, bacterial disease, bellyache, neutralize poison caused by snakebite or sprained, bruise, paralysis, fatigue, dyspepsia, spleen disease (diabetic mellitus), emmenagogue, diuretic, and to refuse black magic (Bouquet *et al*., 1971; Dixit and Bhatt, 1974; Lee and Lin, 1988; Ahmad and Raji, 1992; Bourdy and Walter, 1992; Nasution, 1993; Lin *et al*., 1996; Caniago and Siebert, 1998; Sequiera, 1998; Dalimartha, 1999; Abu-Shamah *et al*., 2000; Harada *et al*., 2002; Batugal *et al*., 2004; Mamedov, 2005; Khare, 2007; Setyawan and Darusman, 2008; Haripriya *et al*., 2010; Irudayaraj *et al*., 2010; Suganya *et al*., 2011; Sivaraman *et al*., 2013).

With the establishment of the genus *Selaginella* by Palisot de Beauvois (1805) enormous research work has been done on this genus in various fields like morphology, anatomy, cytology, palynology, taxonomy, floristics etc. (Mukhopadhyay, 2002). Alston (1945) enumerated 58 species of *Selaginella* from India and Sri Lanka. Of these 45 species were reported from India. Panigrahi and Dixit (1966) recorded twelve species of *Selaginella* in Eastern India. According to Dixit (1984) 18 species are present in South India (Tamil Nadu and Kerala). Twelve species have been reported from the Western Ghats, South India (Manickam and Irudayaraj, 1992). Manickam and Irudayaraj (2003) recorded five species of *Selaginella* from Nilgiris, Western Ghats, South India.

Morphological karyotype of *Selaginella* is firstly published by Manton (1950). Chowdhury (1971) described the anatomy of two Indian xerophytic species viz., *Selaginella wightii* and *Selaginella bryopteris*. The comparative studies on the anatomical and morphological features of *Selaginella apoda* and *Selaginella ludoviciana* A. Br. have been undertaken by Buck and Lucansky (1976). Imaichi and Kato (1989) have examined the developmental anatomy of the shoot apex, rhizophore and root of *Selaginella uncinata.* Morbelli *et al*. (2001) have observed the spore wall structure in *Selaginella* species from Argentina. Mukhopadhyay (2002) has made morpho-anatomical observations on some Indian members of *Selaginella.* Brighigna *et al*. (2002) have studied the structural and ultra structural characterization of *Selaginella lepidophylla*, a desiccation-tolerant plant, during the rehydration process. Seth and Kumar (2005) reported xylem-vessels in *Selaginella adunca* and *Selaginella chrysocaules*. Sarvesh *et al*. (2010) described the ligule morphology in three Indian *Selaginella* species viz., *S. bryopteris*, *Selaginella repanda* and *Selaginella panchghaniana* from Matkuli and Apsravihar (Madya Pradesh). Schulz *et al*. (2010) studied the life cycle of *S. apoda* using morphology and anatomy.

Chromosome number of several *Selaginella* species of India has been investigated by various cytologists Kuriachan (1963); Loyal (1976; 1984); Ghatak (1977) and Vasudeva and Bir (1983), Manickam and Irudayaraj (1988; 1992); Europe (Reese, 1951; Love and Love, 1961; Borgen, 1975), North America (Tryon, 1955, Love and Love, 1976), China (Wang *et al*., 1984; Weng and Qiu, 1988), Taiwan (Tsai and Shieh, 1983; 1988), Japan (Takamiya, 1993) and other countries (Tchermak-Woes and Dolezal-Janish 1959; Zhukova and Petrovsky, 1975). Jermy *et al*. (1967) made extensive investigation on chromosome numbers of 76 species of *Selaginella* collected from the Malay Peninsula, Borneo, New Guinea, Australia, Trinidad and Brazil.

Sharma *et al*. (1992) studied the behavior of chlorophylls, carotenoids and phenols in drought resistance *Selaginella* from Rajasthan. Lin *et al*. (2000) have isolated four new biflavonoids from *Selaginella delicatula* by phytochemical analysis. The activities of 10 enzymes involved in carbohydrate metabolism have been measured in both desiccated and rehydrated fronds of the desiccation tolerant pteridophyte *S. lepidophylla* (Hook. & Grev.) Spring (Harten & Eickmeier, 1986). Cheng *et al*. (2008) isolated and described the two new unusual natural pigments, Selaginellin A and B from *S. tamariscina*. Lee *et al*. (2009) and Juneyoung *et al*. (2009) isolated isocryptomerin from *S. tamariscina* applied in traditional medicine. Paramanick *et al*. (2002) made comparative studies on amino acids in four species of *Selaginella*. Setyawan (2011) have isolated thirteen compounds of flavonoids from *Selaginella* viz., amentoflavone, 2',8"-biapigenin, delicaflavone, ginkgetin, heveaflavone, hinokiflavone, isocryptomerin, kayaflavone, ochnaflavone, podocarpusflavone A, robustaflavone, sumaflavone and taiwaniaflavone. Liu *et al*. (2008) evaluated the nature of desiccation of tolerance in the resurrection plant *Selaginella tamariscina* by comparing the composition of soluble sugars and saturation ratios of phospholipids between hydrated and desiccated tissues of *S. tamariscina*.

Apart from morphology, anatomy and phytochemistry, different species of *Selaginella* have also been subjected to pharmacological studies (Suganya *et al*., 2011) to know the mechanism of action and to know the application of these drugs for the welfare of human beings without any other side effects. Ma *et al*. (2003) reported two new antiviral hormone glycosides, namely Unicoside A and Unicoside B from *S. uncinata*. Hu *et al*. (2004) studied the hepatoprotective effects of *Selaginella doederleinii* Hieron from Taiwan. Gayathri *et al*. (2005) evaluated the immunomodulatory and antioxidant properties of *S. involvens*, *S. delicatula* and *S. wightii*. Janaki (2009) studied the pharmacological effect of an anticancer spike-moss *Selaginella inaequalifolia*. Recently, antibacterial efficacy of *S. inaequalifolia* and *S. involvens* against poultry pathogens and human pathogens has been studied by Haripriya *et al*. (2010) and Irudayaraj *et al*. (2010). Chris Givens (2010) elucidated the ethylene perception in *S. apoda* and *S. moellendorffi*. Sivaraman *et al*. (2013) evaluated the antioxidant potential of *Selaginella* species viz., *S. involvens*, *S. intermedia*, *S. inaequalifolia* and *S. tenera* by scavenging the $DPPH^{+}$, $ABTS^{+}$, FRAP, metal chelating and phosphomolybdenum assays. Research on diversity of *Selaginella* based on isozymic marker for population dynamics is rarely conducted. Setyawan (2011) indicated that enzyme system of esterase (EST), peroxidase (PRX), malate dehydrogenase (MDH), aspartate aminotransferase (AAT) and acid phosphatase (ACP) can be used as diagnostic characteristics for more than 20 species of *Selaginella*.

Molecular studies on *Selaginella*

Rodolfo *et al*. (1999) isolated cDNA, *SlTPS1* encoding a 109-kD protein from the resurrection plant *Selaginella lepidophylla*, which accumulates high levels of treholase complemented with growth and stress tolerance defects in a yeast *tps1* mutant. Protein sequencing comparing showed that *SlTPS1* shares high similarity to treholase-6-phosphate synthase genes from prokaryotes and eukaryotes. *SlTPS1* mRNA was constitutively

expressed in *S. lepidophylla*. The results also suggested that *SlTPS1* protein is a functional plant homolog capable of sustaining treholase biosynthesis that plays a major role in stress tolerance in *S. lepidophylla*. Li *et al*. (2007) explored the genetic relationship among eight medicinal species of *Selaginella* from China using RAPD. A total of 58 amplified bands and RAPD map were obtained. Based on the RAPD map, a Jaccard's genetic similarity matrix and a dendrogram for 8 plants were established. The analyses revealed distinct inter-specific and intra-specific variations among the studied species.

Banks *et al*. (2011) reported the genome sequence of the lycophyte *Selaginella moellendorffii*. The transition from a gametophyte to a sporophyte dominated for fewer new genes than the transition from a non seed vascular to a flowering plant whereas secondary metabolic genes expanded extensively and in parallel to the lycophyte and angiosperm lineages. *Selaginella* differs in post-transcriptional gene regulation, including small RNA regulation of repetitive elements, an absence of the trans-acting small interfering RNA pathway and extensive RNA editing of organellar genes. Hanna *et al*. (2012) identified and analyzed the major intrinsic proteins (MIP's) in the genome of spikemoss *Selaginella moellendorffii*. In total 19 MIP's were found in *S. moellenderffi* belonging to 6 of the 7 MIP subfamilies that was previously identified in the moss *Phycomitrella patens*. Only three of the MIP's were classified as members of the conserved water specific plasma membrane intrinsic protein (PIP) subfamily whereas half belong to the diverse NOD26-like intrinsic protein (NIP).

Yobi *et al*. (2012) compared metabolic profiling between desiccation-sensitive species *Selaginella moellendorffii* and desiccation-tolerant species *Selaginella lepidophylla* using metabolomics profiling technology. A total of 301 metabolites including 170 named and 131 unnamed compounds across both species were observed. *S. lepidophylla* retained significantly higher abundances of sucrose, mono and polysaccharides, and sugar alcohols

than *S. moellendorffii*. Aromatic amino acids, osmoprotectant betaine and flavonoids were present abundantly in *S. lepidophylla*. Notably, levels of γ-glutamyl amino acid, linked with glutathione metabolism in the detoxification of reactive oxygen species and with possible nitrogen remobilization following rehydration were markedly higher in *S. lepidophylla*. Markers for lipoxygenase activity were also greater in *S. lepidophylla*. *S. moellendorffii* contained more than twice the number of unnamed compounds, with only a slightly greater abundance than in *S. lepidophylla*. In contrast, *S. lepidophylla* contained 14 unnamed compounds of fivefold or greater abundance than in *S. moellendorffii*, suggesting that these compounds might play critical roles in desiccation tolerant. Overall, *S. lepidophylla* appears poised to tolerate desiccation in a constitutive manner using a wide range of metabolites with some inducible components, whereas *S. moellendorffii* mounts only limited metabolic responses to dehydration stress

Das *et al*. (2012) optimized a protocol for isolation of genomic DNA from leaves of *Selaginella* species viz., *Selaginella delicatula*, *Selaginella repanda*, *Selaginella bryopteris*, *Selaginella plana* and *Selaginella monospora* using RAPD analysis and studied their genetic variation. The results observed that the Rogers and Benedich (1994) protocol yielded higher quantities of DNA compared to the protocol of Murray and Thompson (1980). On the other hand, the modified Rogers and Benedich (1994) protocol yielded better quantity (2-3 times more) and quality genomic DNA than the original protocol of Rogers and Benedich (1994). The isolation protocols of Dellaporta *et al*. (1983) and Doyle and Doyle (1987) did not yield quantifiable amounts of genomic DNA. The modified DNA extraction protocol of Rogers and Benedich (1994) proved to be most useful as both the quality and quantity of genomic DNA significantly increased and yielded better results in RAPD-PCR analysis compared to the original DNA extraction protocol of Rogers and Benedich (1994).

Lydia *et al*. (2012) explained the expression of MADS-domain proteins in *Selaginella moellendorffii* by means of complete genome sequencing. *S. moellendorffii* revealed 19 putative MADS-box genes (13 Type I, 3 MIKC and 3 MIKC). The results showed that the most recent common ancestor of vascular plants possessed MADS-domain proteins encoded by Type I and Type II genes. Phylogeny tree represents the clades of floral organ identity genes originated in a common ancestor of seed plants after the lineage that led to lycophytes had branched off. The number of MIKC genes and the ratio of MIKC to MIKC genes is lower in *S. moellendorffii* correlated with reduction of the gametophyte in vascular plants.

Drought is one of the most severe limitations to plant growth and productivity which is observed in fern-allies. Resurrection plants have evolved a unique capability to tolerate desiccation in vegetative tissues. Resurrection plants have been studied at structural, physiological and molecular levels (Jiang *et al*., 2007). Proteomic analysis are an excellent approach to study desiccation tolerance mechanisms because they show the actual protein content that is performing enzymatic, regulatory and structural functions (Jiang *et al*., 2007). Lina (2010) investigated the protein expression pattern between fully hydrated and desiccated resurrection plant *Selaginella lepidophylla* from Columbia.

Wang *et al*. (2010) studied the desiccation tolerance in resurrection fern-ally *Selaginella tamariscina* by physiological and proteomic analysis. The results showed that endogenous abscissic acid (ABA) increased to regulate dehydration-responsive genes/proteins. In addition the results showed 138 desiccation-responsive two-dimensional electrophoresis (2-DE) spots, representing 103 unique proteins. Hierarchial clustering analysis revealed that 83% of the proteins were down-regulated upon dehydration. They were mainly involved in photosynthesis, carbohydrate and energy metabolism, stress and defense, protein metabolism, signaling, membrane transport, cell structure and cell division. The dynamic expression changes of desiccation-responsive proteins provide strong evidence that

cell structure modification, photosynthesis reduction, antioxidant system activation and protein-transcriptional/translational modifications are essential to *S. tamariscina* in responsive to dehydration.

Weng and Noel (2013) studied the chemo diversity in *Selaginella* and considered their task as reference system for parallel and convergent metabolic evolution in terrestrial plants. The functional analyses suggested that the specialized metabolites, generally accepted to be restricted to seed plants have been identified in *Selaginella* which arose independently that helps in conventional understanding of the evolution of metabolism.

Korall and Kenrick (2002) developed a phylogenetic framework for the clubmoss family Selaginellaceae based on *rbcL* sequences. The analysis supports to distinguish the monophyly of subgenera *Selaginella* and *Tetragonostachys*; *Stachygyrandrum* and *Heterostachys* are polyphyletic. Korall and Kenrick (2004) evaluated the phylogenetic history of Selaginellaceae based on DNA sequences from the plastid (*rbcL*) and nucleus (26S rDNA gene) sequences. 26S rDNA and *rbcL* regions represent the main elements of species diversity in Selaginellaceae family and analysed by means of maximum parsimony and Bayesian inference. Substitution rates in the 26S rDNA were found to be high (26% informative) but lower than *rbcL* (37% informative). Harholt *et al*. (2012) presented the comparative genomic study of *Selaginella moellendorffi* which occupied an important evolutionary position among land plants with *Physcomitrella patens*, the only non-vascular terrestrial plant in which its genome has been sequenced. Gu *et al*. (2013) explored the utility of ITS2 region for barcoding 103 medicinal plants including 34 species of the Selaginellaceae family. The successful rate of PCR amplification and sequencing of the ITS2 region was 100%. There was significant divergence between the inter-specific and intra-specific genetic distances of the ITS2 regions, while the barcoding gap was more obvious.

To fulfill the objectives of the present investigation, the following *Selaginella* species viz., *Selaginella intermedia* (Bl.) Spring, *Selaginella inaequalifolia* (Hook. et Grev.) Spring, *Selaginella involvens* (Sw.) Spring, *Selaginella tenera* (Hook & Grev.) Spring, *Selaginella wightii* Hieron., *Selaginella brachystachya* (Hook. & Grev.) Spring, *Selaginella repanda* (Desv.) Spring, *Selaginella radicata* (Hook. & Grev.) Spring, *Selaginella bryopteris* (L.) Bak, *Selaginella delicatula* (Desv.) Alston with and without cones (Desv.) Alston were collected from their natural habitats of South India. To know the molecular relationships among the selected species of *Selaginella* following genetic analysis viz., protein separation by means of SDS-PAGE, mass spectroscopic analysis using MALDI-TOF MS, isozymic profile with PAGE and DNA sequencing using *rbcL* gene was carried out.

The detailed methodologies of the present investigation are as follows:

Collection of materials

The young leaves of selected ten *Selaginella* species viz., *S. intermedia, S. inaequalifolia*, *S. involvens*, *S. tenera*, *S. wightii, S. brachystachya*, *S. repanda*, *S. radicata*, *S. bryopteris*, *S. delicatula* with cones and *S. delicatula* without cones were collected from Kaakachi hills (Tirunelveli, Tamil Nadu), Keeriparai and Marunthuvazh Malai (Kanyakumari, Tamil Nadu), Shenbaganur and Eettipallum (Dindigul, Tamil Nadu), Ponmudi (Idukki, Kerala), Nilgiris (Tamil Nadu) and Thenmalai (Kerala), and performed the genetic polymorphism of the selected species (Table 1; Plate I).

Table 1: Geographical details of the selected species of *Selaginella*

Species name	Locality	Latitude and Altitude	Species code	Voucher No. (XCH. No)
S. intermedia (Plate II)	Kaakachi Hills, Tamil Nadu.	8.7152836° N 800-1800 m	S1	25425
S. inaequalifolia (Plate III)	Kaakachi Hills, Tamil Nadu.	8.7152836° N 800-1800 m	S2	25426
S. involvens (Plate IV)	Kaakachi Hills, Tamil Nadu.	8.7152836° N 800-1800 m	S3	25427
S. tenera (Plate V)	Kaakachi Hills, Tamil Nadu.	8.7152836° N 300-2000 m	S4	25428
S. wightii (Plate VI)	Marunthuvazh Malai, Tamil Nadu.	8.1272411° N 350-1500 m	S5	25429
S. brachystachya (Plate VII)	Shenbaganur Hills, Tamil Nadu.	10.2336828° N 1200-2000 m	S6	25430
S. repanda (Plate VIII)	Shenbaganur Hills, Tamil Nadu.	10.2336828° N 1000-1200 m	S7	25431
S. radicata (Plate IX)	Ponmudi Hills, Kerala.	8.7599422° N 110-1200 m	S8	25432
S. bryopteris (Plate X)	Ponmudi Hills, Kerala.	8.7599422° N 110-1200 m	S9	25433
S. delicatula with cones (Plate XI)	Ponmudi Hills, Kerala.	8.7599422° N 0-1000 m	S10	25434
S. delicatula without cones (Plate XII)	Themalai Hills, Kerala.	8.9632448° N 0-950 m	S11	25435

Botanical name: *Selaginella intermedia* (Bl.) Spring

Morphology of *S. intermedia*

Stem erect or sub erect, up to 2mm thick without leaves; rhizophores borne on the basal one-third part of the main stem as stilt roots, entire plants about 30 x 15 cm, distal two-

third part of the main stem bears dichotomously branched lateral branches; leaves dimorphic throughout, lateral leaves oblong-lanceolate, about 5 x 2 mm, base unequal, apex sub acute or obtuse, margin ciliolate at the basal part, entire in the rest; median leaves ovate, 4 x 1.5 mm, apex aristate, margin denticulate; cones about 1 x 0.3 cm, sporophylls uniform, ovate, 2 x 1 mm, acuminate, margin denticulate, microspores yellowish brown, 20 µm in diameter, exine with prominent tubercles (Plate II).

Distribution: *S. intermedia* is confined to Tamil Nadu and Kerala in India. Common on Kothayar hills, Kannikatty hills, Agasthiar hills, Kalakad hills and Sengaltheri hills (Plate II). Occasional on Anamalais and Munnar hills. Terrestrial on forest floor along stream banks or along shaded roadsides between 800-1800 m (Manickam and Irudayaraj, 1992).

Botanical name: *Selaginella inaequalifolia* (Hook. et Grev.) Spring

Morphology of *S. inaequalifolia*

Stem scandent, few feet long; main stem up to 4 mm thick, stramineous, glabrous or with scattered leaves; rhizophores borne at the axis of primary branches, about 10 x 2 mm, bearing roots at the basalmost part; primary branches alternate, about 5 cm apart, patent or slightly ascending, lanceolate or oblanceolate, up to 25 x 7 cm, tripinnate; leaves scattered on main stem, arranged in four rows on other branches, dark green; lateral leaves 5 x 2 mm, ovate-lanceolate, slightly oblique, acute, entire; median leaves 1.5 x 1 mm, acuminate, cones borne on ultimate branches, about 5 x 2 mm, quadrangular; sporophylls uniform, ovate, 2 x 1 mm, acuminate entire; megaspores 400 µm yellowish-brown, microspores 30 µm, exine of both microspores and megaspores are with prominent, elongated outgrowths (Plate III).

Distribution: This is the largest species of *Selaginella* in South India. Occasional on Agasthiar hills, Kothayar hills, Kannikatty hills and Ponmudi hills (Manickam and Irudayaraj, 1992).

Botanical name: *Selaginella involvens* (Sw.) Spring

Morphology of *S. involvens*

Stem erect, rooting at the base only, up to 2 mm thick, terete, up to 2 cm long without branches, stramineous; branched portion up to 28 x 12 cm, quadripinnate; lateral branches about eight pairs, alternate up to 4 cm apart, ascending, lanceolate, about 8 x 2.5 cm, bearing secondary and tertiary branches; leaves on main stem uniform, scattered, appressed, broadly ovate, up to 3 x 2 mm, acute, entire; leaves on distalmost part of the main axis and on lateral branches dimorphic; lateral leaves ovate-lanceolate; slightly oblique, 2 x 1 mm, acute, entire. Leaves pale green to dark green; cones terminal on ultimate branches, up to 5 x 2 mm, quadrangular; sporophylls uniform, ovate, 1 x 1 mm, acuminate, entire; microspores reddish brown in mass, 30 µm in diameter (Plate IV).

Distribution: Common throughout India (Plate IV). Although it is a common species, fertile plants are very rare (Manickam and Irudayaraj, 1992).

Botanical name: *Selaginella tenera* (Hook & Grev.) Spring

Morphology of *S. tenera*

Stem erect, rooting at the base only, up to 3 mm thick without leaves, up to 8 mm wide with leaves, green to pink colour when fresh, stramineous to pink colour when dry, entire plant up to 30 x 15 cm, with about five primary, alternate branches which bear several times branched secondary branches. Leaves dimorphic throughout, contiguous on main stem and on axis of primary branches, spreading and dense on the rest of the branches, lateral leaves 3 x 1.5 mm, oblong-ovate, obtuse or subacute, denticulate on the acroscopic margin, entire on the rest, base unequal; median leaves ovate, 1 x 0.5 mm, aristate, arista less than half the length of the leaf, margin dentate, cones dorsiventral, about 1 x 0.25 cm; sporophylls dimorphic, lateral sporophylls bear megasporangia, others with microsporangia; microspores brick red in mass, 20 µm in diameter with thick wavy leasura; megaspores 150 µm (Plate V).

Distribution: *S. tenera* is confined to the mountains of the extreme South of India includes Kothayar hills, Keeriparai hills, Ponmudi hills and Ooty hills (Plate V). But it is rare in Palni hills. Terrestrial on the forest floor in shade. Rarely seen along shaded roadsides and waysides from 300-2000 m (Manickam and Irudayaraj, 1992).

Botanical name: *Selaginella wightii* Hieron.

Morphology of *S. wightii*

Small plants, about 10 x 4 cm; stem terete, up to 1mm in diameter without leaves, bearing roots occasionally all over the main stem which bears many main branches; main branches branched again many times unequally and irregularly. Leaves dense, spirally arranged, greenish-black, linear, up to 2 x 0.2 mm, apex long acuminate, margin with membranaceous, ciliate border. Cones borne on ultimate branches, very slightly distinct from sterile branches, more or less quadrangular, up to 1.5 x 0.5 mm; sporophylls uniform, spirally arranged, ovate, apex acuminate, base cordate, margin denate, midvein distinct, two short ligule present at the subbasal region; microsporangia and megasporangia borne on the same spike, spherical, up to 1mm in diameter; microspore trilete, 50 x 40 µm, yellowish-brown with prominent, branched ridges which converge to the centre, megaspores up to 250 µm, dark brown (Plate VI).

Distribution: Very rare species. Occasional on Marunthuvazh Malai (Plate VI). Growing on rock crevices in dry places along roadsides between 350-1500 m (Manickam and Irudayaraj, 1992).

Botanical name: *Selaginella brachystachya* (Hook. & Grev.) Spring

Morphology of *S. brachystachya*

Stem sub erect to prostrate, up to 2.5 mm thick, stramineous, branched from the base; rhizophores borne occasionally all over the stem. Leaves dimorphic throughout, arranged in four rows, sparse on main stem, dense on lateral branches; lateral leaves alternate up to 5 mm

apart on main stem, up to 6 x 1 mm aristate, arista more than half the length of the leaf, margin entire, cones flattened, dorsiventral, 1 x 0.2 cm; sporophylls dimorphic, lateral sporophyll spreading, oblanceolate, 2 x 0.5 mm, acute, entire; median sporophyll ovate, up to 2 x 0.75 mm, aristate, arista more than the length of the sporophyll; microspores yellowish-brown, 25 µm in diameter, megaspores 300 µm, exine almost smooth (Plate VII) .

Distribution: *S. brachystachya* is confined to Sri Lanka, Maharashtra and South India. Rare on Anamalais, Kalakad hills, Palni hills and Agasthiar hills (Plate VII). Terrestrial on forest floor or at forest edges in shade between 1200-2000 m (Manickam and Irudayaraj, 1992).

Botanical name: *Selaginella repanda* (Desv.) Spring

Morphology of *S. repanda*

Stem erect, rooting at the base, occasionally near the distal part, stramineous, up to 1 mm thick without leaves, up to 4 mm wide with leaves, entire plant about 20 x 5 cm, primary branches alternate, branched two to six times. Leaves dimorphic throughout lateral leaves ovate-oblong, 3 x 1 mm, apex obtuse or subacute, margin ciliolate on the acroscopic base, denticulate on the rest of the part; median leaves ovate, acute or acuminate, 1 x 0.75 mm, margin sparsely denticulate. Cones about 5 x 2 mm, quadrangular; sporophylls uniform, ovate, 1 x 0.5 mm, acute, margin denticulate; microspores reddish brown, 30 µm in diameter, megaspores light green, 300 µm in diameter (Plate VIII).

Distribution: Occasional on Palni hills. Rare on Muzhiar-Kakki hills (Plate VIII). Terrestrial on shady, moist soil cover between 1000-1200 m (Manickam and Irudayaraj, 1992).

Botanical name: *Selaginella radicata* (Hook. & Grev.) Spring

Morphology of *S. radicata*

S. radicata grows up to 18 x 9 cm; irregular in shape, stem prostrate, rooting occasionally all over. Leaves at the base of main stem uniform, ovate, up to 2 x 1.5 mm, acute, entire, leaves on the rest of the part of main stem and lateral branches dimorphic;

median leaves ascending, ovate, up to 1 x 1 mm, ciliolate; lateral leaves patent, narrowly ovate, apex subacute, margin finely denticulate; leaves pale green, cones 6 x 2 mm, four-sided, sporophylls uniform, 1.5 x 1 mm, broadly ovate, dentate, megasporangia borne at the basal region, microsporangia borne towards the diatal part of the cone; microspores pale brown, triangular with rounded corners, up to 35 µm in diameter, exine with thin densely anastomosed thickenings; megaspore three per sporangium, pale green, up to 300 µm more or less spherical, exine almost smooth (Plate IX).

Distribution: Rare species. *S. radicata* is confined to South India (Plate IX). Terrestrial on moist earth banks along roadsides between 110-1200 m (Manickam and Irudayaraj, 1992).

Botanical name: *Selaginella bryopteris* (L.) Bak.

Morphology of *S. bryopteris*

Stems 5-25 cm, sub-erect to erect, simple in the basal lower third but profusely branched above, branches flabellate. Rhizophores confined at the base. Leaves uniform on the main stem, distant, ovate-lanceolate, denticulate, long-acuminate at tip; heteromorphic on the branches, contiguous, ascending; lateral leaves ovate, oblique at base, acute to acuminate at apex, midrib short, obscure, linear half-leaf dilated, thin, white-transluscent, imbriate, denticulate, outer half-leaf thick, green; axillary leaves ± similar to the leaves; median leaves small, ovate, oblique at base, acute to acuminate at a apex, entire to minutely denticulate. Strobili rare due to prevalence of vegetative propagation, 3-5 x 1-2.5 mm, single at the apex of the branchlets. Sporophylls uniform, ovate, entire to minutely denticulate, acuminate. Megaspores 200-270 µm, dull-yellow, verrucoid. Microspores 15-25 µm, yellow, granulose (Plate X).

Distribution: *S. bryopteris* distributed in India viz., Madhya Pradesh, Uttar Pradesh, Bihar, Orissa, Tamil Nadu, Kerala and Maharashtra (Baishya and Rao, 1982).

Botanical name: *Selaginella delicatula* (Desv.) Alston

Morphology of *S. delicatula*

Stem erect or sub-erect, rooting at the base only, up to 4 mm thick without leaves, stramineous; entire plant up to 45 x 15 cm, lateral branches many, alternate, tripinnate; leaves scattered and oblique on main stem, arranged in four rows on lateral branches; lateral leaves about 2.5 x 1.5 mm, ovate, obtuse, entire; median leaves ovate, 2 x 1 mm, aristate, entire, arista less than half the length of the leaf. Spikes borne on ultimate branches, quadrangular, up to 2 x 0.2 cm; sporophylls uniform, ovate, acuminate, entire, 2 x1 mm; microspores green, 30 µm in diameter, megaspores pale brown, 400 µm in diameter (Plate XI; Plate XII).

Distribution: Common on Kerala Ghats (Plate XI; Plate XII). Common on stone walls or crevices of rocks along roadsides between 0-1000 m (Manickam and Irudayaraj, 1992).

Biochemical studies

Protein isolation

Fresh and young leaves of selected ten species of *Selaginella* viz., *S. intermedia*, *S. inaequalifolia*, *S. involvens*, *S. tenera*, *S. wightii*, *S. repanda*, *S. brachystachya*, *S. radicata*, *S. bryopteris*, *S. delicatula* with cones and *S. delicatula* without cones were collected from various localities of South India. For protein analysis, the collected young leaves of selected *Selaginella* species were washed once in deionized water and mashed in a pre-chilled mortar using 500 µl of phosphate buffer (pH 7.0). The resultant slurry was centrifuged at 10,000 rpm for 10 min at 4°C in an Eppendorf centrifuge and the supernatant was stored at 4°C before use.

Protein separation

SDS-PAGE (Anbalagan, 1999) was carried out at 25°C in the air conditioned room. Separation of protein was carried out at 50V till the tracking dye reaches the separating gel

and at 100V thereafter for 3-5 hours or until the tracking dye had migrated to the bottom of the gel. After electrophoresis, the gels were carefully removed from the mold and stained.

Gel mold

Two precisely cutted glass plates were clamped together with Vaseline coated 1.0 mm spacers on each side and sealed on both sides and bottom.

Gel preparation

Reagents for gel preparation

Solution A - 29.2g Acrylamide and 0.8 g Bis acrylamide in 100 ml of distilled water.

Solution B - Tris HCl 1.5M, pH 8.8

Solution C - Tris HCl 0.5M, pH 6.8

Solution D - 10% (w/v) Ammonium per sulphate

10% (v/v) separation gel consisted of 3.3ml of sol. A, 2.5ml of sol. B, 100µl of sol. D, 4.1ml of distilled water and 10µl TEMED. The solution was mixed and poured in up to three-fourth of the mold and allowed to polymerize. A thin layer of distilled H_2O was dispersed on top of the acrylamide solution to level the surface.

6% (v/v) stacking gel consisted of 0.6 ml of sol. A, 1.25 ml of sol. C, 50 µl of sol. D, 2.1 µl distilled H_2O and 6 µl TEMED. The solution was mixed and added to the top of the separating gel after the removal of water topped over the separating gel. The comb was inserted into the top layer of the solution before it began to polymerize. The gel was allowed to polymerize and the comb was removed immediately after polymerization. The molded gel was clamped on to a vertical slab gel electrophoresis unit. The upper and lower reservoirs of the electrophoresis unit were filled with required quantity of 1.5M Tris-glycine (pH 8.3) tank buffer (Table 2 and 3).

Table 2: Chemical preparation for SDS-PAGE

Chemicals	Preparation
Acrylamide stock 30%	29.2g Acrylamide + 0.8g Bis Acrylamide in 100 ml water
Lower Tris	pH 8.8 for separating gel, 36.34g of Tris made up to 200 ml with distilled water + 1% SDS
Upper Tris	pH 6.8 for stacking gel, 12.1g Tris made up to 200 ml with distilled water + 1% SDS
Ammonium persulphate	0.05g in 0.5ml distilled water
Running Gel Buffer (5X)	Tris 15.0g + 5% SDS + Glycine 72g in 1000 ml distilled water. Dilute 300 ml 5X stock with 1200 ml Distilled water. Do not adjust the pH.
Sample Buffer	7.25ml distilled water + 1.25 ml stocking gel buffer + 1ml of Glycerol + 1% SDS and bromophenol blue.

Table 3: SDS-PAGE preparation

10% Separating Gel : Lower Tris	
30% Acrylamide	10 ml
Lower Tris (8.8 pH)	7.5 ml
Distilled water	12.3 ml
APS	150 µl
TEMED	50 µl
6% Stacking Gel : Upper Tris	
30% Acrylamide	2 ml
Upper Tris (6.8 pH)	3 ml
Distilled water	4.9 ml
APS	75 µl
TEMED	25 µl

Sample loading

50-60 µl of each sample was loaded directly into a well using a syringe. Care was taken to avoid mixing of the sample with the reservoir buffer and also to avoid cross

contamination of samples. After electrophoresis the gels were stained using the method described by Mortz *et al*. (2001) and Sorensen *et al*. (2002).

SILVER STAINING

Methodology for silver staining

- After electrophoresis, the gel was removed from the cassette and placed into a tray containing appropriate volume of fixing solution, and then the gel was soaked in fixing solution for 2 h.
- The fixative solution was discarded and the gel was washed in 20% ethanol for 20 min. The solution was changed three times to remove the remaining detergent ions as well as fixation acid from the gel.
- The ethanolic solution was discarded and enough volume of the sensitizing solution was added and incubated for 2 min with gentle rotation.
- The sensitizing solution was discarded and the gel was washed twice, 1 min each time, in deionized water. Then the water was discarded.
- The cold silver staining solution was added and shaked for 20 min to allow the silver ions to bind with proteins.
- After staining, the staining solution was poured off and the gel was rinsed with a large volume of deionized water for 20-60 sec to remove the excess of unbound silver ions. Repeat the washing once more.
- Again the gel was shortly rinsed with the developing solution and then the solution was discarded.
- To stop the reduction reaction 50 ml of terminating solution was added directly to the gel that is still immersed into developing solution. The gel was gently agitated for 10 mins. As soon as “boubling” of the solution is over, the development is stopped.

- Moist gels was kept in 12% acetic acid at 4°C in sealed plastic bags or placed in the drying solution for 2 h prior to vacuum drying.
- After staining the gel was viewed using a Vilber Loubermat gel documentation system and banding profiles of protein was determined by the migration from the origin towards the anode. The chemicals needed for silver staining were prepared as illustrated in Table 4.

To reveal the inter-specific relationship among the studied *Selaginella* species, the SDS-PAGE protein profiles of the studied *Selaginella* species converted in to "1" and "0" matrix, to indicate the presence or absence of bands, respectively. Genetic similarities (GS) were estimated according to Nei and Li (1979) algorithm. To demonstrate the inter-specific relationship, a cladogram was constructed by UPGMA using NTSYSpc- 2.0 software.

Table 4: Chemical preparation for silver staining

Reagents	Preparation of chemicals
Fixation solution	50% ethanol + 12% glacial acetic acid + 0.05% formalin + 100 ml distilled water
Washing solution	20% ethanol + 100 ml distilled water
Sensitizing solution	0.02% sodium thiosulphate + 100 ml distilled water
Deionized water	Washing
Staining	0.2% silver nitrate + 0.076% formalin + 100 ml distilled water
Developing solution	6% sodium carbonate + 0.0004% sodium thiosulphate + 0.05% formalin + 100 ml distilled water
Terminating solution	12% acetic acid
Drying solution	20% ethanol

MALDI-TOF MS analysis

MALDI spectrum of *Selaginella* species were recorded using Applied Biosystems MALDI-TOF Voyager De-Pro spectrometer. The MALDI sample was prepared by mixing 1µL of protein sample solution and sinapic acid matrix solution (5 mg/mL sinapic acid in 50% ACN/0.1% TFA). 0.75µL of the resulting mixture was spotted onto a freshly cleaned stain less steel MALDI target plate. After air drying, the crystallized spots were processed with a MALDI-TOF mass spectrometer (voyager DE PRO, Applied Biosystem). MS was recorded in the positive and negative mode within a mass range from 500 to 1, 00, 000 kDa, using a nitrogen laser (337 nm). The acceleration voltages applied for MS was 25 kV.

Isozymic variation

Five enzyme systems resolved in the selected *Selaginella* species were: peroxidase (PO), esterase (EST), acid phosphatase (ACP), alkaline phosphatase (ALP) and polyphenol oxidase (PPO).

Preparation of enzyme extract

To know the isozymic variation of the selected *Selaginella* species, the collected young leaves were washed once in deionized water. For isozymic analysis, young 2-3 month old plants from the natural habitat were collected in the wet paper and brought to the laboratory. After wash in tap water, the leaves were dissected out and the crude enzyme extracts were prepared by grinding in different buffers at varied pH based on enzymatic system (Table 5) as described by Sadasivam and Manickam (1991). The slurry was centrifuged at varied rpm at 4°C in a cooling centrifuge and the supernatant is stored at 4°C before use. The isolated enzymes then separated using PAGE.

Separation of isozyme

- Polyacrylamide Gel Electrophoresis (PAGE) is carried out at 4°C using vertical gel electrophoresis unit.

Separation of isozymes were carried out at 50V till the tracking dye reaches the separating gel and at 100V thereafter for 3-5 hours or until the tracking dye had migrated to the bottom of the gel.

Table 5: Isolation buffers based on enzyme system

Enzymes	Buffer with varied pH	rpm
Esterase	0.1M phosphate buffer (pH-7.0)	10,000 for 10 minutes
Peroxidase	1M sodium acetate buffer (pH-5.0)	10,000 for 10 minutes
Acid phosphatase	50 mM citrate buffer (pH-5.3)	10,000 for 10 minutes
Alkaline phosphatase	50 mM glycine buffer (pH-10.4)	10,000 for 10 minutes
Polyphenol oxidase	0.01 M phosphate buffer (pH-7.0) and 1% Tween 80	20,000 for 15 minutes

Gel mold

Two precisely cut glass plates are clamped together with Vaseline coated 1.0 mm spacers on each side and sealed on both sides and bottom.

Gel preparation

Reagents for gel preparation

Solution A - 29.2 g Acrylamide and 0.8 g Bis acrylamide in 100 ml of distilled water.

Solution B - Tris HCl 1.5 M, pH 8.8

Solution C - Tris HCl 0.5 M, pH 6.8

Solution D - 10% (w/v) Ammonium per sulphate

10% (v/v) separation gel consisted of 3.9 ml of Sol. A, 3 ml of Sol. B, 4.9 ml of distilled water, 60 µl of Sol. D and 47 µl of TEMED. The solution was mixed and poured in up to three-fourth of the mold and allowed to polymerize. Disperse a thin layer of ethanol on top of the acrylamide solution to level the surface.

6% (v/v) stacking gel consisted of 0.6 ml of Sol. A, 1 ml of Sol. C, 1.5 ml of distilled water, 33 µl of Sol. D and 23 µl of TEMED. The solution was mixed and added to the top of the separating gel after the removal of ethanol topped over the separating gel. The comb was

then inserted into the top layer of the solution before it began to polymerize. The gel was allowed to polymerize and the comb was removed immediately after polymerization. The molded gel was clamped on to a vertical slab gel electrophoresis unit. The upper and lower reservoirs of the electrophoresis unit were filled with required quantity of 1.5 M Tris - glycine tank buffer (Table 6 and 7).

Table 6: Chemical preparation for PAGE

Chemicals	Preparation
Acrylamide stock 30%	29.2g Acrylamide + 0.8g Bis Acrylamide + 100ml water
Lower Tris	pH 8.8 for separating gel, 36.34g of Tris made up to 200ml with distilled water
Upper Tris	pH 6.8 for stacking gel, 12.1g Tris made up to 200ml with distilled water
Ammonium persulphate	0.05g in 0.5ml distilled water
Running Gel Buffer(5X)	Tris 15.0g + Glycine 72g in 1000 ml distilled water. Dilute 300 ml 5 x stock with 1200 ml Distilled water. Do not adjust the pH.
Sample Buffer	7.25ml distilled water +1.25ml stocking gel buffer + 1ml of Glycerol and bromophenol blue.

Table 7: PAGE preparation

10% Separating Gel : Lower Tris	
30% Acrylamide	10 ml
Lower Tris (8.8 pH)	7.5 ml
Distilled water	12.3 ml
APS	150 µl
TEMED	50 µl
6% Stacking Gel : Upper Tris	
30% Acrylamide	2 ml
Upper Tris (6.8 pH)	3 ml
Distilled water	4.9 ml
APS	75 µl
TEMED	25 µl

Sample loading

50-60 µl of each sample was loaded directly into a well using a micropipette. Care was taken to avoid mixing of the sample with the tank buffer and also to avoid cross contamination of samples. Staining procedures were varied depending upon the specific assays.

Esterase assay

- The enzymes separated in the gel are actively stained with the specific reaction mixture for the isozyme esterase (Table 8).

Table 8: Staining reagents for esterase activity

Reagents	**Volume**
1M phosphate buffer (pH-7.0)	10 ml
Fast blue B salt	100 mg
α-napthyl acetate	80 mg
Distilled water	4 ml
Methanol	60 ml

- The gel was incubated in the dark at 37°C for 20-30 minutes.
- After incubation the gel was fixed in methanol: water: acetic acid: ethanol for 15 minutes.

Peroxidase assay

- The enzymes separated in the gel are actively stained with the specific reaction mixture for the isoperoxidase (Table 9).

Table 9: Staining reagents for peroxidase activity

Reagents	Volume
0.1 M phosphate buffer (pH-7.0)	28 ml
O- dianizidine	100 mg
Distilled water	4 ml
H_2O_2	6 ml
Methanol	60 ml

- The gel was incubated in the dark at 30°C for 30 minutes.
- After incubation the gel was fixed in 7% acetic acid for 15 minutes.

Acid phosphatase assay

- The enzymes separated in the gel are actively stained with the specific reaction mixture for the isozyme acid phosphatase (Table 10).

Table 10: Staining reagents for acid phosphatase activity

Reagents	Volume
50 mM Tris buffer (pH-5.3)	100 ml
Fast blue RR salt	100 mg
α-napthyl phosphate	50 mg
Magnesium chloride	230 mg

- The gel was incubated in the dark at 37°C for 3-4 hrs.

Alkaline phosphatase assay

- The enzymes separated in the gel are actively stained with the specific reaction mixture for the isozyme alkaline phosphatase (Table 11).
- The gel was incubated in the dark at 37°C for 3-4 hrs.

Table 11: Staining reagents for alkaline phosphatase activity

Reagents	Volume
0.1 N NaOH buffer (pH-10.5)	42 ml
Glycine	375 mg
p-nitrophenyl phosphate	165 mg
Magnesium chloride	10 g

Polyphenol oxidase assay

- The enzymes separated in the gel are actively stained with the specific reaction mixture for the isozyme polyphenol oxidase (Table 12).

Table 12: Staining reagents for polyphenol oxidase activity

Reagents	Volume
0.1 M sodium phosphate buffer (pH-6.8)	100 ml
Catechol	15 mg
Sulphanilic acid	50 mg

- The gel was incubated in the dark at 37°C for 5-10 minutes.

DNA BARCODING

For the present study, ten selected species of *Selaginella* were examined for DNA polymorphism. DNA was extracted from (0.01-0.05 g) liquid nitrogen grounded leaves sample using a modified Cetyl Trimethyl Ammonium Bromide (CTAB) extraction method described by Murray and Thompson (1980); Doyle and Doyle (1987); Rogers and Benedich (1994).

Procedure:

Step I

- The young leaves samples were grounded using liquid nitrogen at -196°C to a fine powder by using mortar and pestle.
- 1 gm of Liq.N_2 grinded leaves samples of *Selaginella* taken for the isolation of DNA.
- 1% β-mercaptoethanol was added to the extraction buffer and warmed at 65°C for 5-10 mins.
- 1 ml of warm extraction buffer was added to the ground sample.
- Then it was thoroughly mixed to make it slurry and transferred to 3 ml eppendorf tubes.
- These tubes were incubated at 65°C for 1-2 hrs.

Chloroform extraction

- Chloroform: isopropanol mixture was prepared in 24:1 ratio and it was added in equal volume to the slurry.
- The slurry was centrifuged at 10,000 rpm for 10 min at 4°C.
- Supernatant was taken and $1/10^{th}$ volume of CTAB/NaCl and equal volume of chloroform was added.
- It was centrifuged at 10,000 rpm for 5 min at 4°C.
- To the clear (greenish yellow) supernatant, double the volume of CTAB precipitation buffer was added.
- The mixture was incubated at 37°C overnight in a water bath.

Step II

- The precipitation mixture was centrifuged at 8000 rpm for 8-10 min at 4°C.
- The pellet was collected and the supernatant was discarded.
- 1 ml of high salt TE was added to re-suspend the pellet.

- The suspended pellets were transferred to glass tubes.
- 1.0 ml (0.6 volumes) of isopropanol was added and refrigerated at -20°C for 30 min to get the pellets.
- After the precipitates were observed, the tubes were centrifuged at 8,000 rpm for 5 min at 4°C.
- The pellets were washed with 1ml of 80% ethanol by centrifuged at 10,000 rpm for 5 min at 4°C to remove the residual CTAB.
- The pellets were resuspended in 0.5 ml 1X TE.
- The suspended pellets were collected in micro-vials.

Step III

- The overnight incubated vials were spun at 12,000 rpm for 15 min at 4°C.
- The pellets were collected and washed with 0.5 ml of 70% ethanol.
- Centrifugation was done at 10,000 rpm for 5 min at 4°C.
- The pellets were re-suspended in 100 µl 1X TE.
- The re-suspended pellets were collected and stored at -20°C.

Verification of DNA levels in Agarose gel electrophoresis

- The sides of the gel tray were taped to hold the gel while setting and well forming combs were placed with tray.
- 0.8% agarose gel was prepared by mixing 1.5 g agarose with 150 ml 1X TBE Buffer. It was boiled in a microwave oven until the agarose dissolves (1-2 minutes).
- Cooled down by holding under a running cold tap; once cooled and 6 µl of ethidium bromide was added (make sure to work in the fume hood) and swirlled to mix.
- Then the gel was poured into the tray and allowed to stand for at least 30 minutes before loading the samples. The combs are removed and placed the gel into the electrophoresis tank.

- 5 µl of Loading/Tracking dye - sucrose mixture (Bromophenol blue and Xylene cyanol) was spotted for each 25 µl of the DNA sample on a strip of parafilm.
- 5 µl of DNA was mixed using a pipette with the loading buffer and the mix was loaded into a well on the gel and allowed to run for about 30 minutes at 110 Milli Amps.
- After electrophoresis the gel was viewed under the ultraviolet light box and the gel was photographed.

The chemicals required for DNA isolation and identification are depicted in Table 13.

Table 13: Reagents used for DNA isolation

Extraction Buffer (pH 8.0)	
CTAB	2.0 gm
1M Tris HCl	10.0 ml
0.5 M EDTA	4.0 ml
1.4 M NaCl	8.2 gm
PVP	1.2%
Made up to 100 ml with distilled water	
Precipitation Buffer (pH 8.0)	
CTAB	1.0 gm
50 mM Tris HCl	5.0 ml
10 mM EDTA	2.0 ml
Made up to 10 ml with distilled water	
Stock Precipitation Buffer (TE Buffer)	
1M Tris HCl	100 ml
0.5M EDTA	50 ml
1X TE Buffer	
1M Tris HCl	1.0 ml
0.5 M EDTA	0.2 ml
Made up to 100 ml with distilled water	
High Salt TE	

1M NaCl 10mM Tris HCl (pH 8.0) 0.1mM EDTA (pH 8.0)	5.85 gm 1.0 ml 20µl
Made up to 100 ml with distilled water	
10X TBE (pH 8.0)	
Tris base Boric acid 0.5 M EDTA (pH 8.0)	21.6 gm 11.0 gm 8.0 ml
Made up to 200 ml with distilled water and the pH was adjusted with NaOH	
CTAB/ NaCl (10 % CTAB/0.2M NaCl)	
CTAB NaCl	5.0 gm 2.04 gm
Made up to 50 ml with distilled water	

PCRs were conducted in a total reaction volume of 30 µL containing 6 µL of autoclaved ion-exchanged water, 5µL of dNTP mixture (stock of 10 mM of each dNTP), 13µL of 10x Taq reaction buffer (200 mM Tris pH 8.8, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 20mM $MgSO_4$.7H2O, 1% (v/v) Triton X-100, 50% (w/v) sucrose, 0.25% (w/v) cresol red), 2µL of *rbcL* primer (0.67 mM final concentration), 2µL of Taq DNA polymerase and 2 µL of genomic DNA. The amplicon size of *rbcL* ranges from 654 bp; primers used (5'–3') were ATGTCACCACAAACAGAGACTAAAGC and GAAACGGTCTCTCCAACGCAT (Kress and Erickson, 2007; Fazekas *et al*., 2008). PCR Samples were loaded after mixing with gel loading dye along with 10µl of DNA Ladder (1000bp DNA Ladder viz., 100bp, 200bp, 300bp, 400bp, 500bp, 600bp, 700bp, 800bp, 900bp, 1000bp) (Table 14).

The PCR for *rbcL* was performed with an initial denaturation of 30 sec at 94°C followed by 35 cycles under the following conditions: 94°C for 30 seconds, 58°C for 30 sec and 72°C for 30 sec, terminated by an extension of 72°C for 5 min.

Table 14: Composition of PCR mixture

Components	Quantity
In PCR vial Master mix	20µl
rbcL Primer mix (5pmoles/µl)	2µl
Genomic DNA	2µl
Water, nuclease free	6µl
Total volume	30µl

Cycle sequencing

PCR *rbcL* products were sequenced after simple purification and the DNA samples were sequenced according to the method originally described by Sanger's method 1977 on an ABI Prism 3100-Avant Genetic Analyzer (USA). The electrophoresis was performed at 50V till the dye reaches three fourth distance of the gel.

Gel viewed in UV Transilluminator and the banding pattern was observed and the obtained sequence was aligned using the BioEdit sequence alignment editor version 7.0.4.1.

Sequencing and alignment

The sequences from each DNA region were aligned using CLUSTAL W and MULTALIN software tools. The nucleotide sequence data of the partial *rbcL* sequence spacer were deposited in the Genbank nucleotide sequence databases (NCBI). The genetic distance was calculated using the similatity indices and the phylogenetic trees were constructed using MEGA 5.0 software (Tamura *et al*., 2011).

Phylogenetic analysis

Phylogenetic analysis involved sequences of ten *Selaginella* species collected from different localities of South India. The analysis involved the selected nucleotide sequences. Codon positions included were 1^{st}, 2^{nd}, 3^{rd} and Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 567, 548, 537, 401, 480, 593, 570, 582, 571, 563 and 574 positions in the final dataset of inter-specific sequence analysis of ten

species of *Selaginella*. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA 5 (Tamura *et al*., 2011) in four different methods viz., Neighbor-Joining method, Maximum parsimony, minimum evolution method and UPGMA method.

Neighbor-Joining method

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.45655325 is shown. The evolutionary distances were computed using the Maximum composite likelihood method (Tamura *et al*., 2004). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. There were a total of 468 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011).

Maximum parsimony analysis

The evolutionary history was inferred using the maximum parsimony method. The most parsimonious tree with length 165 is shown. The consistency index is 0.890909, the retention index is 0.946429, and the composite index is 0.843182 for all sites and parsimony informative sites The maximum parsimony tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm and branch lengths were calculated using the average pathway method (Nei and Kumar, 2000) with search level 0 in which the initial trees were obtained by the random addition of sequences (10 replicates).

Minimum evolution method

The evolutionary history was inferred using the minimum evolution method (Rzhetsky and Nei, 1992). The optimal tree with the sum of branch length = 0.45729602 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the

evolutionary distances used to infer the phylogenetic tree. The confidence probability (multiplied by 100) that the interior branch length is greater than 0, as estimated using the bootstrap test (500 replicates) is shown next to the branches (Dopazo, 1994; Rzhetsky, 1992). The evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al*., 2004). The minimum evolution tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (Nei and Kumar, 2000) at a search level of 1. The Neighbor-Joining algorithm (Saitou and Nei, 1987) was used to generate the initial tree.

UPGMA method

The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The optimal tree with the sum of branch length is 0.45504821. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al*., 2004).

The biochemical and molecular analysis of the selected ten species of *Selaginella* were done using the techniques of SDS-PAGE (protein separation), MALDI-TOF MS (mass spectroscopic analysis), PAGE (isozymic profile) and DNA barcoding using *rbcL* gene to identify the molecular relationship among them.

Protein profiling of *Selaginella* species

The relative positions of the protein bands of the studied *Selaginella* species viz., *S. intermedia*, *S. inaequalifolia*, *S. involvens*, *S. tenera*, *S. wightii*, *S. brachystachya*, *S. repanda*, *S. radicata*, *S. bryopteris*, *S. delicatula* with and without cones collected from various localities of South India were revealed by SDS-PAGE. Multiple regions of activity were observed in the protein electrophoretic system of *Selaginella* species. A total of 190 bands with various Rf values and molecular weight were displayed in the SDS-PAGE gel system of *Selaginella* (Table 15; Plate XIII) Among the ten species of *Selaginella*, *S. repanda* showed maximum number of protein bands (25) followed by *S. tenera* (24), *S. radicata* (21), *S. intermedia* (19), *S. brachystachya* (18), *S. wightii* (17). *S. involvens* and *S. delicatula* without cones expressed equal number of bands (14). *S. inaequalifolia* and *S. bryopteris* depicted equal number of protein bands (13). *S. delicatula* showed minimum number of bands (12). The recorded data illustrated the role of protein in expressing the genetic similarity and/or variation between the studied species of *Selaginella*. Each region expressed different proteins which act as representative of the expression of a particular gene.

Based on the occurrence of proteins in the *Selaginella* gel system, the protein profiles were classified into ten regions. Region 1 observed with seven positions and eighteen bands. $PP1^{1}$ (Rf- 0.02; MW-281.8 kDa) showed its presence in *S. intermedia*, *S. inaequalifolia*, *S. involvens*, *S. tenera*, *S. wightii*, *S. radicata* and *S. bryopteris*. $PP1^{2}$ (Rf-0.03; MW-272.6 kDa)

was shared by *S. brachystachya* and *S. repanda*. $PP1^3$ (Rf-0.04; MW-251.1 kDa), $PP1^4$ (Rf-0.05; MW-223.8 kDa) and $PP1^7$ (Rf-0.09; 169.8 kDa) expressed its unique presence in *S. brachystachya*, *S. bryopteris* and *S. repanda* respectively. $PP1^5$ (Rf-0.07; MW-199.5 kDa) dispersed mutually by *S. intermedia* and *S. wightii*. $PP1^6$ (Rf-0.08; MW-177.8 kDa) displayed its common occurrence in *S. involvens*, *S. tenera*, *S. delicatula* with and without cones. Region 1 described the similarity among the studied species of *Selaginella*.

Region 2 displayed twenty two bands in nine positions. $PP2^1$ (Rf-0.10; MW-162.2 kDa) was shared by *S. intermedia* and *S. bryopteris*. Similarly, $PP2^2$ (Rf-0.11; MW-158.4 kDa) expressed its mutual presence in *S. radicata* and *S. bryopteris*. $PP2^3$ (Rf-0.12; MW-154.8 kDa) represented its common occurrence in *S. inaequalifolia*, *S. brachystachya* and *S. repanda*. $PP2^4$ (Rf-0.13; MW-141.2 kDa) was universal to *S. intermedia* and *S. involvens*. $PP2^5$ (Rf-0.14; MW-135.8 kDa) illustrated its identical existence in *S. inaequalifolia*, *S. tenera* and *S. radicata*. $PP2^6$ (Rf-0.16; MW-123 kDa) displayed its presence in *S. intermedia*, *S. wightii* and *S. bryopteris*. $PP2^7$ (Rf-0.17; MW-111.3 kDa) observed mutually in *S. tenera* and S. *radicata*. $PP2^8$ (Rf-0.18; MW-100 kDa) occupied its occurrence equally in *S. brachystachya* and *S. repanda*. $PP2^9$ (Rf-0.19; MW-99.1 kDa) was evenly distributed in *S. involvens* and *S. tenera* respectively. No unique band was found in region 2. Thus, region 2 clearly explained the biochemical similarity and phylogenetic relationships among the studied *Selaginella* species.

Region 3 depicted twenty one bands in seven positions. $PP3^1$ (Rf-0.21; MW-98.7 kDa) expressed its common occurrence in *S. brachystachya*, *S. repanda* and *S. radicata*. $PP3^2$ (Rf-0.22; MW-96.2 kDa) was shared by *S. delicatula* with and without cones. $PP3^3$ (Rf-0.23; MW-95.4 kDa) was unique to *S. brachystachya*. $PP3^4$ (Rf-0.25; MW-93.3 kDa) observed commonly in *S. tenera*, *S. wightii*, *S. radicata* and *S. bryopteris*. $PP3^5$ (Rf-0.26; MW-92.5 kDa) represented its presence in *S. intermedia*, *S. tenera* and *S. radicata*. $PP3^6$ (Rf-0.27; 89.1

kDa) illustrated its common existence in *S. wightii*, *S. brachystachya* and *S. repanda*. PP3[7] (Rf-0.29; MW-87 kDa) was displayed by five species of *Selaginella* viz., *S. intermedia*, *S. involvens*, *S. tenera*, *S. brachystachya* and *S. radicata*.

Region 4 represented seventeen bands with eight positions. PP4[1] (Rf-0.30; MW-81.2 kDa) was common to *S. repanda* and *S. bryopteris*. PP4[2] (Rf-0.32; MW-79.4 kDa) was uniformly distributed in *S. intermedia*, *S. tenera* and *S. radicata*. PP4[3] (Rf-0.33; MW-70.7 kDa) was observed jointly in *S. tenera* and *S. radicata*. Similarly, PP4[4] (Rf-0.34; MW-63 kDa) explained its mutual presence in *S. brachystachya* and *S. repanda*. PP4[5] (Rf-0.35; MW-61.6 kDa) expressed its unique existence in *S. intermedia*. *S. wightii* illustrated two distinct bands with positions PP4[6] (Rf-0.36; MW-59 kDa) and PP4[8] (Rf-0.39; MW-55.9 kDa) which explained its variation with the other studied species of *Selaginella*. PP4[7] (Rf-0.38; MW-57.5 kDa) displayed its occurrence in *S. intermedia*, *S. tenera*, *S. brachystachya*, *S. repanda* and *S. radicata*. Region 5 depicted fifteen bands with five positions only. PP5[1] (Rf-0.40; MW-53.8 kDa) was distinct to *S. repanda* that explained its special occurrence in the region 5. PP5[2] (Rf-0.41; MW-52.1 kDa) was observed in *S. tenera*, *S. repanda* and *S. bryopteris*. PP5[3] (Rf-0.43; MW-50.1 kDa) was common to *S. brachystachya* and *S. repanda*. PP5[4] (Rf-0.45; MW-47.4 kDa) expressed commonly in *S. involvens*, *S. tenera*, *S. wightii*, *S. repanda*, and *S. delicatula*. PP5[5] (Rf-0.47; MW-46.1 kDa) displayed its common existence in *S. intermedia*, *S. repanda*, *S. bryopteris* and *S. delicatula* without cones.

Region 6 represented twenty three protein bands with six different positions. PP6[1] (Rf-0.50; MW-44.6 kDa) was distributed in *S. wightii*, *S. brachystachya* and *S. repanda*. PP6[2] (Rf-0.51; MW-43.2 kDa) was commonly expressed in *S. intermedia*, *S. inaequalifolia*, *S. involvens*, *S. radicata*, *S. delicatula* with and without cones. PP6[3] (Rf-0.52; MW-41.9 kDa) was displayed its unique presence in *S. tenera*. PP6[4] (Rf-0.54; MW-39.8 kDa) was observed in *S. wightii*, *S. repanda*, *S. bryopteris* and *S. delicatula*. PP6[5] (Rf-0.57; MW-38 kDa) was

found common in *S. intermedia*, *S. brachystachya* and *S. radicata*. PP6[6] (Rf-0.58; MW-37.1 kDa) showed its presence uniformly in *S. involvens*, *S. tenera*, *S. brachystachya*, *S. repanda*, *S. delicatula* with and without cones.

Region 7 expressed fourteen bands with five different positions. PP7[1] (Rf-0.60; MW-35.6 kDa) was shared by *S. inaequalifolia* and *S. wightii*. PP7[2] (Rf-0.61; MW-33.1 kDa) showed its exclusive presence in *S. delicatula* without cones which explained its variation with other studied species of *Selaginella*. PP7[3] (Rf-0.63; 30.9 kDa) displayed uniformly in *S. tenera*, *S. brachystachya*, *S. repanda* and *S. radicata*. PP7[4] (Rf-0.67; MW-29.1 kDa) showed its common occurrence in *S. involvens*, *S. tenera* and *S. wightii*. PP7[5] (Rf-0.69; MW-28 kDa) displayed its presence in *S. intermedia*, *S. brachystachya*, *S. repanda* and *S. delicatula* without cones.

Region 8 occupied twenty one bands with eight positions. PP8[1] (Rf-0.70; MW-27.3 kDa) and PP8[4] (Rf-0.75; MW-22 kDa) showed its unique presence in *S. inaequalifolia* and *S. tenera* respectively which explained its variation with other studied species of *Selaginella*. PP8[2] (Rf-0.72; MW-25.1 kDa) showed its mutual existence in *S. involvens* and *S. tenera*. PP8[3] (Rf-0.73; MW-24.8 kDa) elucidated its common occurrence in *S. intermedia*, *S. inaequalifolia*, *S. wightii*, *S. repanda*, *S. radicata*, *S. delicatula* with and without cones. PP8[5] (Rf-0.76; MW-21.6 kDa) was shared by *S. inaequalifolia* and *S. bryopteris*. PP8[6] (Rf-0.77; MW-17.7 kDa) represented its uniform presence in *S. intermedia*, *S. involvens*, *S. wightii*, *S. radicata*, *S. delicatula* with and without cones. PP8[7] (Rf-0.78; MW-19 kDa) and PP8[8] (Rf-0.79; MW-18.4 kDa) displayed its unique occurrence in *S. brachystachya* and *S. tenera* respectively.

Region 9 displayed twenty four protein bands with seven different regions. PP9[1] (Rf-0.8; MW-17.9 kDa) showed its common existence in *S. repanda* and *S. radicata*. Similarly, PP9[2] (Rf-0.82; MW-16.5 kDa) displayed its expression equally in *S. involvens*, *S. bryopteris*

and *S. delicatula*. PP9[3] (Rf-0.83; MW-15.9 kDa) depicted its common occurence in *S. intermedia*, *S. inaequalifolia*, *S. tenera*, *S. repanda* and *S. radicata*. PP9[4] (Rf-0.85; MW-14.1 kDa) depicted its presence jointly in *S. wightii* and *S. bryopteris*. PP9[5] (Rf-0.86; MW-13.8 kDa) displayed its occurrence in *S. inaequalifolia*, *S. tenera*, *S. radicata*, *S. delicatula* with and without cones. PP9[6] (Rf-0.88; MW-12.5 kDa) dispersed uniformly in *S. intermedia*, *S. wightii* and *S. brachystachya*. Similarly, PP9[7] (Rf-0.89; MW-11.9 kDa) showed it's jointly presence in *S. inaequalifolia*, *S. involvens* and *S. repanda*. Region 10 illustrated fifteen bands with four positions. PP10[1] (Rf-0.91; MW-11.4 kDa) represented its joint distribution in *S. tenera*, *S. radicata* and *S. delicatula*. PP10[2] (Rf-0.92; MW-11 kDa) exhibited its existence in *S. inaequalifolia*, *S. repanda* and *S. delicatula* without cones. PP10[3] (Rf-0.95; MW-10.4 kDa) was demonstrated its occurrence in *S. intermedia*, *S. inaequalifolia*, *S. tenera*, *S. wightii*, *S. radicata* and *S. delicatula*. PP10[4] (Rf-0.97; MW-9.9 kDa) described its occurrence in *S. involvens*, *S. radicata* and *S. delicatula* without cones. Regions 9 and 10 were failed to express distinct bands in the studied *Selaginella* species.

Table 15: Protein profile of *Selaginella* species in SDS-PAGE gel system

Rf	Mol. Wt (kDa)	R	P	S1	S2	S3	S4	S5	S6	S7	S8	S9	S 10	S 11
0.02	281.8	1	PP1[1]	+	+	+	+	+			+	+		
0.03	272.6		PP1[2]						+	+				
0.04	251.1		PP1[3]						+					
0.05	223.8		PP1[4]									+		
0.07	199.5		PP1[5]	+				+						
0.08	177.8		PP1[6]			+	+						+	+
0.09	169.8		PP1[7]							+				
0.10	162.2	2	PP2[1]	+								+		
0.11	158.4		PP2[2]								+	+		
0.12	154.8		PP2[3]		+				+	+				
0.13	141.2		PP2[4]	+		+								
0.14	135.8		PP2[5]		+		+				+			+
0.16	123.0		PP2[6]	+				+				+		
0.17	111.3		PP2[7]				+				+			
0.18	100.0		PP2[8]						+	+				
0.19	99.1		PP2[9]			+	+							
0.21	98.7	3	PP3[1]						+	+	+			
0.22	96.2		PP3[2]										+	+
0.23	95.4		PP3[3]						+					
0.25	93.3		PP3[4]				+	+			+	+		
0.26	92.5		PP3[5]	+			+				+			
0.27	89.1		PP3[6]					+	+	+				
0.29	87.0		PP3[7]	+		+	+		+		+			
0.30	81.2	4	PP4[1]							+		+		
0.32	79.4		PP4[2]	+			+				+			
0.33	70.7		PP4[3]				+				+			
0.34	63.0		PP4[4]						+	+				
0.35	61.6		PP4[5]	+										
0.36	59.0		PP4[6]					+						
0.38	57.5		PP4[7]	+			+		+	+	+			
0.39	55.9		PP4[8]					+						
0.40	53.8	5	PP5[1]							+				
0.41	52.1		PP5[2]				+			+		+		
0.43	50.1		PP5[3]						+	+				
0.45	47.4		PP5[4]			+	+	+		+			+	
0.47	46.1		PP5[5]	+						+		+		+
0.50	44.6	6	PP6[1]					+	+	+				
0.51	43.2		PP6[2]	+	+	+					+		+	+
0.52	41.9		PP6[3]				+							
0.54	39.8		PP6[4]					+		+		+	+	
0.57	38.0		PP6[5]	+					+		+			
0.58	37.1		PP6[6]			+	+		+	+			+	+
0.60	35.6	7	PP7[1]		+			+						

0.61	33.1		$PP7^2$											+
0.63	30.9		$PP7^3$				+		+	+	+			
0.67	29.1		$PP7^4$			+	+	+						
0.69	28.0		$PP7^5$	+					+	+				+
0.70	27.3	**8**	$PP8^1$		+									
0.72	25.1		$PP8^2$			+	+							
0.73	24.8		$PP8^3$	+	+			+		+	+		+	+
0.75	22.0		$PP8^4$				+							
0.76	21.6		$PP8^5$		+							+		
0.77	19.9		$PP8^6$	+		+		+			+		+	+
0.78	19.0		$PP8^7$						+					
0.79	18.4		$PP8^8$				+							
0.80	17.9	**9**	$PP9^1$							+	+			+
0.82	16.5		$PP9^2$			+						+	+	
0.83	15.9		$PP9^3$	+	+		+			+	+			
0.85	14.1		$PP9^4$					+				+		
0.86	13.8		$PP9^5$		+		+				+		+	+
0.88	12.5		$PP9^6$	+				+	+					
0.89	11.9		$PP9^7$		+	+				+				
0.91	11.4	**10**	$PP10^1$				+				+		+	
0.92	11.0		$PP10^2$		+					+				+
0.95	10.4		$PP10^3$	+	+		+	+			+		+	
0.97	9.9		$PP10^4$			+				+				+
Total				**19**	**13**	**14**	**24**	**17**	**18**	**25**	**21**	**13**	**12**	**14**

Note: R- Region, P- Position, S1- *S. intermedia*, S2- *S. inaequalifolia*, S3- *S. involvens*, S4- *S. tenera*, S5- *S. wightii*, S6- *S. brachystachya*, S7- *S. repanda*, S8- *S. radicata*, S9- *S. bryopteris*, S10- *S. delicatula* with cones, S11- *S. delicatula* without cones.

The similarity indices were calculated and cladogram was constructed based on the protein profiles of *Selaginella*. It revealed the similarities and variation among the studied *Selaginella* species (Table 16; Fig. 1). The evolutionary tree which constructed based on the protein profile expressed two clusters (C_1 and C_2). The cluster (C_1) includes eight species of *Selaginella* viz., *S. intermedia*, *S. tenera*, *S. radicata*, *S. inaequalifolia*, *S. involvens*, *S. delicatula* with and without cones, *S. wightii* and *S. bryopteris*. The cluster (C_2) includes two species, *S. brachystachya* and *S. repanda*. The cluster C_1 is again divided into two nodes (C_1N^1 and C_1N^2). The node C_1N^1 is further branched into two branches includes $C_1N^1B_1$ and $C_1N^1B_2$. The branch $C_1N^1B_1$ is again branched into two small branches ($C_1N^1B_1b_1$ and $C_1N^1B_1b_2$). The small branch $C_1N^1B_1b_1$ showed the uniqueness of *S. intermedia* from the other nine studied species of *Selaginella*. The small branch $C_1N^1B_1b_2$ is promoted into two sub branches $C_1N^1B_1b_2A$ and $C_1N^1B_1b_2B$. The sub branch $C_1N^1B_1b_2A$ explained the

similarity and variation among *S. tenera* and *S. radicata* whereas the sub branch $C_1N^1B_1b_2B$ depicted the exclusive character of *S. inaequalifolia* from the other studied species of *Selaginella*. The branch $C_1N^1B_2$ is branched into two small branches $C_1N^1B_2b_1$ which is distinct from the other species of *Selaginella*. The small branch $C_1N^1B_2b_2B$ represented the closeness and divergence between *S. delicatula* with and without cones. The node C_1N^2 is branched into two division $C_1N^2B_1$ and $C_1N^2B_2$ that illustrated the similarity and variation between *S. wightii* and *S. bryopteris*. The cluster (C_2) is simply divided into two nodes C_2N^1 and C_2N^2 which showed the closeness and divergence between *S. brachystachya* and *S. repanda*.

Table 16: Similarity indices of *Selaginella* species based on SDS-PAGE profile

Taxon	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
S1	1.00										
S2	0.15	1.00									
S3	0.15	0.11	1.00								
S4	0.16	0.13	0.21	1.00							
S5	0.19	0.13	0.12	0.12	1.00						
S6	0.13	0.03	0.06	0.09	0.08	1.00					
S7	0.11	0.13	0.10	0.13	0.11	0.27	1.00				
S8	0.27	0.20	0.11	0.28	0.13	0.10	0.10	1.00			
S9	0.12	0.07	0.07	0.08	0.17	0.00	0.10	0.08	1.00		
S10	0.12	0.16	0.23	0.16	0.17	0.00	0.10	0.18	0.08	1.00	
S11	0.15	0.18	0.17	0.10	0.06	0.03	0.17	0.17	0.07	0.26	1.00

Note: S1 - *S. intermedia*, S2 - *S. inaequalifolia*, S3 - *S. involvens*, S4 - *S. tenera*, S5 - *S. wightii*, S6 - *S. brachystachya*, S7 - *S. repanda*, S8 - *S. radicata*, S9 - *S. bryopteris*, S10 - *S. delicatula* with cones, S11 - *S. delicatula* without cones.

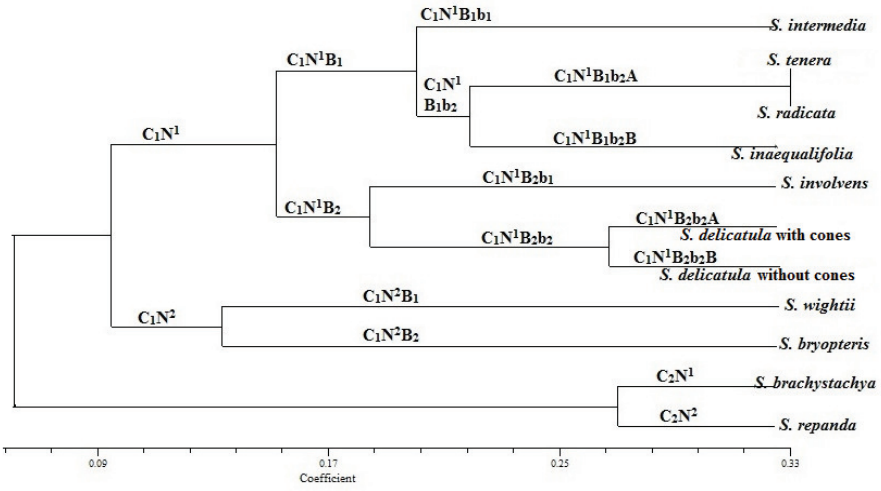

Fig. 1: Cladogram of studied *Selaginella* species based on SDS-PAGE profile

MALDI-TOF mass spectrum analysis

MALDI-TOF MS characterization of *Selaginella* species collected from various localities of South India expressed different ion peaks ranged from 0 - 1, 00,000 kDa (Plate XIV; XV). The results of MALDI- TOF MS analysis showed both positive and negative peaks. For the present study, positive peaks were selected to identify the similarities and variation among the studied *Selaginella* species. MALDI-TOF MS analysis of ten *Selaginella* species showed totally 483 spectral peak values (m/z values) and they were represented in Table 17. Among the spectral profile of ten *Selaginella* species, *S. involvens* displayed maximum number (86) of spectral peaks ranged from 527 to 99215 m/z values. Next to that, *S. tenera* represented 77 spectral peaks with m/z values 719 to 95316. *S. inaequalifolia* showed 73 spectral peaks displayed from 501 to 96748 m/z values. *S. delicatula* with cones expressed 69 spectral peaks (511 to 94366 m/z values) while *S. delicatula* without cones depicted 45 spectral peaks ranged from 528 to 92003 m/z values. *S. bryopteris* established 54 spectral peaks with m/z values 594 to 95279. Subsequently, *S. brachystachya* exhibited 52 defined spectral peaks varied from 515 to 99633 m/z values. *S. repanda* demonstrated 50 spectral peaks with m/z values 536 to 99939. *S. wightii* recognized 42 spectral peaks differed from 522 to 96955 m/z values. *S. intermedia* predicted 36 spectral peaks with m/z values 491 to 96955. *S. radicata* depicted minimum number (21) of spectral peaks varied from 694 to 91754 m/z values (Table 17). Among the 483 spectral peak values of the studied ten *Selaginella* species, 71 spectral peaks were observed with unique presence, which distinguished the genetic similarities and variation of studied *Selaginella* species.

Based on the MALDI-TOF mass spectral peaks of studied *Selaginella* species, the similarity indices were calculated and the cladogram was constructed (Table 18; Fig. 2). The cladogram displayed two clades C_1 and C_2. The clade C_1 includes five species of *Selaginella* viz., *S. intermedia*, *S. inaequalifolia*, *S. wightii*, *S. involvens* and *S. tenera*. Similarly, the

clade C_2 includes five species of *Selaginella* viz., *S. brachystachya*, *S. radicata*, *S. repanda*, *S. bryopteris*, *S. delicatula* with and without cones. The clade C_1 is divided into two nodes, C_1N^1 and C_1N^2. The node C_1N^2 showed the unique existence in *S. tenera*. The node C_1N^1 is branched into $C_1N^1B_1$ and $C_1N^1B_2$. The branch $C_1N^1B_1$ is further divided into two small branches $C_1N^1B_1b_1$ and $C_1N^1B_1b_2$, whereas the branch $C_1N^1B_2$ expressed the exclusive character of *S. involvens*. The small branch $C_1N^1B_1b_1$ displayed the similarity between *S. intermedia* and *S. inaequalifolia*. The small branch $C_1N^1B_1b_2$ depicted the distinct character of *S. wightii*. The clade C_2 is divided into two nodes C_2N^1 and C_2N^2. The C_2N^1 is branched into $C_2N^1B_1$ and $C_2N^1B_2$. The branch $C_2N^1B_1$ is again divided into two small branches $C_2N^1B_1b_1$ and $C_2N^1B_1b_2$. The branch $C_2N^1B_2$ illustrated the unique occurrence in *S. bryopteris*. The small branch $C_2N^1B_1b_1$ explained the similarity between *S. brachystachya* ($C_2N^1B_1b_1A$) and *S. radicata* ($C_2N^1B_1b_1B$). The small branch $C_2N^1B_1b_2$ displayed the restricted distribution of *S. repanda*. The clade C_2N^2 is branched into $C_2N^2B_1$ and $C_2N^2B_2$ that confirmed the similarity between *S. delicatula* with and without cones. The distingished character of *S. tenera*, *S. involvens*, *S. wightii*, *S. brachystachya* and *S. repanda* is confirmed in the cladogram by the presence of unique m/z values (Fig. 2). These specific m/z values can be act as a biochemical marker to identify the specific *Selaginella* species in chemosystematics and pharmaceutical industries.

Table 17: MALDI-TOF MS analysis of *Selaginella* species

S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
491	501	527	719	522	515	536	694	594	511	528
522	506	667	1020	594	536	555	11424	677	555	555
527	522	10045	10750	610	599	669	16000	694	599	574
636	625	10135	11061	667	694	694	16145	822	694	615
651	667	10190	11610	916	698	918	19545	11269	698	694
667	693	10335	13041	920	711	15937	20879	11487	876	698
688	833	10356	13549	921	798	16145	21460	11830	992	711
693	905	10574	13787	1165	3675	16238	21481	13280	2257	6103
729	10356	10615	14887	1170	5325	16382	23596	15512	2588	7068
895	10362	11460	20920	10149	10367	16725	24425	16145	9369	10284
916	11874	11466	21065	10252	15678	16995	27785	16694	10554	10367
920	12579	11874	21459	10278	16145	17710	31357	16974	10875	10554
10360	12616	12331	21832	10398	16766	17897	33637	16995	11258	10688
13200	13223	12336	22387	10424	17046	19898	37618	17202	11849	11070
20111	13435	12616	22809	10563	18602	20299	42933	17472	11923	11269
20112	14114	13435	23906	10589	21481	20610	43492	18405	12410	12130
21729	15794	13611	25648	10817	23596	23036	43533	18892	12783	13674
22062	19962	13871	26850	11025	31046	23596	47586	19473	14491	15077
22081	20112	14114	27928	11522	31357	25379	50257	21294	14566	15149
25815	20380	14316	28011	11652	31979	25628	51108	22393	15429	16145
25835	21251	14814	28364	11668	32870	25649	91754	22870	15771	16497
31189	21729	20112	29318	11895	34301	26582		23596	16145	17046
31232	22435	21749	29815	12435	37162	28116		24052	16818	17654
39193	23388	21792	30174	13829	38240	31357		24301	16995	19410
54052	23887	22062	30754	13887	40092	31439		24985	17046	20610
66271	23906	22435	31189	15794	41543	33824		27723	17181	20651
68469	24455	23906	31624	18469	43492	34239		28116	17482	33139
72579	25419	24611	32040	20380	44529	36561		29256	18405	39816
91294	25815	24776	32910	21045	45919	36955		29692	19048	50630
93699	26083	27182	32994	21066	48365	37972		30382	19136	52476
93741	27121	27888	34134	21251	49589	42933		31357	20610	53098
94052	27765	27928	34570	21832	61916	50133		32282	20651	57245
96374	28157	28054	36103	21895	71936	53637		32517	20879	59485
96685	29775	28115	36498	22062	72435	54031		34798	21066	60444
96748	29796	28281	38841	22081	72953	54301		38240	21771	62414
96955	31543	29796	39982	22413	73181	57773		40092	22268	63264
	32579	29815	40796	22435	74322	58013		40506	23761	63860
	33011	30071	41438	23802	77183	59941		42538	25649	63887
	34537	32742	42247	25835	79775	61916		43471	26209	65213
	40837	33233	45087	39193	81428	61979		46312	26707	65590
	40859	34735	45835	39215	81801	64715		54301	29423	67307
	41314	36103	48364	96955	84786	69485		62808	32062	71066
	41688	36167	49234		86737	71066		66250	33326	86488
	43057	37059	50339		89640	75886		69381	33575	88933
	47080	37182	51002		91090	83709		70962	33637	92003
	48260	37888	51272		91754	87110		72601	36561	

S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
	48261	39610	51956		92003	88147		74591	38635	
	49464	40257	54217		95237	92003		75337	41460	
	53264	40286	55212		95279	95528		78770	42890	
	54052	46602	55897		97436	99939		86473	42933	
	58824	47370	56239		98389			86488	45358	
	59179	47390	57390		99633			88685	50962	
	66416	54154	58157					92400	51190	
	66912	54177	60256					95279	51895	
	66913	57173	62310						59672	
	67661	57742	64445						62284	
	68261	60984	66208						63450	
	73368	62103	68633						65379	
	73866	63347	69877						70382	
	74135	63512	70629						74715	
	77723	65472	71127						77411	
	79173	67121	72662						79235	
	80299	67370	73781						80224	
	84446	69340	75067						82341	
	84840	71418	75503						83045	
	87245	74612	78903						85159	
	90610	75234	79630						88187	
	92435	76539	80816						90799	
	93699	76561	82683						94366	
	95025	79091	85523							
	95047	84465	85979							
	95370	84466	86394							
	96748	87722	87482							
		87743	88198							
		90610	88903							
		91709	91956							
		91810	95316							
		96748								
		96955								
		97265								
		97687								
		99152								
		99215								

Note: S1 - *S. intermedia*, S2 - *S. inaequalifolia*, S3 - *S. involvens*, S4 - *S. tenera*, S5 - *S. wightii*, S6 - *S. brachystachya*, S7 - *S. repanda*, S8 - *S. radicata*, S9 - *S. bryopteris*, S10 - *S. delicatula* with cones, S11 - *S. delicatula* without cones.

Table 18: Similarity indices of *Selaginella* species based on MALDI-TOF MS analysis

Taxon	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
S1	1.00										
S2	0.10	1.00									
S3	0.05	0.09	1.00								
S4	0.01	0.06	0.02	1.00							
S5	0.10	0.05	0.02	0.02	1.00						
S6	0.01	0.07	0.00	0.08	0.00	1.00					
S7	0.03	0.02	0.01	0.08	0.02	0.07	1.00				
S8	0.02	0.01	0.00	0.00	0.00	0.08	0.06	1.00			
S9	0.01	0.07	0.07	0.00	0.01	0.06	0.04	0.05	1.00		
S10	0.01	0.06	0.00	0.01	0.08	0.04	0.06	0.04	0.03	1.00	
S11	0.02	0.02	0.00	0.00	0.01	0.07	0.05	0.02	0.03	0.06	1.00

Note: S1 - *S. intermedia*, S2 - *S. inaequalifolia*, S3 - *S. involvens*, S4 - *S. tenera*, S5 - *S. wightii*, S6 - *S. brachystachya*, S7 - *S. repanda*, S8 - *S. radicata*, S9 - *S. bryopteris*, S10 - *S. delicatula* with cones, S11 - *S. delicatula* without cones.

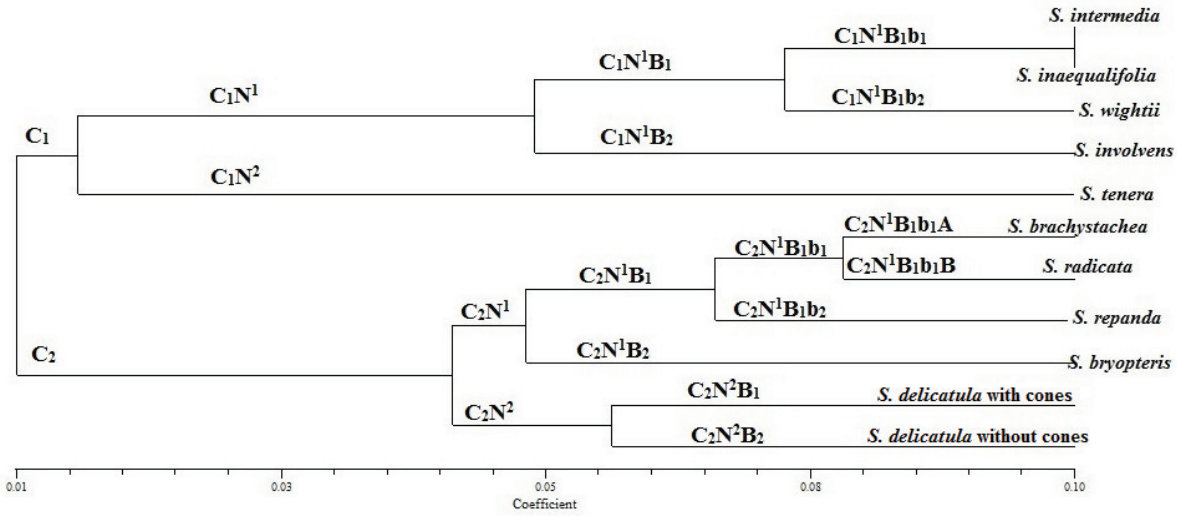

Fig. 2: Cladogram of *Selaginella* species based on MALDI-TOF MS analysis

Isozyme analysis

To know the isozymic variation among the selected ten *Selaginella* species from South India, five enzyme systems were resolved viz., esterase (EST), peroxidase (PO), acid phosphatase (ACP), alkaline phosphatase (ALP) and polyphenol oxidase (PPO).

Esterase

Multiple regions of activity with a total of thirty eight esterase bands were obtained in the esterase system of studied *Selaginella* species (Table 19; Plate XVI). *S. brachystachya* displayed maximum number (5) of esterase bands and *S. inaequalifolia* expressed minimum number (2) of esterase bands. Esterase enzyme system of *Selaginella* showed eight active

regions (Table 19). Region 1 depicted four active bands. EST 1^1 (0.01) was common to *S. intermedia*, *S. involvens*, *S. repanda*, *S. radicata* and *S. bryopteris*. EST 1^2 (0.03) showed its presence jointly in *S. tenera*, *S. brachystachya*, *S. delicatula* with and without cones. EST 1^3 (0.05) depicted its common existence in *S. involvens*, *S. wightii* and *S. repanda*. EST 1^4 (0.09) represented its occurrence in *S. intermedia*, *S. bryopteris* and *S. delicatula* without cones. Region 2 displayed three active bands of esterase enzymes. EST 2^1 (0.10) was observed in *S. tenera*, *S. repanda* and *S. delicatula*. EST 2^2 (0.14) established its mutual presence in *S. repanda* and *S. radicata*. EST 2^3 (0.16) was shared by *S. involvens* and *S. wightii*. Region 3 illustrated with three active esterase enzymes. EST 3^1 (0.20) was common in *S. repanda* and *S. delicatula* without cones. EST 3^2 (0.22), EST 3^3 (0.23) and EST 3^4 (0.25) were distinct to *S. inaequalifolia*, *S. intermedia* and *S. delicatula* with cones. EST 3^5 (0.27) established its mutual presence in *S. tenera* and *S. radicata*. Region 4 and 5 depicted with a single enzymatic band, EST 4^1 with MW-Rf value (0.34) was universal to *S. radicata* and EST 5^1 (0.49) showed its common presence in *S. involvens*, *S. repanda* and *S. bryopteris*. Region 6 expressed three active enzymatic bands. EST 6^1 (0.50), EST 6^2 (0.54) and EST 6^3 (0.58) exhibited its restricted character in *S. brachystachya*, *S. involvens* and *S. repanda*. Region 7 and 8 displayed with a single esterase band, EST 7^1 with MW-Rf (0.69) and EST 8^1 (0.76) were distinct to *S. intermedia* and *S. wightii* respectively.

Table 19: Esterase enzymatic profile of *Selaginella* species

MW Rf	R	Enzyme position	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
0.01	1	EST 1^1	+		+				+	+	+		
0.03		EST 1^2				+		+				+	+
0.05		EST 1^3			+		+		+				
0.09		EST 1^4	+								+		+
0.10	2	EST 2^1				+		+				+	
0.14		EST 2^2						+		+			
0.16		EST 2^3		+			+						
0.20	3	EST 3^1						+					+
0.22		EST 3^2		+									
0.23		EST 3^3	+										
0.25		EST 3^4										+	
0.27		EST 3^5				+				+			
0.34	4	EST 4^1								+			
0.49	5	EST 5^1			+				+		+		
0.50	6	EST 6^1						+					
0.54		EST 6^2			+								
0.58		EST 6^3							+				
0.69	7	EST 7^1	+										
0.76	8	EST 8^1					+						
Total			**4**	**2**	**4**	**3**	**3**	**5**	**4**	**4**	**3**	**3**	**3**

Note:R- Region, S1- *S. intermedia*, S2- *S. inaequalifolia*, S3- *S. involvens*, S4- *S. tenera*, S5- *S. wightii*, S6- *S. brachystachya*, S7- *S. repanda*, S8- *S. radicata*, S9- *S. bryopteris*, S10- *S. delicatula* with cones, S11- *S. delicatula* without cones.

Based on the esterase profile of the *Selaginella* species studied, the similarity indices were calculated and the cladogram was constructed (Table 20; Fig. 3). The evolutionary tree which was constructed based on the esterase profile expressed two clusters (C_1 and C_2). The cluster (C_1) includes eight species of *Selaginella* viz., *S. intermedia*, *S. involvens*, *S. repanda*, S. *bryopteris*, *S. radicata*, *S. tenera*, *S. brachystachya*, *S. delicatula* with and without cones. The cluster (C_2) includes two *Selaginella* species, *S. inaequalifolia* and *S. wightii*. The cluster C_1 is again divided into two nodes (C_1N^1 and C_1N^2). The node C_1N^1 is branched into $C_1N^1B_1$ and $C_1N^1B_2$. The branch $C_1N^1B_1$ is again divided into two small branches ($C_1N^1B_1b_1$ and $C_1N^1B_1b_2$). The small branch $C_1N^1B_1b_1$ showed the unique presence of *S. intermedia* from the other studied species of *Selaginella*. The small branch $C_1N^1B_1b_2$ is promoted into two sub branches $C_1N^1B_1b_2A$ and $C_1N^1B_1b_2B$. The sub branch $C_1N^1B_1b_2A$ depicted the exclusive character of *S. involvens* whereas the sub branch $C_1N^1B_1b_2B$ explained the

similarity between *S. repanda* and *S. bryopteris*. The branch $C_1N^1B_2$ showed the distinct nature of *S. radicata* from the other studied species of *Selaginella*. The node C_1N^2 is branched into two division $C_1N^2B_1$ and $C_1N^2B_2$. The branch $C_1N^2B_1$ represented the exclusive character of *S. tenera*. The branch $C_1N^2B_2$ is again divided into two small branches $C_1N^2B_2b_1$ and $C_1N^2B_2b_2$. The small branch $C_1N^2B_2b_1$ illustrated the similarity between *S. delicatula* with cones ($C_1N^2B_2b_1A$) and without cones ($C_1N^2B_2b_1B$). The small branch $C_1N^2B_2b_2$ explained the unique nature of *S. brachystachya* from the other studied *Selaginella* species. The cluster (C_2) is simply divided into two nodes C_2N^1 and C_2N^2 which showed the closeness between *S. inaequalifolia* and *S. wightii*.

Table 20: Similarity indices of *Selaginella* species based on esterase profile

Taxon	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
S1	1.00										
S2	0.00	1.00									
S3	0.11	0.00	1.00								
S4	0.00	0.00	0.00	1.00							
S5	0.00	0.14	0.11	0.00	1.00						
S6	0.00	0.00	0.00	0.16	0.00	1.00					
S7	0.14	0.00	0.37	0.00	0.14	0.00	1.00				
S8	0.12	0.00	0.11	0.08	0.00	0.12	0.14	1.00			
S9	0.33	0.00	0.28	0.00	0.00	0.00	0.40	0.16	1.00		
S10	0.00	0.00	0.00	0.18	0.00	0.28	0.00	0.00	0.00	1.00	
S11	0.14	0.00	0.00	0.09	0.00	0.28	0.00	0.00	0.20	0.16	1.00

Note: S1 - *S. intermedia*, S2 - *S. inaequalifolia*, S3 - *S. involvens*, S4 - *S. tenera*, S5 - *S. wightii*, S6 - *S. brachystachya*, S7 - *S. repanda*, S8 - *S. radicata*, S9 - *S. bryopteris*, S10 - *S. delicatula* with cones, S11 - *S. delicatula* without cones.

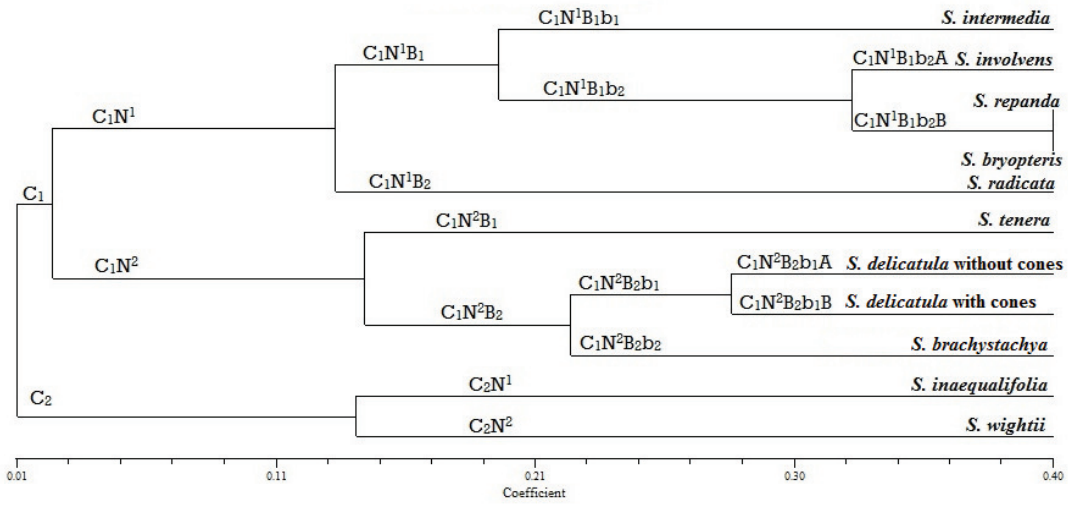

Fig. 3: Cladogram of *Selaginella* species based on esterase profile

Peroxidase

Peroxidase enzyme system of *Selaginella* produced sixty six peroxidase bands with ten active regions (Table 21). *S. brachystachya* displayed highest number (11) of peroxidase bands and *S. inaequalifolia* expressed lowest number (2) of peroxidase bands (Table 21; Plate XVI). Region 1 illustrated with six active bands. PRX 1^1 (0.01) was shared by *S. wightii* and *S. bryopteris*. PRX 1^2 (0.02), PRX 1^3 (0.04) and PRX 1^4 (0.05) were distinct to *S. brachystachya*, *S. involvens* and *S. inaequalifolia* respectively. PRX 1^5 (0.08) represented its presence in *S. delicatula* with and without cones. PRX 1^6 (0.09) showed its common presence in *S. intermedia*, *S. brachystachya* and *S. radicata*. Region 2 depicted six active enzymatic bands. PRX 2^1 (0.11), PRX 2^4 (0.16) and PRX 2^6 (0.19) exhibited its restricted character in *S. repanda*, *S. involvens* and *S. radicata* respectively. PRX 2^2 (0.12) was shared by *S. bryopteris* and *S. delicatula*. PRX 2^3 (0.15) was showed its common existence in *S. brachystachya* and *S. delicatula* without cones. PRX 2^5 (0.18) represented its mutual presence in *S. repanda* and *S. bryopteris*. Region 3 illustrated with five active enzymatic bands. PRX 3^1 (0.21) and PRX 3^5 (0.28) exhibited its restricted character in *S. tenera* and *S. brachystachya*. PRX 3^2 (0.22) was demonstrated its common occurence in *S. intermedia*, *S. delicatula* with and without cones. PRX 3^3 (0.23) was showed it's jointly presence in *S. involvens*, *S. brachystachya* and *S. bryopteris*. PRX 3^4 (0.26) established its mutual presence in *S. tenera* and *S. radicata*. Region 4 expressed four peroxidase bands. PRX 4^1, PRX 4^3 and PRX 4^4 with MW-Rf values 0.32, 0.36 and 0.38 were represented its unique existence in *S. repanda*, *S. radicata* and *S. tenera* respectively. PRX 4^2 (0.35) showed its common presence in *S. wightii*, *S. brachystachya* and *S. bryopteris*. Region 5 illustrated with three enzymatic bands. PRX 5^1 (0.42) and PRX 5^3 (0.47) were expressed their unique presence only in *S. involvens*. PRX 5^2 (0.46) established its mutual presence in *S. tenera* and *S. wightii*. Region 6 showed four active peroxidase bands. PRX 6^1, PRX 6^2, PRX 6^3 and PRX 6^4 with MW-Rf values 0.52,

0.53, 0.56 and 0.59 were displayed its unique presence in *S. brachystachya*, *S. radicata*, *S. wightii* and *S. involvens* respectively. Similarly, region 7 displayed four peroxidase bands. PRX 7^1 (0.60) and PRX 7^3 (0.66) exhibited its restricted expression in *S. bryopteris* and *S. wightii*. PRX 7^2 (0.64) was observed in *S. radicata*, *S. delicatula* with and without cones. PRX 7^4 (0.69) was shared by *S. involvens* and *S. tenera*. Similar to region 6 and 7, region 8 also observed with four enzymatic bands. PRX 8^1, PRX 8^3 and PRX 8^4 with MW-Rf values 0.70, 0.76 and 0.78 were depicted its unique character in *S. brachystachya*, *S. intermedia* and *S. repanda* correspondingly. PRX 8^2 (0.74) was commonly shared by *S. brachystachya* and *S. radicata*. Region 9 expressed with a single enzymatic band PRX 9^1 (0.88) was restricted to *S. delicatula* without cones. Region 10 produced four active enzymatic bands. PRX 10^1 (0.91) and PRX 10^3 (0.95) were distinct to *S. tenera* and *S. radicata* respectively. PRX 10^2 showed it's jointly distribution in *S. brachystachya* and *S. bryopteris*. PRX 10^4 (0.97) was dispersed commonly in six species of *Selaginella* viz., *S. inaequalifolia*, *S. involvens*, *S. wightii*, *S. brachystachya*, *S. repanda* and *S. bryopteris*.

Table 21: Peroxidase profile of *Selaginella* species

MW Rf	R	Enzyme position	S1	S2	S3	S4	S5	S6	S7	S8	S9	S 10	S 11
0.01	1	PRX 1^1					+				+		
0.02		PRX 1^2						+					
0.04		PRX 1^3			+								
0.05		PRX 1^4		+									
0.08		PRX 1^5										+	+
0.09		PRX 1^6	+					+		+			
0.11	2	PRX 2^1							+				
0.12		PRX 2^2									+	+	
0.15		PRX 2^3						+					+
0.16		PRX 2^4			+								
0.18		PRX 2^5							+		+		
0.19		PRX 2^6								+			
0.21	3	PRX 3^1				+							
0.22		PRX 3^2	+									+	+
0.23		PRX 3^3			+			+			+		
0.26		PRX 3^4				+				+			
0.28		PRX 3^5						+					
0.32	4	PRX 4^1							+				
0.35		PRX 4^2					+	+			+		
0.36		PRX 4^3								+			
0.38		PRX 4^4				+							
0.42	5	PRX 5^1			+								
0.46		PRX 5^2				+	+						
0.47		PRX 5^3			+								
0.52	6	PRX 6^1						+					
0.53		PRX 6^2								+			
0.56		PRX 6^3					+						
0.59		PRX 6^4			+								
0.60	7	PRX 7^1									+		
0.64		PRX 7^2								+		+	+
0.66		PRX 7^3					+						
0.69		PRX 7^4			+	+							
0.70	8	PRX 8^1						+					
0.74		PRX 8^2						+		+			
0.76		PRX 8^3	+										
0.78		PRX 8^4							+				
0.88	9	PRX 9^1											+
0.91	10	PRX 10^1				+							
0.92		PRX 10^2						+			+		
0.95		PRX 10^3								+			
0.97		PRX 10^4		+	+		+	+	+		+		
Total			**3**	**2**	**8**	**6**	**6**	**11**	**5**	**8**	**8**	**4**	**5**

Note: R- Region, S1- *S. intermedia*, S2- *S. inaequalifolia*, S3- *S. involvens*, S4- *S. tenera*, S5- *S. wightii*, S6- *S. brachystachya*, S7- *S. repanda*, S8- *S. radicata*, S9- *S. bryopteris*, S10- *S. delicatula* with cones, S11- *S. delicatula* without cones.

Based on the peroxidase profile of studied *Selaginella* species, the similarity indices were calculated and the cladogram was constructed (Table 22; Fig. 4). The phylogentic tree was constructed based on the peroxidase profile of studied *Selaginella* species expressed two clusters, C_1 and C_2. The cluster C_1 displayed the similarity and variation between three species of *Selaginella* viz., *S. intermedia*, *S. radicata*, *S. delicatula* with and without cones. The cluster C_2 represented the closeness and divergence among seven species of *Selaginella* viz., *S. inaequalifolia*, *S. repanda*, *S. involvens*, *S. wightii*, *S. bryopteris*, *S. brachystachya* and *S. tenera*. The cluster C_1 is divided into two nodes, C_1N^1 and C_1N^2. The node C_1N^1 is branched into $C_1N^1B_1$ and $C_1N^1B_2$ which explained the similarity between *S. intermedia* and *S. radicata*. Similarly, the node C_1N^2 is branched into $C_1N^2B_1$ and $C_1N^2B_2$ that established the similarity between *S. delicatula* with and without cones. The cluster C_2 is divided into two nodes C_2N^1 and C_2N^2. The node C_2N^1 is branched into $C_2N^1B_1$ and $C_2N^1B_2$. The branch $C_2N^1B_1$ is again branched into two small branches $C_2N^1B_1b_1$ and $C_2N^1B_1b_2$. Both small branches viz., $C_2N^1B_1b_1$ and $C_2N^1B_1b_2$ represented the similarity between *S. inaequalifolia* and *S. repanda*. The branch $C_2N^1B_2$ is further divided into two small branches, $C_2N^1B_2b_1$ and $C_2N^1B_2b_2$. The small branch $C_2N^1B_2b_1$ showed the unique expression of *S. involvens*. The small branch $C_2N^1B_2b_2$ is again sub branched into $C_2N^1B_2b_2A$ and $C_2N^1B_2b_2B$. $C_2N^1B_2b_2A$ determined the closeness between *S. wightii* and *S. bryopteris*. $C_2N^1B_2b_2B$ expressed the distinct nature of *S. brachystachya*. The node C_2N^2 simply depicted the exclusive character of *S. tenera* from the other studied species of *Selaginella*.

Table 22: Similarity indices of *Selaginella* species based on peroxidase profile

Taxon	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
S1	1.00										
S2	0.00	1.00									
S3	0.00	0.10	1.00								
S4	0.00	0.00	0.07	1.00							
S5	0.00	0.12	0.08	0.08	1.00						
S6	0.07	0.07	0.10	0.00	0.10	1.00					
S7	0.00	0.14	0.07	0.00	0.09	0.06	1.00				
S8	0.09	0.00	0.00	0.07	0.00	0.10	0.00	1.00			
S9	0.00	0.10	0.12	0.00	0.21	0.21	0.15	0.00	1.00		
S10	0.16	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.09	1.00	
S11	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.09	0.00	0.14	1.00

Note: S1- *S. intermedia*, S2- *S. inaequalifolia*, S3- *S. involvens*, S4- *S. tenera*, S5- *S. wightii*, S6- *S. brachystachya*, S7- *S. repanda*, S8- *S. radicata*, S9- *S. bryopteris*, S10- *S. delicatula* with cones, S11- *S. delicatula* without cones.

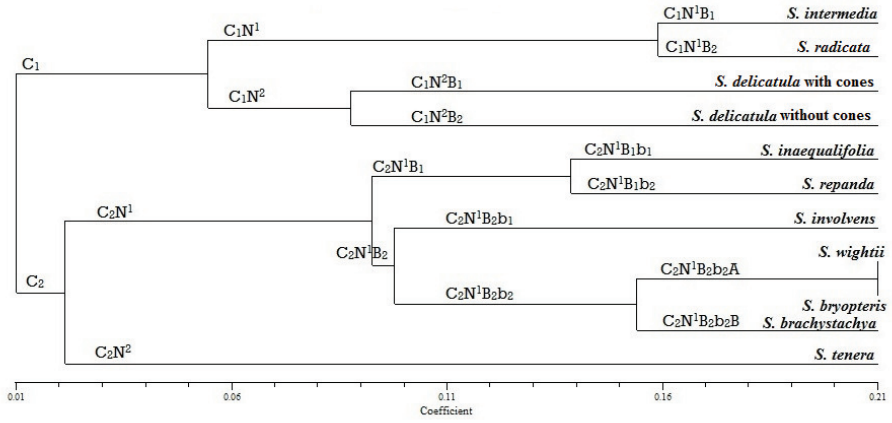

Fig. 4: Cladogram of *Selaginella* species based on peroxidase profile

Acid phosphatase

A total of sixty three bands with ten regions were observed in the acid phosphatase enzyme system (Table 23; Plate XVII). Region 1 displayed with four active bands. *S. intermedia* showed high number (12) of acid phosphatase enzyme bands. *S. involvens* and *S. brachystachya* expressed low number (1) of enzymatic bands (Table 5). ACP 1^1 (0.01) established its mutual presence in *S. intermedia* and *S. tenera*. ACP 1^2 (0.03) was common to *S. radicata*, *S. bryopteris* and *S. delicatula* with cones. ACP 1^3 (0.04) was shared by *S. intermedia* and *S. inaequalifolia*. ACP 1^4 (0.07) showed its common presence in *S. involvens*, *S. wightii*, *S. brachystachya*, *S. bryopteris* and *S. delicatula* without cones. Region 2 depicted five active acid phosphatase bands. ACP 2^1 (0.11) was distributed jointly in *S. tenera* and

S. radicata. Similarly, ACP 2^2 (0.12) was mutually shared by *S. bryopteris* and *S. delicatula*. ACP 2^3 (0.14) showed its presence only in *S. wightii*. ACP 2^4 (0.15) displayed its universal distribution in *S. intermedia*, *S. inaequalifolia*, *S. radicata* and *S. delicatula* without cones. ACP 2^5 (0.19) was widespread in *S. intermedia*, *S. tenera* and *S. bryopteris*. Region 3 expressed four active enzymatic bands. ACP 3^1, ACP 3^2, ACP 3^3 and ACP 3^4 with MW-Rf values 0.20, 0.25, 0.26 and 0.28 were distinct to *S. delicatula* with and without cones, *S. intermedia* and *S. bryopteris* correspondingly. Region 4 showed five active acid phosphatase bands. ACP 4^1 (0.30), ACP 4^2 (0.31), ACP 4^3 (0.36) and ACP 4^5 (0.39) were exhibited its restricted expression in *S. inaequalifolia*, *S. radicata*, *S. delicatula* without cones and *S. delicatula* with cones respectively. ACP 4^4 (0.38) was shared by *S. intermedia* and *S. bryopteris*. Region 5 displayed four active enzymatic bands. ACP $5^1$and ACP 5^4 with MW-Rf values 0.44 and 0.49 were established their unique presence in *S. wightii*. ACP 5^2 (0.46) was commonly observed in *S. intermedia*, *S. inaequalifolia* and *S. delicatula* with cones. ACP 5^3 (0.47) displayed its distinct presence in *S. repanda*. Region 6 represented two active enzymatic bands. ACP 6^1 (0.55) and ACP 6^2 (0.57) were established its unique existence in *S. intermedia* and *S. delicatula* with cones respectively. Region 7 described five acid phosphatase bands. ACP 7^1, ACP 7^3, ACP 7^4 and ACP 7^5 with respective MW-Rf values 0.61, 0.65, 0.68 and 0.69 were exhibited its restricted expression in *S. radicata*, *S. inaequalifolia*, *S. wightii* and *S. intermedia* correspondingly. ACP 7^2 (0.63) was uniformly distributed in *S. intermedia* and *S. bryopteris*. Region 8 delivered four active acid phosphatase bands. ACP 8^1 (0.71) established its mutual presence in *S. wightii* and *S. repanda*. ACP 8^2 (0.74), ACP 8^3 (0.76) and ACP 8^4 (0.77) were displayed their distinct presence in *S. intermedia*, *S. delicatula* with cones and *S. inaequalifolia* correspondingly. Region 9 showed a single enzymatic band, ACP 9^1 with MW-Rf value 0.88 was observed its presence jointly in *S. inaequalifolia*, *S. radicata* and *S. delicatula* without cones. Region 10

illustrated three active bands of acid phosphatase. ACP 10^1 (0.90) was shared by *S. intermedia* and *S. wightii*. Similarly, ACP 10^2 (0.96) established its mutual existence in *S. repanda* and *S. delicatula* with cones. ACP 10^3 (0.98) was observed in *S. tenera*, *S. delicatula* with and without cones.

Table 23: Acid phosphatase profile of *Selaginella* species

MW	R	Enzyme	S1	S2	S3	S4	S5	S6	S7	S8	S9	S 10	S 11
0.01	1	ACP 1^1	+			+							
0.03		ACP 1^2								+	+	+	
0.04		ACP 1^3	+	+									
0.07		ACP 1^4			+		+	+			+		+
0.11	2	ACP 2^1				+				+			
0.12		ACP 2^2									+	+	
0.14		ACP 2^3					+						
0.15		ACP 2^4	+	+						+			+
0.19		ACP 2^5	+			+					+		
0.20	3	ACP 3^1										+	
0.25		ACP 3^2											+
0.26		ACP 3^3	+										
0.28		ACP 3^4									+		
0.30	4	ACP 4^1		+									
0.31		ACP 4^2								+			
0.36		ACP 4^3											+
0.38		ACP 4^4	+								+		
0.39		ACP 4^5										+	
0.44	5	ACP 5^1					+						
0.46		ACP 5^2	+	+								+	
0.47		ACP 5^3							+				
0.49		ACP 5^4					+						
0.55	6	ACP 6^1	+										
0.57		ACP 6^2										+	
0.61	7	ACP 7^1								+			
0.63		ACP 7^2	+								+		
0.65		ACP 7^3		+									
0.68		ACP 7^4					+						
0.69		ACP 7^5	+										
0.71	8	ACP 8^1					+		+				
0.74		ACP 8^2	+										
0.76		ACP 8^3										+	
0.77		ACP 8^4		+									
0.88	9	ACP 9^1		+						+			+
0.90	10	ACP 10^1	+				+						
0.96		ACP 10^2							+			+	
0.98		ACP 10^3				+						+	+
Total			**12**	**7**	**1**	**4**	**7**	**1**	**3**	**6**	**7**	**9**	**6**

Note: R- Region, S1- *S. intermedia*, S2- *S. inaequalifolia*, S3- *S. involvens*, S4- *S. tenera*, S5- *S. wightii*, S6- *S. brachystachya*, S7- *S. repanda*, S8- *S. radicata*, S9- *S. bryopteris*, S10- *S. delicatula* with cones, S11- *S. delicatula* without cones.

Based on the acid phosphatase profile of studied *Selaginella* species, the similarity indices were calculated and the cladogram was constructed (Table 24; Fig. 5). The evolutionary tree showed two clusters, C_1 and C_2. The cluster C_1 displayed the similarity and variation among five species of *Selaginella* viz., *S. intermedia*, *S. inaequalifolia*, *S. radicata*, *S. tenera*, *S. delicatula* with and without cones. Similarly, the cluster C_2 represented the closeness and divergence among five species of *Selaginella* viz., *S. involvens*, *S. brachystachya*, *S. bryopteris*, *S. wightii* and *S. repanda*. The cluster C_1 is divided into two nodes, C_1N^1 and C_1N^2. The node C_1N^1 is branched into $C_1N^1B_1$ and $C_1N^1B_2$. The node C_1N^2 displayed the unique character of *S. delicatula* with cones. The branch $C_1N^1B_1$ is further divided into $C_1N^1B_1b_1$ and $C_1N^1B_1b_2$. The branch $C_1N^1B_2$ represented the exclusive nature of *S. tenera*. $C_1N^1B_1b_1$ presented the similarity between *S. intermedia* ($C_1N^1B_1b_1A$) and *S. inaequalifolia* ($C_1N^1B_1b_1B$). $C_1N^1B_1b_2$ demonstrated the closeness between *S. radicata* ($C_1N^1B_1b_2A$) and *S. delicatula* without cones ($C_1N^1B_1b_2B$). The cluster C_2 is divided into two nodes, C_2N^1 and C_2N^2. The node C_2N^1 is branched into $C_2N^1B_1$ and $C_2N^1B_2$.The branch $C_2N^1B_1$ explained the similarity between *S. involvens* and *S. brachystachya*. The branch $C_2N^1B_2$ exposed the distinct occurrence of *S. bryopteris*. The node C_2N^2 is branched into $C_2N^2B_1$ and $C_2N^2B_2$ which depicted the similarity between *S. wightii* and *S. repanda*.

Table 24: Similarity indices of *Selaginella* species based on acid phosphatase profile

Taxon	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
S1	1.00										
S2	0.15	1.00									
S3	0.00	0.00	1.00								
S4	0.12	0.00	0.00	1.00							
S5	0.05	0.00	0.12	0.00	1.00						
S6	0.00	0.00	0.50	0.00	0.12	1.00					
S7	0.00	0.00	0.00	0.00	0.10	0.00	1.00				
S8	0.05	0.15	0.00	0.10	0.00	0.00	0.00	1.00			
S9	0.15	0.00	0.12	0.09	0.06	0.12	0.00	0.09	1.00		
S10	0.04	0.06	0.00	0.07	0.00	0.00	0.08	0.06	0.11	1.00	
S11	0.05	0.15	0.14	0.10	0.07	0.14	0.00	0.16	0.07	0.06	1.00

Note: S1- *S. intermedia*, S2- *S. inaequalifolia*, S3- *S. involvens*, S4- *S. tenera*, S5- *S. wightii*, S6- *S. brachystachya*, S7- *S. repanda*, S8- *S. radicata*, S9- *S. bryopteris*, S10- *S. delicatula* with cones, S11- *S. delicatula* without cones.

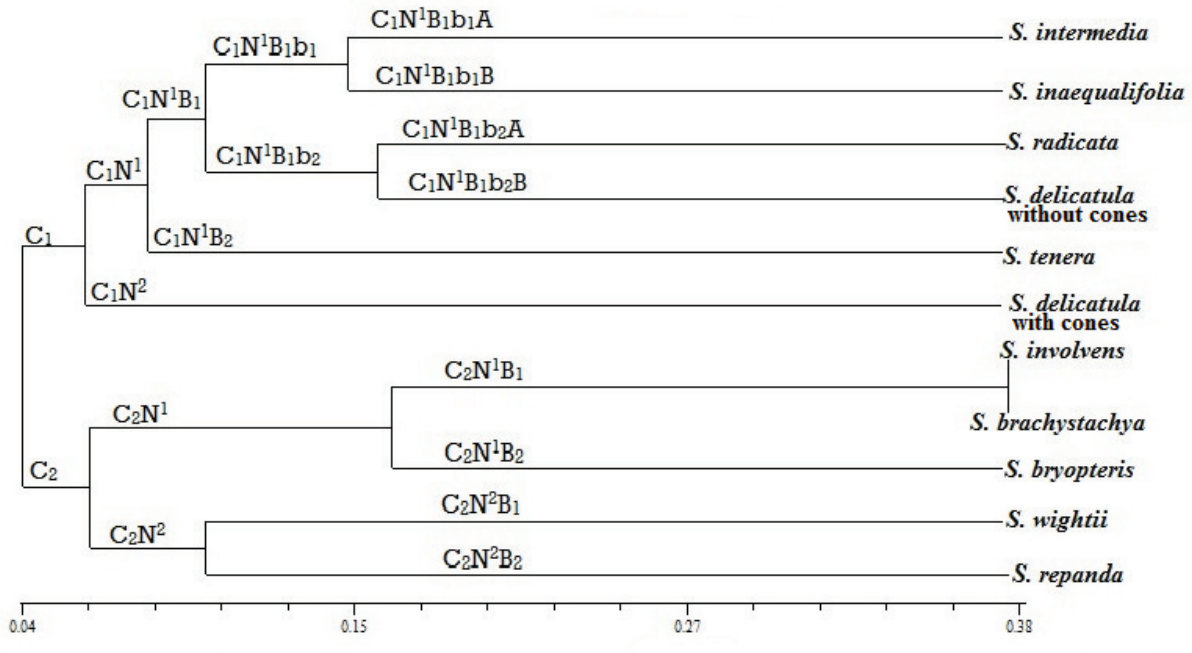

Fig. 5: Cladogram of *Selaginella* species based on acid phosphatase profile

Alkaline phosphatase

In the alkaline phosphatase enzyme system, ten regions of activity with forty eight bands were obtained (Table 25; Plate XVII). *S. bryopteris* displayed maximum number (10) of alkaline phosphatase bands expression and *S. radicata* represented with a single enzymatic band. Region 1 illustrated with four enzymatic bands (Table 25). ALP 1^1 (0.01) was restricted to *S. bryopteris*. ALP 1^2 (0.02) showed its common presence in *S. involvens*, *S. tenera* and *S. wightii*. ALP 1^3 with MW-Rf value 0.08 was shared by *S. brachystachya* and *S. radicata*. ALP 1^4 (0.09) was common to *S. bryopteris*, *S. delicatula* with and without cones. Region 2 exposed five alkaline phosphatase bands. ALP 2^1 (0.11) was uniformly distributed in *S. involvens* and *S. tenera*. ALP 2^2 (0.12) was shared by *S. inaequalifolia* and *S. wightii*. ALP 2^3 (0.14) was observed commonly in *S. intermedia*, *S. inaequalifolia*, *S. tenera* and *S. repanda*. ALP 2^4 (0.16) was distinct to *S. brachystachya*. ALP 2^5 (0.18) was mutually shared by *S. involvens* and *S. bryopteris*. Region 3 represented three active alkaline phosphatase bands. ALP 3^1 (0.22) was restricted to *S. brachystachya*. ALP 3^2 and ALP 3^3 with MW-Rf values

0.23 and 0.28 were expressed only in *S. tenera*. Region 4 displayed four active enzymatic bands. ALP 4^1 (0.30) was shared by *S. intermedia* and *S. tenera*. ALP 4^2 (0.33), ALP 4^3 (0.37) and ALP 4^4 (0.38) were showed their unique presence in *S. involvens*, *S. bryopteris* and *S. tenera* respectively. Region 5 displayed three enzymatic bands. ALP 5^1, ALP 5^2 and ALP 5^3 with MW-Rf values 0.46, 0.48 and 0.49 were restricted to *S. involvens*, *S. bryopteris* and *S. brachystachya* correspondingly. Region 6 observed with a single enzymatic band, ALP 6^1 (0.58) was distinct to *S. brachystachya*. Region 7 illustrated with three alkaline phosphatase bands. ALP 7^1 (0.60) and ALP 7^3 (0.64) were showed its unique existence in *S. delicatula* and *S. brachystachya* respectively. ALP 7^2 (0.61) was uniformly distributed in *S. involvens* and *S. bryopteris*. Region 8 depicted three alkaline phosphatase bands. ALP 8^1, ALP 8^2 and ALP 8^3 with MW-Rf values 0.75, 0.77 and 0.79 were expressed its exclusive character in *S. bryopteris*, *S. wightii* and *S. involvens* respectively. Region 9 delivered three enzymatic bands. ALP 9^1 (0.81) and ALP 9^2 (0.83) were restricted to *S. bryopteris* and *S. delicatula* with cones. ALP 9^3 (0.85) was shared by *S. involvens* and *S. bryopteris*. Region10 showed two alkaline phosphatase bands. ALP 10^1 (0.90) established its unique occurrence in *S. intermedia*. ALP 10^2 (0.92) was observed in *S. involvens*, *S. wightii*, *S. repanda*, *S. bryopteris* and *S. delicatula* without cones.

Table 25: Alkaline phosphatase profile of *Selaginella* species

MW Rf	R	Enzyme position	S1	S2	S3	S4	S5	S6	S7	S8	S9	S 10	S 11
0.01	1	ALP 1^1									+		
0.02		ALP 1^2			+	+	+						
0.08		ALP 1^3						+		+			
0.09		ALP 1^4									+	+	+
0.11	2	ALP 2^1			+	+							
0.12		ALP 2^2		+			+						
0.14		ALP 2^3	+	+		+			+				
0.16		ALP 2^4						+					
0.18		ALP 2^5			+						+		
0.22	3	ALP 3^1						+					
0.23		ALP 3^2				+							
0.28		ALP 3^4				+							
0.30	4	ALP 4^1	+			+							
0.33		ALP 4^2			+								
0.37		ALP 4^3									+		
0.38		ALP 4^4				+							
0.46	5	ALP 5^1			+								
0.48		ALP 5^2									+		
0.49		ALP 5^3						+					
0.58	6	ALP 6^1						+					
0.60	7	ALP 7^1										+	
0.61		ALP 7^2			+						+		
0.64		ALP 7^3						+					
0.75	8	ALP 8^1									+		
0.77		ALP 8^2					+						
0.79		ALP 8^3			+								
0.81	9	ALP 9^1									+		
0.83		ALP 9^2										+	
0.85		ALP 9^3			+						+		
0.90	10	ALP 10^1	+										
0.92		ALP 10^2			+		+		+		+		+
Total			**3**	**2**	**9**	**7**	**4**	**6**	**2**	**1**	**10**	**3**	**2**

Note: R- Region, S1- *S. intermedia*, S2- *S. inaequalifolia*, S3- *S. involvens*, S4- *S. tenera*, S5- *S. wightii*, S6- *S. brachystachya*, S7- *S. repanda*, S8- *S. radicata*, S9- *S. bryopteris*, S10- *S. delicatula* with cones, S11- *S. delicatula* without cones.

Based on the alkaline phosphatase profile of studied *Selaginella* species, the similarity indices were calculated and the cladogram was constructed (Table 26; Fig. 6). The evolutionary tree displayed two clusters, C_1 and C_2. The cluster C_1 is divided into two nodes, C_1N^1 and C_1N^2. The node C_1N^1 is branched into $C_1N^1B_1$ and $C_1N^1B_2$. The branch $C_1N^1B_1$ is further divided into $C_1N^1B_1b_1$ and $C_1N^1B_1b_2$. $C_1N^1B_1b_1$ represented the exclusive nature of *S. intermedia* from the other studied species of *Selaginella*. $C_1N^1B_1b_2$ explained the similarity

between *S. inaequalifolia* and *S. repanda*. The branch $C_1N^1B_2$ exposed the distinct occurrence of *S. tenera*. The node C_1N^2 is branched into $C_1N^2B_1$ and $C_1N^2B_2$. The branch $C_1N^2B_1$ is again branched into $C_1N^2B_1b_1$ and $C_1N^2B_1b_2$. $C_1N^2B_1b_1$ presented the similarity between *S. involvens* ($C_1N^2B_1b_1A$) and *S. bryopteris* ($C_1N^2B_1b_1B$). The branch $C_1N^2B_1b_2$ displayed the unique presence of *S. wightii.* The branch $C_1N^2B_2$ demonstrated the closeness between *S. delicatula* with cones ($C_1N^2B_2b_1$) and without cones ($C_1N^2B_2b_2B$). The cluster C_2 is divided into two nodes C_2N^1 and C_2N^2 which displayed the closeness between *S. brachystachya* and *S. radicata*.

Table 26: Similarity indices of *Selaginella* species based on alkaline phosphatase profile

Taxon	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
S1	1.00										
S2	0.20	1.00									
S3	0.00	0.00	1.00								
S4	0.20	0.11	0.12	1.00							
S5	0.00	0.16	0.15	0.09	1.00						
S6	0.00	0.00	0.00	0.00	0.00	1.00					
S7	0.20	0.25	0.09	0.11	0.16	0.00	1.00				
S8	0.00	0.00	0.00	0.00	0.00	0.14	0.00	1.00			
S9	0.00	0.00	0.21	0.00	0.07	0.00	0.08	0.00	1.00		
S10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	
S11	0.00	0.00	0.09	0.00	0.16	0.00	0.25	0.00	0.16	0.20	1.00

Note: S1- *S. intermedia*, S2- *S. inaequalifolia*, S3- *S. involvens*, S4- *S. tenera*, S5- *S. wightii*, S6- *S. brachystachya*, S7- *S. repanda*, S8- *S. radicata*, S9- *S. bryopteris*, S10- *S. delicatula* with cones, S11- *S. delicatula* without cones.

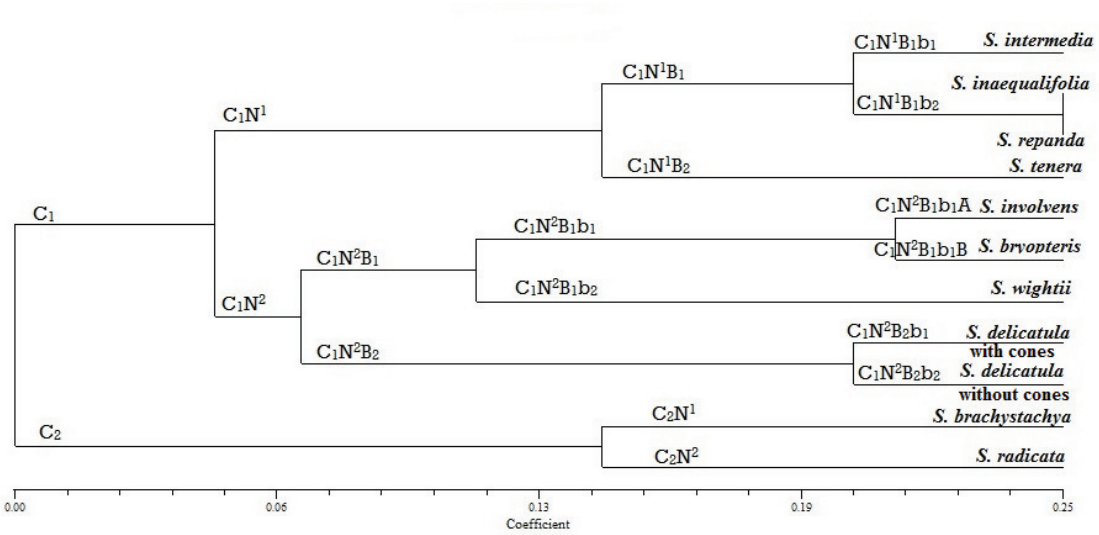

Fig. 6: Cladogram of *Selaginella* species based on alkaline phosphatase profile

Polyphenol oxidase

Two regions of activity with twelve polyphenol oxidase bands were observed in the polyphenol oxidase enzyme system of studied *Selaginella* species (Table 27; Plate XVII). Region 1 showed three active enzymatic bands. PPO 1^1 (0.01) was restricted to *S. bryopteris*. PPO 1^2 (0.02) was distributed in *S. radicata*, *S. delicatula* with and without cones. PPO 1^3 (0.05) was observed in *S. intermedia*, *S. inaequalifolia*, *S. involvens*, *S. tenera*, *S. wightii*, *S. brachystachya* and *S. repanda*. Region 2 failed to show the active polyphenol oxidase enzymes. Region 3 observed with a single enzymatic band, PPO 3^1 (0.29) was distinct to *S. brachystachya*.

Table 27: Polyphenol oxidase profile of *Selaginella* species

MW Rf	R	Enzyme position	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
0.01	1	PPO 1^1									+		
0.02		PPO 1^2								+		+	+
0.05		PPO 1^3	+	+	+	+	+	+	+				
0.29	3	PPO 3^1						+					
Total			**1**	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**1**	**1**	**1**

Note: R- Region, S1- *S. intermedia*, S2- *S. inaequalifolia*, S3- *S. involvens*, S4- *S. tenera*, S5- *S. wightii*, S6- *S. brachystachya*, S7- *S. repanda*, S8- *S. radicata*, S9- *S. bryopteris*, S10- *S. delicatula* with cones, S11- *S. delicatula* without cones.

The similarity indices were calculated and phylogenetic tree was constructed based on the polyphenol oxidase profile of studied *Selaginella* species (Table 28; Fig. 7). In the cladogram, four clades were displayed viz., C_1, C_2, C_3 and C_4. The clade C_1 is divided into two nodes, C_1N^1 and C_1N^2. The node C_1N^1 is branched into $C_1N^1B_1$ and $C_1N^1B_2$. The branch $C_1N^1B_1$ explained the similarities among the four species of *Selaginella* viz., *S. intermedia*, *S. inaequalifolia*, *S. involvens* and *S. tenera*. The branch $C_1N^1B_2$ showed the closeness between *S. wightii* and *S. repanda*. The clade C_2 expressed the distinct nature of *S. radicata*. The clade C_3 represented the exclusive character of *S. bryopteris*. The clade C_4 illustrated the similarity between *S. delicatula* with and without cones.

Table 28: Similarity indices of *Selaginella* species based on polyphenol oxidase profile

Taxon	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
S1	1.00										
S2	0.50	1.00									
S3	0.50	0.50	1.00								
S4	0.50	0.50	0.50	1.00							
S5	0.50	0.50	0.50	0.33	1.00						
S6	0.33	0.33	0.33	0.25	0.33	1.00					
S7	0.50	0.50	0.50	0.33	0.50	0.33	1.00				
S8	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00			
S9	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00		
S10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00	
S11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.50	0.00	0.50	1.00

Note: S1- *S. intermedia*, S2- *S. inaequalifolia*, S3- *S. involvens*, S4- *S. tenera*, S5- *S. wightii*, S6- *S. brachystachya*, S7- *S. repanda*, S8- *S. radicata*, S9- *S. bryopteris*, S10- *S. delicatula* with cones, S11- *S. delicatula* without cones.

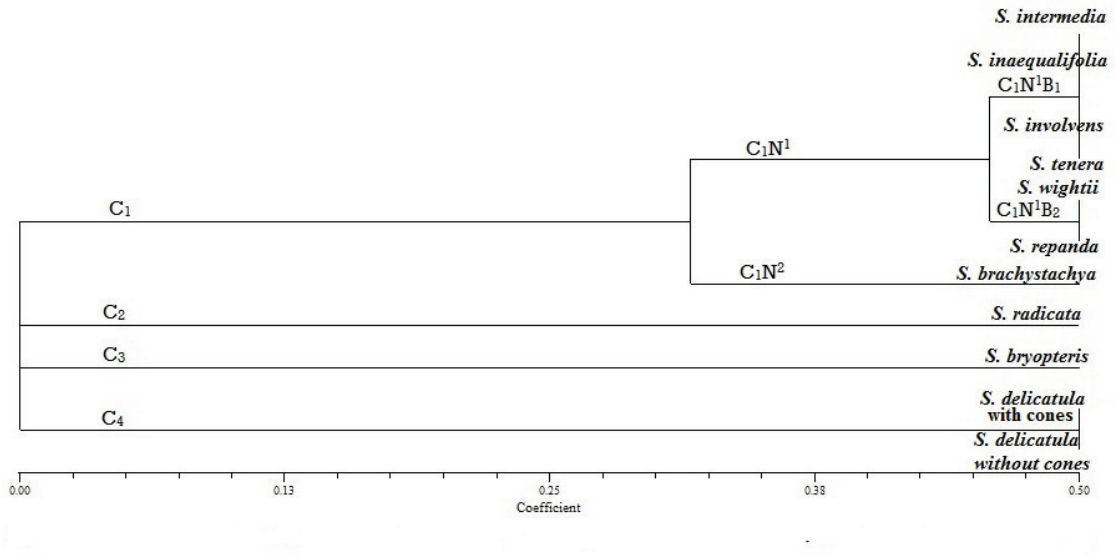

Fig. 7: Cladogram of *Selaginella* species based on polyphenol oxidase profile

Amalgamated isozymic profile

Based on the five enzymatic profiles viz., peroxidase, esterase, acid phosphatase, alkaline phosphatase and polyphenol oxidase of studied *Selaginella* species the similarity indices were calculated and the amalgamated cladogram was constructed (Table 29; Fig. 8). The evolutionary tree represents two main clusters C_1 and C_2. The cluster C_1 is diverged into two nodes C_1N^1 and C_1N^2. The node C_1N^1 is branched into $C_1N^1B_1$ and $C_1N^1B_2$ where C_1N_2 showed the unique existence of *S. bryopteris*. $C_1N^1B_1$ is again branched into $C_1N^1B_1b_1$ and $C_1N^1B_1b_2$. $C_1N^1B_1b_1$ displayed the similarity between two sub branches denoted by

$C_1N^1B_1b_1A$ (*S. intermedia*) and $C_1N^1B_1b_1B$ (*S. inaequalifolia*). The branch $C_1N^1B_1b_2$ showed two sub branches $C_1N^1B_1b_2A$ and $C_1N^1B_1b_2B$. $C_1N^1B_1b_2A$ expressed the similarity between *S. involvens* and *S. repanda*. $C_1N^1B_1b_2B$ displayed the individuality of *S. wightii*. $C_1N^1B_2$ displayed the similarity between *S. tenera* ($C_1N^1B_2b_1$) and *S. brachystachya* ($C_1N^1B_2b_2$). The cluster C_2 is divided into two nodes C_2N^1 and C_2N^2. The node C_2N^1 showed the distinct character of *S. radicata*. C_2N^2 displayed the similarity between *S. delicatula* with cones and *S. delicatula* without cones.

Table 29: Similarity indices of *Selaginella* species using amalgamated isozymic profile

Taxon	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
S1	1.00										
S2	0.17	1.00									
S3	0.12	0.12	1.00								
S4	0.16	0.12	0.13	1.00							
S5	0.11	0.18	0.19	0.10	1.00						
S6	0.08	0.08	0.18	0.13	0.11	1.00					
S7	0.16	0.17	0.20	0.08	0.19	0.07	1.00				
S8	0.05	0.03	0.02	0.05	0.00	0.07	0.03	1.00			
S9	0.09	0.02	0.14	0.02	0.07	0.06	0.12	0.05	1.00		
S10	0.04	0.01	0.00	0.05	0.00	0.05	0.02	0.11	0.04	1.00	
S11	0.04	0.03	0.04	0.04	0.04	0.09	0.05	0.15	0.08	0.21	1.00

Note: S1- *S. intermedia*, S2- *S. inaequalifolia*, S3- *S. involvens*, S4- *S. tenera*, S5- *S. wightii*, S6- *S. brachystachya*, S7- *S. repanda*, S8- *S. radicata*, S9- *S. bryopteris*, S10- *S. delicatula* with cones, S11- *S. delicatula* without cones.

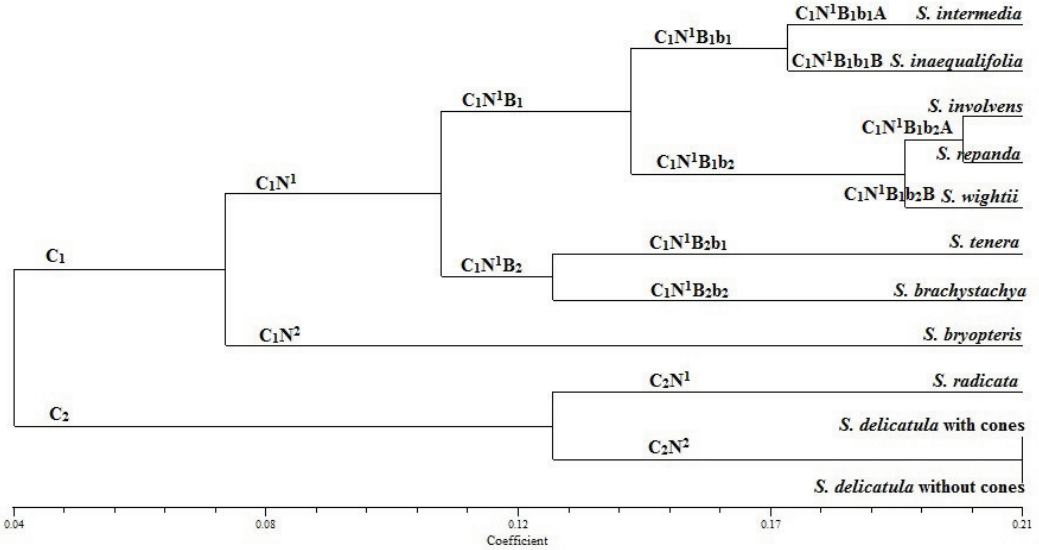

Fig. 8: Amalgamated cladogram of *Selaginella* species based on isozymic profiles

DNA isolation and PCR amplification

Among the three DNA isolation methods, the modified CTAB extraction method described by Murray and Thompson (1980) yielded a good DNA isolation. The genomic DNA of selected ten species of *Selaginella* collected from various localities of South India was then amplified using *rbcL* primer. The results were illustrated in Plate XVIII. The PCR amplification efficiency was good for the studied species and molecular weight ranges up to 500-600 base pairs and the amplicons were sequenced (Plate XIX-XXIV). The obtained sequences were aligned, annotated and submitted in GenBank. The GenBank accession numbers for the submitted *Selaginella* sequences with geographical details were tabulated in Table 30.

Table 30: Geographical location and Genbank details of studied *Selaginella* species

Species name	Locality	Latitude and Altitude	Species code	Voucher number (XCH. No)	Genbank Accession number
S. intermedia	Kaakachi Hills, Tamil Nadu.	8.7152836° N 800-1800 m	S1	25425	KF917189
S. inaequalifolia	Kaakachi Hills, Tamil Nadu.	8.7152836° N 800-1800 m	S2	25426	KF929424
S. involvens	Kaakachi Hills, Tamil Nadu.	8.7152836° N 800-1800 m	S3	25427	KF917190
S. tenera	Kaakachi Hills, Tamil Nadu.	8.7152836° N 300-2000 m	S4	25428	KF917191
S. wightii	Marunthuvazh Malai, Tamil Nadu.	8.1272411° N 350-1500 m	S5	25429	KF929423
S. brachystachya	Shenbaganur Hills, Tamil Nadu.	10.2336828° N 1200-2000 m	S6	25430	KF929421
S. repanda	Shenbaganur Hills, Tamil Nadu.	10.2336828° N 1000-1200 m	S7	25431	KF917188
S. radicata	Ponmudi Hills, Kerala.	8.7599422° N 110-1200 m	S8	25432	KF917192
S. bryopteris	Ponmudi Hills, Kerala.	8.7599422° N 110-1200 m	S9	25433	KF929422
S.delicatula with cones	Ponmudi Hills, Kerala.	8.7599422° N 0-1000 m	S10	25434	KF917193
S. delicatula without cones	Themalai Hills, Kerala.	8.9632448° N 0-950 m	S11	25435	KF917194

Sequence Alignment

Multiple sequence alignment was performed for the studied ten species of *Selaginella* using MULTALIN (version 5.4.1) and Clustal W tool and aligned sequences were illustrated in Plate XXV. Phylogenetic tree was constructed based on the results of Clustal W (Fig. 9).

The goal of molecular systematics is to determine the phylogeny of a species or a group of related species. Phylogeny is commonly represented in the form of cladogram. Clade is a unit of common evolutionary descent which is classified into monophyletic, paraphyletic and polyphyletic. A valid clade is monophyletic which consists of the ancestor species and all its descendants. Paraphyletic group is one consisting of a common ancestor but not all descendants of that common ancestor. Polyphyletic clade includes many species that lack a common ancestor.

The cladogram constructed using Clustal W displayed two main clades C_1 and C_2. The clade 1 & 2 included the monophyletic and paraphyletic expression (Fig. 8). The clade C_1 is divided into two nodes (C_1N^1 and C_1N^2). C_1N^1 expressed monophyletic taxon group viz., *S. repanda*, *S. radicata*, *S. involvens*, *S. delicatula* with and without cones, *S. inaequalifolia* and C_1N^2 includes paraphyletic taxon *S. intermedia*. The clade C_2 expressed the monophyletic taxon groups which comprised *S. wightii*, *S. bryopteris*, *S. brachystachya* and C_2N^1 includes paraphyletic taxon *S. tenera*. C_1N^1 is branched into $C_1N^1B_1$ and $C_1N^1B_2$. The branch $C_1N^1B_1$ is sub branched into $C_1N^1B_1b_1$ and $C_1N^1B_1b_2$. The sub branch $C_1N^1B_1b_1$ explained the similarity between *S. repanda* and *S. radicata* where as $C_1N^1B_1b_2$ showed the distinct character of *S. involvens*. $C_1N^1B_2$ is also sub branched into $C_1N^1B_2b_1$ and $C_1N^1B_2b_2$. The sub branch $C_1N^1B_2b_1$ showed the similarity between *S. delicatula* with and without cones where, $C_1N^1B_2b_2$ explained the exclusive character of *S. inaequalifolia*. The clade C_2 is divided into two nodes (C_2N^1 and C_2N^2). The node C_2N^2 showed the distinct occurrence of *S. tenera*. The node C_2N^1 is branched into $C_2N^1B_1$ and $C_2N^1B_2$. The branch $C_2N^1B_1$ includes *S.*

brachystachya and *S. bryopteris* which explained the closeness between them and the branch $C_2N^1B_2$ illustrated the exclusive trait of *S. wightii*.

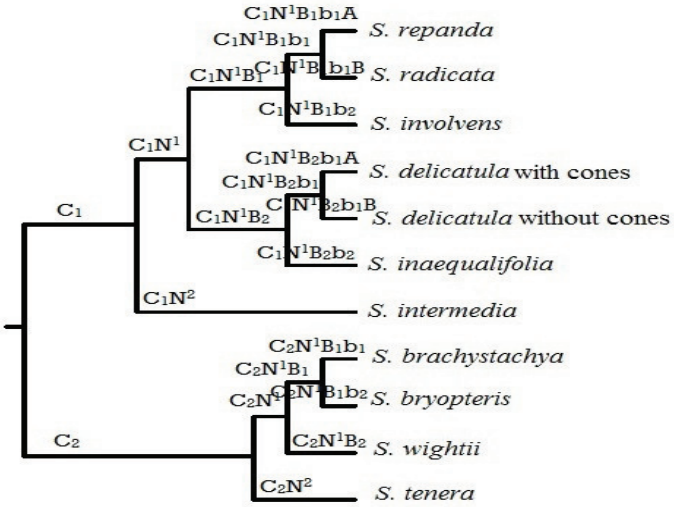

Fig. 9: Rooted phylogenetic cladogram of *Selaginella* species based on Clustal W

Molecular taxonomy studies using statistical tool

To study the molecular taxonomy of selected species of *Selaginella*, it is necessary to know some basic statistical information such as nucleotide frequencies, codon frequencies, transition/transversion bias, amino acid composition and phylogenetic relationships using standard statistical tool namely MEGA 5.2 version.

Nucleotide composition

The nucleotide base frequencies for ten *Selaginella* species were represented in Table 31. The nucleotide bases were found to be AT rich (58.7%) in *S. wightii* and low (46.5%) in *S. inaequalifolia*. The nucleotide base GC is rich in *S. inaequalifolia* (53.4%) and low (41.3%) in *S. wightii*.

Maximum and minimum frequency of thymine was found in *S. wightii* (30.6%) and *S. delicatula* without cones (20.7%). The higher and lower frequency of cytosine content was observed in *S. delicatula* with cones (29.4%) and *S. wightii* (18.8%). Adenine concentration was higher in *S. brachystachya* (28.3%) and lower in *S. inaequalifolia* (25%). Guanine

frequency was found to be high in *S. tenera* (25.8%) and low in *S. brachystachya* (22.4%) respectively. The average frequencies of A, T, G and C were displayed as 26.6%, 24.3%, 23.8% and 25.2%.

Table 31: Nucleotide composition of studied *Selaginella* species

Domain	T(U)	C	A	G	AT	GC	Total
S. intermedia	23.2	26.5	25.8	24.4	49.0	50.9	581
S. inaequalifolia	21.5	28.8	25.0	24.6	46.5	53.4	548
S. involvens	22.0	27.4	27.1	23.5	49.1	50.9	**605**
S. tenera	27.0	21.3	25.9	25.8	52.9	47.1	555
S. wightii	30.6	18.8	28.1	22.5	58.7	41.3	**480**
S. brachystachya	29.2	20.1	28.3	22.4	57.5	42.5	593
S. repanda	22.1	27.4	26.5	24.0	48.6	51.4	570
S. radicata	22.2	27.7	26.5	23.7	48.7	51.4	582
S. bryopteris	29.1	20.0	28.0	22.9	57.1	42.9	571
S. delicatula with cones	21.0	29.4	25.8	23.9	46.7	53.3	582
S. delicatula without cones	20.7	29.3	26.0	24.0	46.7	53.3	574

Nucleotide substitution pattern

Maximum likelihood estimation

The nucleotide substitution pattern for ten *Selaginella* species can be estimated using maximum likelihood method that is represented in Table 32. The overall transition/transversion bias for the studied *Selaginella* species is R = 9.709, where R = [A*G*k_1 + T*C*k_2]/[(A+G)*(T+C)].

Table 32: Maximum composite likelihood estimation of *Seleginella* species

Base pairs	A	T	C	G
A	-	1.23	1.13	7.69
T/U	1.25	-	35.45	1.07
C	1.25	38.49	-	1.07
G	9.01	1.23	1.13	-

Composite distance pattern

The composite distance pattern deals with the differences in base composition even when the substitution patterns are homogeneous among lineages. In addition, the compositional distance will correlate with the number of differences between 10 nucleotide sequences. The composite difference among ten nucleotide sequences of *Selaginella* was displayed in Table 33. Among the ten *Selaginella* species, the highest composite difference (3.59) was observed among *S. wightii* and *S. delicatula* with cones, *S. wightii* and *S. delicatula* without cones, *S. bryopteris* and *S. delicatula* with cones, *S. bryopteris* and *S. delicatula* without cones. The lowest composite difference (0.00) was observed among *S. involvens* and *S. repanda*, *S. involvens* and *S. radicata*, *S. wightii* and *S. brachystachya*, *S. repanda* and *S. radicata*, *S. wightii* and *S. bryopteris*, *S. brachystachya* and *S. bryopteris*, *S. delicatula* with and without cones.

Table 33: Estimation of composite distance of *Selaginella* species

Taxon	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
S1	1.00										
S2	0.50	1.00									
S3	0.26	0.08	1.00								
S4	0.63	2.05	1.50	1.00							
S5	1.34	3.29	2.48	0.19	1.00						
S6	1.25	3.13	2.34	0.16	0.00	1.00					
S7	0.23	0.07	0.00	1.44	2.43	2.29	1.00				
S8	0.23	0.07	0.00	1.44	2.43	2.29	0.00	1.00			
S9	1.34	3.29	2.48	0.19	0.00	0.00	2.43	2.43	1.00		
S10	0.64	0.01	0.12	2.30	3.59	3.42	0.12	0.12	3.59	1.00	
S11	0.64	0.01	0.12	2.30	3.59	3.42	0.12	0.12	3.59	0.00	1.00

Note: S1- *S. intermedia*, S2- *S. inaequalifolia*, S3- *S. involvens*, S4- *S. tenera*, S5- *S. wightii*, S6- *S. brachystachya*, S7- *S. repanda*, S8- *S. radicata*, S9- *S. bryopteris*, S10- *S. delicatula* with cones, S11- *S. delicatula* without cones.

Nucleotide pair frequencies for directional sequences

The evolutionary rate of the base substitutions present in nucleotide sequences can be estimated using Kimura (1980) 2-parameter mode. The transition/transversion bias (R) for directional sequences (16 pairs) of ten *Selaginella* species was estimated as 2.52. The nucleotide pair frequencies were high in AA (133) followed by GG (118), CC (114) and TT

(110). The average identical pairs (ii) of ten nucleotides were 475, transitional pairs (Si) were 53 and transversional pairs (Sv) were 22 displayed in the Table 34.

Table 34: Nucleotide pair frequencies for directional sequences of *Selaginella* species

Frequencies	Identical pairs (ii)	Transitional pairs (si)	Transversional pairs (sv)	Ratio (si/sv)	AA	TT	CC	GG	Total
Average	475	53	22	2.45	133	110	114	118	548.89
1st codon	169	12	2	7.39	53	32	49	34	182.96
2nd codon	135	34	15	2.23	37	46	30	22	183.31
3rd codon	171	7	5	1.40	43	31	36	61	182.62

Statistical domain

The statistical domain among 605 base pairs of ten *Selaginella* species was estimated using MEGA 5.2 (Table 35). The statistical domain for overall species of ten *Selaginella* species includes 411 conserved regions, 187 variable regions, 347 0-fold degenerate sites, 67 two-fold degenerate sites and 62 four-fold degenerate sites (Table 35).

Table 35: Statistical domain for *Selaginella* species

Taxon	Conserved Region	Variable region	0-fold degenerate sites	2-fold degenerate sites	4-fold degenerate sites
S1	525	49	373	108	95
S2	537	4	353	93	100
S3	540	34	394	110	99
S4	416	139	343	119	84
S5	377	103	308	103	66
S6	449	125	375	133	80
S7	527	36	371	101	92
S8	538	36	377	105	94
S9	446	125	364	125	78
S10	574	-	372	104	103
S11	574	-	366	102	102
Total species	**411**	**187**	**347**	**67**	**62**

Note: S1- *S. intermedia*, S2- *S. inaequalifolia*, S3- *S. involvens*, S4- *S. tenera*, S5- *S. wightii*, S6- *S. brachystachya*, S7- *S. repanda*, S8- *S. radicata*, S9- *S. bryopteris*, S10- *S. delicatula* with cones, S11- *S. delicatula* without cones.

Genetic distance estimation

The evolutionary divergence between nucleotide sequences of ten *Selaginella* species conducted using the maximum composite likelihood model (Table 36). The maximum evolutionary distance (0.34) was observed among *S. tenera* and *S. delicatula* with and without cones. The minimum evolutionary distance (0.00) was found between *S. wightii* and *S. brachystachya*; *S. wightii* and *S. bryopteris*; *S. brachystachya* and *S. bryopteris*; *S. repanda* and *S. radicata*; *S. delicatula* with and without cones.

Table 36: Genetic distance estimation for studied *Selaginella* species

Taxon	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
S1	1.00										
S2	0.10	1.00									
S3	0.11	0.07	1.00								
S4	0.33	0.33	0.33	1.00							
S5	0.32	0.32	0.32	0.07	1.00						
S6	0.32	0.32	0.32	0.07	0.00	1.00					
S7	0.11	0.07	0.01	0.33	0.31	0.31	1.00				
S8	0.11	0.07	0.01	0.33	0.31	0.31	0.00	1.00			
S9	0.32	0.32	0.32	0.07	0.00	0.00	0.31	0.31	1.00		
S10	0.10	0.01	0.06	0.34	0.33	0.32	0.07	0.07	0.33	1.00	
S11	0.10	0.01	0.06	0.34	0.33	0.32	0.07	0.07	0.33	0.00	1.00

Note: S1- *S. intermedia*, S2- *S. inaequalifolia*, S3- *S. involvens*, S4- *S. tenera*, S5- *S. wightii*, S6- *S. brachystachya*, S7- *S. repanda*, S8- *S. radicata*, S9- *S. bryopteris*, S10- *S. delicatula* with cones, S11- *S. delicatula* without cones.

Amino acid composition

The amino acid compositions of the selected *Selaginella* species were analysed using MEGA 5.2 and the results of amino acid composition were recorded in Table 36. *S. involvens* displayed maximum number of amino acids (196) and *S. wightii* showed minimum number of amino acids (154). Totally twenty amino acids were expressed in the studied ten *Selaginella* species viz., alanine (Ala), cysteine (Cys), aspartic acid (Asp), glutamic acid (Glu), phenylalanine (Phe), glycine (Gly), histamine (His), isoleucine (Ile), lysine (Lys), leucine (Leu), methionine (Met), asparagine (Asn), proline (Pro), glutamine (Gln), arginine (Arg), serine (Ser), threonine (Thr), valine (Val), tryptophan (Trp) and tyrosine (Tyr). Among the

twenty amino acids, leucine showed highest percentage (22.15%) in *S. tenera* and tyrosine expressed lowest percentage (0.53%) in *S. intermedia* (Table 37).

Table 37: Amino acid composition of studied *Selaginella* species

Amino acids	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
Ala	1.61	2.23	1.53	2.84	1.29	1.05	2.18	2.13	1.09	2.10	2.13
Cys	2.15	2.79	2.56	3.40	2.59	2.11	2.73	2.67	2.19	2.10	2.13
Asp	2.68	2.23	2.56	3.40	3.89	3.70	2.73	2.67	3.29	2.63	2.67
Glu	4.30	3.91	4.10	3.40	2.59	3.70	3.82	3.74	3.84	3.68	3.74
Phe	2.15	2.23	2.56	2.84	1.94	2.64	2.73	2.67	2.74	2.63	2.13
Gly	3.22	3.35	3.58	5.68	3.24	3.17	3.27	3.20	3.29	3.15	3.20
His	2.15	1.67	2.56	0.56	1.94	2.11	2.18	2.67	2.19	2.63	2.67
Ile	6.45	6.14	6.66	3.97	5.84	5.82	6.55	6.41	5.49	6.31	6.41
Lys	5.37	6.14	5.64	8.52	7.79	7.40	6.01	5.88	7.69	5.78	5.88
Leu	9.67	9.49	9.23	**22.1**	21.4	21.1	9.28	9.62	20.89	10.00	10.16
Met	3.22	3.35	4.61	4.54	5.84	4.23	4.37	4.27	4.94	3.15	3.20
Asn	4.83	5.02	5.12	4.54	4.54	4.76	4.91	4.81	4.94	5.26	4.81
Pro	14.51	16.76	14.87	6.81	5.19	4.23	15.30	14.97	4.39	15.78	16.04
Gln	3.76	2.23	3.58	1.70	1.94	3.17	3.82	3.74	2.74	2.10	2.13
Arg	9.67	10.05	9.74	6.81	3.89	5.82	8.74	9.09	6.04	11.05	10.69
Ser	6.98	5.58	6.66	2.84	4.54	4.76	4.91	5.34	4.94	6.31	6.41
Thr	5.91	6.14	4.10	5.11	5.19	5.29	4.91	4.81	4.39	5.26	5.34
Val	8.60	8.37	7.69	6.25	9.74	7.93	8.19	8.02	8.24	7.36	7.48
Trp	2.15	1.67	1.53	2.27	2.59	2.64	2.18	2.13	2.74	1.57	1.60
Tyr	***0.53***	0.55	1.02	2.27	3.89	4.23	1.09	1.06	3.84	1.05	1.06
Total	186	179	**195**	176	***154***	189	183	187	182	190	187

Note: S1- *S. intermedia*, S2- *S. inaequalifolia*, S3- *S. involvens*, S4- *S. tenera*, S5- *S. wightii*, S6- *S. brachystachya*, S7- *S. repanda*, S8- *S. radicata*, S9- *S. bryopteris*, S10- *S. delicatula* with cones, S11- *S. delicatula* without cones.

Phylogenetic analysis using inference methods

The evolution of genetically related group of organisms can be estimated using phylogenetic relationships obtained by MEGA 5.2. The phylogenetic analysis of ten *Selaginella* species collected from different localities of South India was carried out using four methods viz., minimum evolution, neighbor joining, UPGMA and maximum parsimony.

The phylogenetic analysis of studied *Selaginella* species using minimum evolution, neighbor joining and UPGMA methods showed similar trees whether maximum parsimony tree displayed varied tree. The evolutionary tree using minimum evolution, neighbor joining and UPGMA methods represents two main clusters C_1 and C_2 (Fig. 10, 11 and 12). The

cluster C_1 is made of monophyletic taxon group viz., *S. delicatula* with and without cones, *S. inaequalifolia*, *S. involvens*, *S. repanda*, *S. radicata* and a paraphyletic taxon *S. intermedia* with the distance value 0.11. The cluster C_2 consist of monophyletic taxon group viz., *S. brachystachya*, *S. wightii* and *S. bryopteris* and a paraphyletic taxon *S. tenera* with highest distance range 0.12. The cluster C_1 is divided into two nodes C_1N^1 and C_1N^2. The node C_1N^1 is branched into $C_1N^1B_1$ and $C_1N^1B_2$ where C_1N^2 showed the unique character of *S. intermedia* with the distance value 0.05. $C_1N^1B_1$ is again branched into $C_1N^1B_1b_1$ and $C_1N^1B_1b_2$. $C_1N^1B_1b_1$ displayed the similarity between $C_1N^1B_1b_1A$ (*S. delicatula* with cones) and $C_1N^1B_1b_1B$ (*S. delicatula* without cones). The branch $C_1N^1B_1b_2$ expressed the individuality of *S. inaequalifolia* with the distance value 0.002. $C_1N^1B_2$ is then branched into $C_1N^1B_2b_1$ and $C_1N^1B_2b_2$. The branch $C_1N^1B_2b_1$ displayed the diverged character of *S. involvens* with the distance of 0.007. $C_1N^1B_2b_2$ represented 100% similarity between *S. repanda* ($C_1N^1B_2b_2A$) and *S. radicata* ($C_1N^1B_2b_2B$) with 0.00 distance value. The cluster C_2 is divided into two nodes C_2N^1 and C_2N^2. The node C_2N^1 showed the distinct character of *S. tenera* with distance range 0.04. The node C_2N^2 is branched into $C_2N^2B_1$ and $C_2N^2B_2$. The $C_2N^2B_1$ expressed the individuality of *S. brachystachya* with lowest (0.00) distance value. The branch $C_2N^2B_2$ is again divided into two branches $C_2N^2B_2b_1$ and $C_2N^2B_2b_2$ which displayed the closeness between *S. wightii* and *S. bryopteris* with least (0.00) distance range.

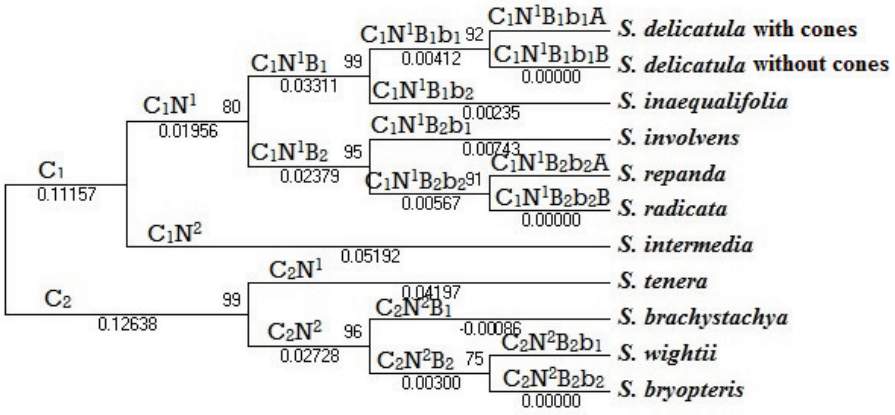

Fig. 10: Phylogenetic tree of *Selaginella* species based on minimum evolution method

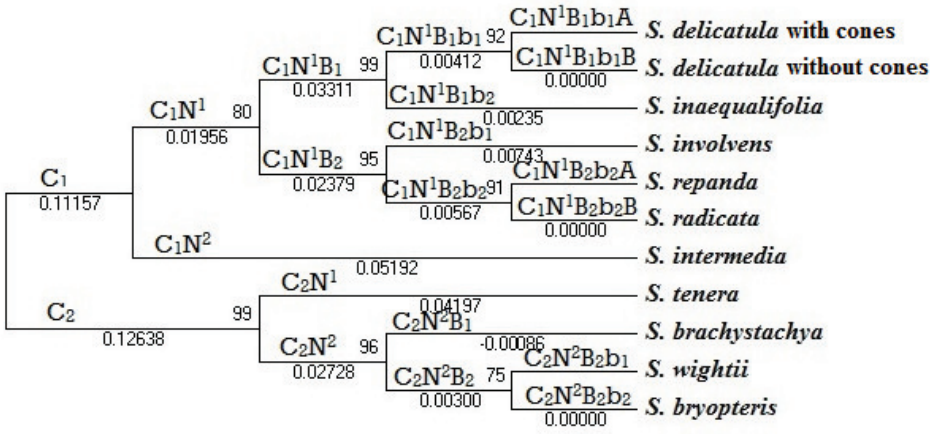

Fig. 11: Phylogenetic tree of *Selaginella* species based on NJ method

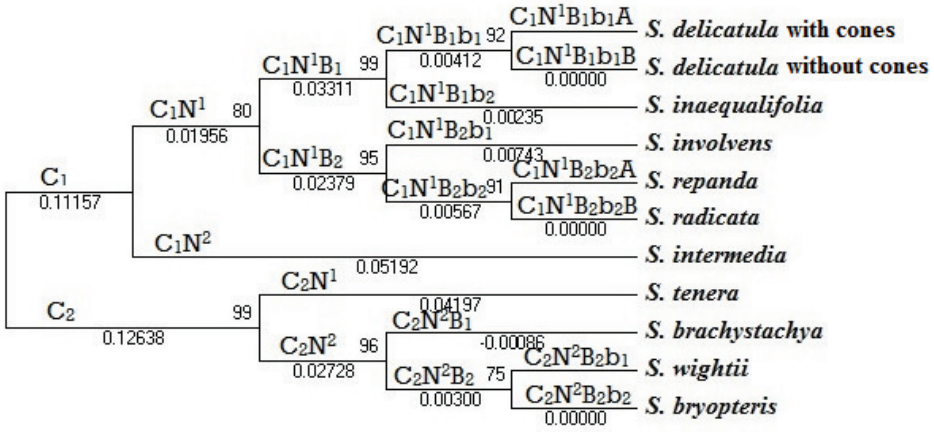

Fig. 12: UPGMA tree for *Selaginella* species

Maximum parsimony tree

The evolutionary history was inferred using the Maximum Parsimony method. The most parsimonious tree with length is 165 (Fig. 13). The consistency index, retention index and composite index for all substitution sites and parsimony-informative sites of the studied ten *Selaginella* species are 0.890909, 0.946429 and 0.843182 respectively.

The evolutionary tree represents two main clusters C_1 and C_2. The cluster C_1 is made of monophyletic taxon group viz., *S. delicatula* with and without cones, *S. inaequalifolia*, *S. involvens*, *S. repanda*, *S. radicata* and a paraphyletic taxon *S. intermedia* with the distance value 24.33. The cluster C_2 involves monophyletic taxon groups namely *S. brachystachya*, *S. wightii* and *S. bryopteris* and a paraphyletic taxon *S. tenera* with highest distance range 41.00.

The cluster C_1 is diverged into two nodes C_1N^1 and C_1N^2. The node C_1N^1 is branched into $C_1N^1B_1$ and $C_1N^1B_2$ where C_1N_2 showed the unique existence of *S. intermedia* with the distance value 18.00. $C_1N^1B_1$ is again branched into $C_1N^1B_1b_1$ and $C_1N^1B_1b_2$. $C_1N^1B_1b_1$ displayed the similarity between two sub branches denoted by $C_1N^1B_1b_1A$ (*S. delicatula* with cones) and $C_1N^1B_1b_1B$ (*S. delicatula* without cones) with least distance value 0.00 between them. The branch $C_1N^1B_1b_2$ expressed the individuality of *S. inaequalifolia* compared with other studied *Selaginella* species with distance 1.50. $C_1N^1B_2$ is then branched into $C_1N^1B_2b_1$ and $C_1N^1B_2b_2$. The branch $C_1N^1B_2b_1$ displayed the diverged character of *S. involvens* with distance range 2.50. $C_1N^1B_2b_2$ represented the similarity between *S. repanda* ($C_1N^1B_2b_2A$) and *S. radicata* ($C_1N^1B_2b_2B$) with 0.00 distance value. The cluster C_2 is divided into two nodes C_2N^1 and C_2N^2. The node C_2N^1 showed the distinct character of *S. tenera* with distance range of 15.33. The node C_2N^2 is diverged into two branches $C_2N^2B_1$ and $C_2N^2B_2$. The $C_2N^2B_1$ expressed the individuality of *S. brachystachya* with lowest distance value 0.00. The branch $C_2N^2B_2$ is again branched into $C_2N^2B_2b_1$ and $C_2N^2B_2b_2$ which displayed the similarity between *S. wightii* and *S. bryopteris* with least distance range 0.00 respectively.

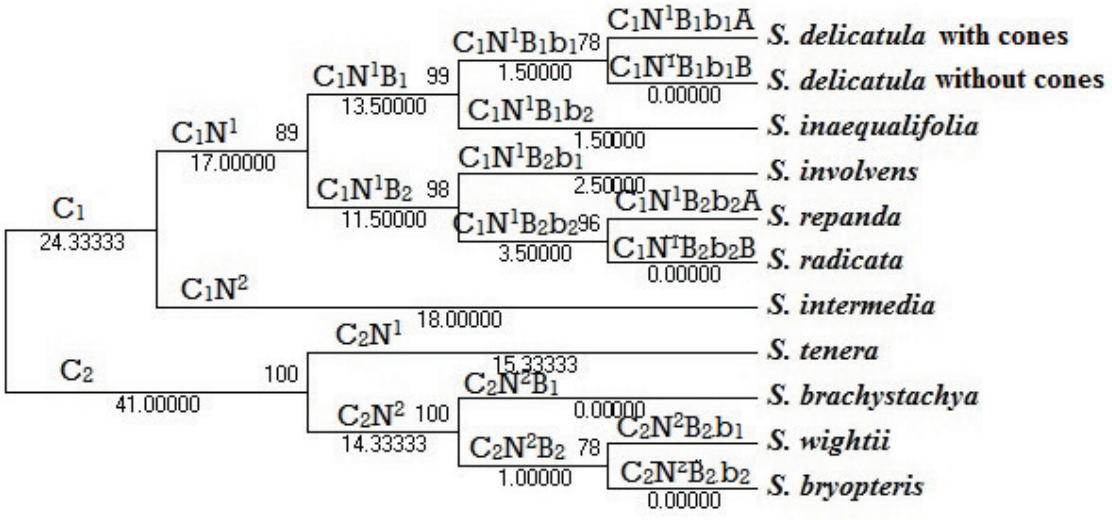

Fig. 13: Maximum parsimony tree of studied *Selaginella* species

Systematics includes the classification of living organisms into hierarchical series of groups emphasizing their phylogenetic relationships. It is the science of arranging species which reflects their evolutionary relationships. Based on this concept, taxonomy would be just a part of systematics but systematic includes both taxonomy and phylogeny (Wagele, 2005). Phylogenetics is the science of the reconstruction of phylogeny and phylogenetic systematics is a method of classification based on the study of evolutionary relationships between groups of organisms and the integration of proper species of groups into a hierarchical system which reflects their phylogeny (Garcia *et al*., 2008).

The concept of "species" is perhaps the most debated subject in evolutionary biology as demonstrated by the existence of more than twenty definitions based on different methods and criteria (Hey, 2001). The morphological differences among the species are the result of the extent of genetic divergence. Numerous taxonomic methods have been used to measure these differences. Genetic information provided by morphological characters is often limited and expression of quantitative trait is subjected to strong environmental influences. The molecular approach for the identification of plant varieties or genotypes seems to be more effective than the use of traditional morphological markers because it allows direct access to the genetic material and makes it possible to understand the relationships between the individuals (Karp *et al*., 1997). Thus molecular approaches have found a niche in taxonomy. The molecular techniques now have become less time consuming and help us to establish the genetic relationship between the members of different taxonomic categories (Singh, 2012). The application of molecular data on the field of plant systematics can be used to infer phylogeny. The molecular phylogenetic approach is most useful at different levels ranging from kingdom to species. In the majority of cases, different molecular approaches tend to confirm the taxonomic schemes established for plants (Wilson, 1995).

Assessment of genetic variability within and between species or populations for purpose of conservation and to seek useful genotypes can be achieved by employing a variety of techniques. Among these, use of molecular markers finds greater utility due to its ability to reveal polymorphism at the genetic level. They have become increasingly popular in plant research since they can potentially provide a much higher number of markers. Among the different types of markers available, biochemical and molecular markers have become exceedingly popular for estimation of genetic changes within and between the species (Williams *et al*., 1990). With this knowledge, the present study aimed to reveal inter-specific relationships among the selected species of *Selaginella* using biochemical (SDS-PAGE and PAGE), spectroscopic (MALDI-TOF MS) and molecular analysis (DNA sequencing using *rbcL* gene).

As molecular phylogenetic studies have accumulated, it has become evident that different molecular tools are required for answering different questions because of varying rates of sequence evolution among genomes, genes and gene regions. The choice of molecular tools plays a vital role to ensure that an appropriate level of variation is recovered to answer the phylogenetic problems. Most species display a complex of genetic variations along their range of distribution. In the present study, molecular relationships among the ten species of *Selaginella* viz., *S. intermedia*, *S. inaequalifolia*, *S. involvens*, *S. tenera*, *S. wightii*, *S. brachystachya*, *S. repanda*, *S. radicata*, *S. bryopteris*, *S. delicatula* with cones and *S. delicatula* without cones collected from various localities of South India which includes Kaakachi hills (Tirunelveli, Tamil Nadu), Keeriparai and Maruthuvalmalai (Kanyakumari, Tamil Nadu), Shenbaganur and Eettipallum (Dindigul, Tamil Nadu), Ponmudi (Idukki, Kerala), Nilgiris (Tamil Nadu) and Thenmalai (Kerala) were examined.

Study of genetic variation is one such important and powerful procedure that often been employed for taxonomic studies. Electrophoresis of proteins using SDS-PAGE has been

successfully used for the characterization of different taxonomic, evolutionary and genetic relationship studies in plants (Johnson *et al*., 2009; Malik *et al*., 2009; Johnson *et al*., 2012). SDS-PAGE technique is a powerful tool for estimating the molecular weights of proteins. It simultaneously exploits differences in molecular size to resolve proteins differed by as little as 1% in their electrophoretic mobility through the gel matrix (Smila *et al*., 2007).

Tateno *et al*. (2003) purified and characterized the expression of lectins (proteins) which is approximately 15 kDa from rhizomes of the fern *Phlebodium aureum* by affinity chromatography. Chang *et al*. (2005) analyzed various proteins of *Arabidopsis thaliana* by use of high-speed centrifugation, sucrose gradient fractionation, one and two dimensional gel electrophoresis, liquid chromatography purification and mass spectrometry. Lina (2010) isolated and compared the protein expression between hydrated and desiccated *Selaginella lepidophylla* tissues using two-dimensional difference polyacrylamide gel electrophoresis. Wang *et al*. (2010) studied the desiccation tolerance in resurrection fern-ally *Selaginella tamariscina* revealed by physiological and proteomic analysis. The results showed that endogenous abscissic acid (ABA) increased to regulate dehydration-responsive genes/proteins. In addition the results showed 138 desiccation-responsive two-dimensional electrophoresis (2-DE) spots, representing 103 unique proteins. Banks *et al*. (2011) reported the genome sequence of the lycophyte *Selaginella moellendorffii*. Lydia *et al*. (2012) explained the expression of MADS-domain proteins in *Selaginella moellendorffii* by means of complete genome sequencing. In the present study, SDS-PAGE proteins of *Seleaginella* were isolated and separated, the protein profile was used to reveal inter-specific relationships among the studied *Selaginella* species collected from various localities of South India.

Similar to the present study, the similarity and variation among the plant species using SDS-PAGE have been carried out by many researchers (Smila *et al*., 2007; Johnson *et al*., 2009; Babu *et al*., 2010; Nanthini *et al*., 2011; Johnson *et al*., 2012). The present study

explores the existing polymorphic proteins through SDS-PAGE to facilitate the characterization of *Selaginella* species. SDS-PAGE revealed the inter-specific variability among the selected *Selaginella* species in which, a total of 190 bands with various Rf values and molecular weight were displayed in the SDS-PAGE gel system of *Selaginella*. Each region expressed different proteins which act as representative of the expression of a particular gene of the studied *Selaginella* species. Among the ten species of *Selaginella*, *S. repanda* showed maximum number (25) of protein bands and *S. delicatula* with cones showed minimum number (12) of protein bands. Unique banding patterns observed among the ten *Selaginella* species acts as fingerprint of the selected *Selaginella* species. Such finger printing is useful in differentiating the species and act as biochemical markers for such species in plant systematic studies. These banding profiles will also facilitate the identification of the medicinally important *Selaginella* species.

Singh *et al*. (2004) observed in *Ocimum* that, UPGMA cluster analysis of genetic similarity indices helped to group all the accessions into two major clusters corresponding to the botanical sections. Inter clustering within the two clusters precisely grouped one species in one sub cluster as expected from their genetic background. Similar to Singh *et al.* (2004) observation, in the present study the protein profile based cladogram exhibited two clusters in which cluster C_1 exhibited eight species of *Selaginella* which includes *S. intermedia*, *S. tenera*, *S. radicata*, *S. inaequalifolia*, *S. involvens*, *S. wightii* and *S. bryopteris*, *S. delicatula* with and without cones. The cluster C_2 contains only two species that includes *S. brachystachya* and *S. repanda*. The protein profile based cladogram explained the genetic variability among the ten studied *Selaginella* species. The protein profiles presents an interbreed depiction of the relationship among the ten species of *Selaginella*.

Matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry (TOF MS) has been applied for the analysis of a wide range of biomolecules.

Initial applications were almost exclusively used for the qualitative analysis of biopolymers because MALDI-TOF MS provided a fast and accurate approach to molecular mass and purity information. Because of the speed of analysis, ease of use, relative low equipment cost, ease of data interpretation and limited potential for cross-contamination between samples. MALDI profiling offers exceptionally high-throughput and has therefore become an attractive tool for the discovery and validation of biomarkers. In addition, the method must be sufficiently precise to observe relative differences between distinct populations. Profiling has now been applied in thousands of studies includes the quantification of amino acids, lipids, natural products, drugs, polymers, herbicides, metabolites, toxins, oligonucleotides, carbohydrates, peptides and proteins.

Analyzing proteins with MALDI–TOF MS has several advantages. MALDI–TOF MS is fast; ionization, separation by size and detection of proteins takes milliseconds to complete. By contrast, conventional electrophoretic methods for separating and detecting proteins can take hours to complete. The results are absolute, being based on the intrinsic property of the mass-to-charge ratio (m/z). This is inherently more accurate than electrophoresis-based or hybridization array based methods, which are both susceptible to complications from secondary-structure formation in proteins (Park, 2004). Furthermore, the absolute nature of detection, combined with the detection of predominantly singly charged molecular ions, makes the analysis of complex mixtures and great potential for high-throughput protein analysis applications are now possible by MALDI-TOF MS (Nakumara and Oda, 2007).

Analysis by MALDI-TOF MS delivers fast determination, characterization and measurement of accurate molecular weight of proteins. This systematic study allows the analysis of proteomes or gene products in various tissues and physiological states of cells. Protein characterization using MALDI TOF MS has become a major field of functional genomics. MALDI-TOF MS analysis in proteomic research is advanced in animals, bacteria

and fungi (Kersten *et al.*, 2002). In plants, MALDI-TOF MS was carried out to reveal the protein profile of *Arabidopsis thaliana* (Peltier, 2001; Datta *et al*., 2013); spanich (Yamaguchi and Subramanian, 2000); rice (Koller *et al*., 2002; Zhao *et al*., 2012); *Helianthus annus* (Castillejo *et al*., 2008); *Artemisia vulgaris* (Kumar and Kumari, 2009); wheat (Kamal *et al*., 2010); *Ananas cosmos* and *Kalanchoe pinnata* (Hong *et al*., 2012); *Medicago truncatula* (Lee *et al*., 2013).

In the present study, MALDI-TOF MS analysis of ten *Selaginella* species collected from different localities of South India expressed different ion peaks ranged from 0-1,00,000 Da. The results showed totally 483 spectral peak values (m/z values) and revealed the biochemical similarity and variations among the selected species of *Selaginella*. Among the spectral profile of ten *Selaginella* species, *S. involvens* displayed maximum number (86) of spectral peaks ranged from 527 to 99215 m/z values. Next to that, *S. tenera* represented 77 spectral peaks with m/z values 719 to 95316. *S. radicata* depicted minimum number (21) of spectral peaks varied from 694 to 91754 m/z values. Among the 483 spectral peak values of the studied ten *Selaginella* species, 71 spectral peaks were observed with unique presence, which distinguished the genetic similarities and variation of studied *Selaginella* species. The resulting peak lists of individual samples were submitted to NTSYS software to produce a taxonomic tree to reveal inter-specific variation. The morphologically distinguished character of *S. tenera*, *S. involvens*, *S. wightii*, *S. brachystachya* and *S. repanda* is confirmed in the cladogram by the presence of unique m/z values. Thus, the present m/z values of MALDI-TOF MS analysis acted as a spectroscopic tool to recognize the inter-specific variation among the selected *Selaginella* species.

Variations in isozyme expression of tissues reflect changes in metabolic activities during growth, development and differentiation of plant by means of biochemical indices. Changes in isozyme action during development of a tissue can be detected by pattern shifts

on PAGE profile. Isozyme markers have been useful in determining genetic relationships among closely related species and cultivars. Isozyme electrophoresis is chosen for its relative simplicity because it provides direct visualization of gene products and can provide an unique fingerprint for each genetically distinct clone (Lebot *et al.*, 1991). Isozyme polymorphism has been successfully used for demonstrating inter and intra-specific variations in different species and cultivars (Barta *et al.*, 2003; Mukhlesur *et al.*, 2004; Johnson *et al.*, 2010; Angelov and Ivanova, 2012).

Characterization of purified enzyme is important to reveal physiological functions and action of particular enzymes. Investigation of enzyme catabolism using molecular approaches is increased in recent trends (Rea *et al*., 2004). The functional characterization of plant esterase has been explored (Sukor *et al*., 2006; Johnson and Raja, 2007; Hammad, 2009; Nanthini *et al*., 2011). An increase in peroxidase activity has been reported as an early response to different stresses and may provide cells with resistance against formation of H_2O_2 which is formed when plants are exposed to stress factors and so cause change in plant metabolism (Castillo, 1992). Peroxidase is also involved in a large number of biochemical and physiological processes and may change quantitatively and qualitatively during growth and development (Zhi, 2003).

Phosphorus is one of the major elements found in plants. The available phosphate concentration in many soils is rather low because it is commonly bound to many soil constituents (Morales *et al*., 2012). As the sources of phosphorus run out, improvements in the absorption and efficiency of phosphorus become necessary. Plants have developed several mechanisms to overcome phosphorus deficiency by induction of extracellular and intracellular phosphatases. The purified acid phosphatase was sensitive to ions at various degrees depending on the ions nature and isoenzymes. The requirement of metallic ions for acid phosphatase activity has also varied according to plant species, development stage and

isoenzymes heterogeneity. However, Mg^{2+}, Zn^{2+} and EDTA were inhibitors and K^{+}, Cu^{2+} and Ba^{2+} were activators for the specific enzyme. The acid phosphatase activity has been previously reported in other plant species such as pea (Mizuta and Suda, 1980), spinach (Pan, 1985), tobacco (Pan *et al*., 1987; Chen, 1992), peanut seedlings (Gonnety *et al*., 2006) and rice (Asaduzzaman, 2011). Alkaline phosphatases are enzymes that catalyze the hydrolysis of phosphate ester bonds in extracellular organic molecules, permitting the cellular uptake of inorganic phosphate (Pi) for metabolism. Their properties include low substrate specificity and an alkaline pH optimum. The active site of most ALPs contains a single Mg and two Zn atoms. Alkaline phosphatases in plants play a major role in the supply and metabolism of inorganic phosphate for the maintenance of cellular metabolism (Coleman, 1992).

Polyphenol oxidase has been shown to exist in multiple and inter convertible forms and is widely distributed in plant kingdom. It is well known that the enzyme plays an important role in the browning reaction in fruits and vegetables. It has been suggested that the polyphenol oxidase enzyme might be associated with many important physiological functions such as growth and differentiation (Nitesh *et al*., 2010).

Isozymic analysis using electrophoresis has been carried out in both higher plants and pteridophytes (Woodin *et al*. 1978; Gastony, 1982; Haufler and Soltis, 1986; Shigeo and Yasuyuki, 1994; Hooper and Haufler, 1997; Santiago, 1999; Herrero, 2001; Pajaron *et al*., 2005; Sukor *et al*., 2006; Hammad, 2009; Irudayaraj *et al.,* 2009; Nandhini *et al*., 2011; Johnson *et al*., 2012; Owk *et al*., 2013). Johnson *et al*. carried out few isozymic studies on South Indian pteridophytes viz., Johnson *et al*. (2010) in *Trichomonas*; Johnson *et al*. (2010) in *Pteris*; Johnson *et al*. (2010) in *Cheilanthes viridis*, *Phlebodium aureum*, *Pronephrium triphyllum* and *Sphaerostephanos unitus*; Johnson *et al*. (2010) in *Tectaria*; Johnson *et al*. (2010) in *Adiantum*.

Irudayaraj and Johnson (2011) identified the phylogenetic relationships among *Sphaerostephanos arbuscula*, *S. unitus* and *S. subtruncatus* using isoperoxidase analysis. A total of six different bands in five different positions with different molecular weight were obtained. Johnson *et al*. (2012) exposed inter-specific variation on three species of tree fern *Cyathea* by means of isoperoxidase analysis. A total of seven bands with three active regions were expressed in the *Cyathea* spp. enzyme system. The banding pattern revealed hundred percentage genetic differentiations between the three *Cyathea* species which belong to three different intra generic taxonomical groups. Johnson *et al*. (2012) assessed the genetic variation between different populations of *Thelypteris ciliata* collected from different localities on Tirunelveli hills, using isoperoxidase profiling. A total of six bands in six different positions with five active regions were observed in the isoperoxidase system of *T. ciliata*. The isoperoxidase enzyme systems confirms inter and intra biochemical difference of *T. ciliata*.

Similar to the previous observations in the present study also isozymic analysis viz., esterase, peroxidase, acid phosphatase, alkaline phosphatase and polyphenol oxidase were carried out to know inter-specific relationship among the selected ten species of *Selaginella*. The evolutionary tree of studied *Selaginella* species based on the esterase profile showed the distinct nature of *S. intermedia*, *S. involvens*, *S. radicata*, *S. tenera* and *S. brachystachya*. These will be an additional supportive evident to distinguish the morphologically unique species of *Selaginella* viz., *S. intermedia*, *S. involvens*, *S. radicata*, *S. tenera* and *S. brachystachya*. In peroxidase system of *Selaginella*, twenty five distinct bands were observed. The phylogenetic tree based on the peroxidase profile of studied *Selaginella* species explained the uniqueness of *S. involvens*, *S. brachystachya* and *S. tenera*. Isozymes such as esterase and peroxidase have been utilized to assess the genetic similarity and differences at the various taxonomic levels (Onus, 2000; Sabu *et al*., 2001; Manjunatha *et al*.,

2003). Similarly in the present study also, these isozymes are used as fingerprint for the identification of *Selaginella* species. They revealed the variation at species level.

Acid phosphatase system of studied *Selaginella* species was observed with a total of sixty three enzymatic bands with twenty one classified bands in the acid phosphatase profile. The cladistic tree based on the acid phosphatase profile displayed the restricted character of *S. tenera*, *S. delicatula* with cones and *S. bryopteris*. In addition, the acid phosphatase system revealed the developmental stage variation in *S. delicatula* with and without cones. In morphotaxonomy, the cones of fern allies play an important role to distinguish the fern allies at species level. In the present study, the result of the acid phospahstase profiles provided an alternative for the identification of *S. delicatula* without cones. In the alkaline phosphatase enzyme system of studied *Selaginella* species, twenty exceptional bands were obtained. The evolutionary tree displayed the distinctive character of *S. intermedia*, *S. tenera* and *S. wightii*. Polyphenol oxidase enzyme system of studied *Selaginella* species was observed with twelve active bands and showed only two distinct enzymatic bands. The phylogenetic tree of *Selaginella* species displayed the uniqueness of *S. brachystachya*, *S. radicata* and *S. bryopteris* based on polyphenol oxidase profile. The amalgamated cladogram of isozymic profiles displayed the distinct character of *S. wightii*, *S. bryopteris* and *S. radicata*. Unique banding profiles of esterase, peroxidase, acid phosphatase, alkaline phosphatase and polyphenol oxidase were observed in the studied *Selaginella* species which were used to distinguish the selected *Selaginella* species and these profiles will act as biochemical fingerprint for the studied species in plant systematic studies.

Genetic markers represent genetic differences between individual organisms or species. Such markers themselves do not affect the trait of interest because they are located only near or linked to genes controlling the trait. All genetic markers occupy specific genomic positions within chromosomes called loci. All methods for the identification of

species that rely on DNA or protein sequence analysis presuppose the neutral theory of molecular evolution, in which different lineages diverge over evolutionary times by the accumulation of molecular changes (Pereira, 2008). These methods are based on the assumption that individuals from the same species carry specific protein or DNA sequences that are different from those found in individuals from other species. However, the distribution of a given molecular variant in time and in space will be influenced by the reproductive success of individuals, migratory events and random genetic drift. Therefore, it should be realized that a continuous genetic variability does exist among individuals of a species.

Molecular markers have been looked upon as tools for a large number of applications ranging from localization of a gene to improvement of plant varieties by marker assisted selection. They have also become extremely popular markers for phylogenetic analysis adding new dimensions to the evolutionary theories. Genome analysis based on molecular markers has generated a vast amount of information and a number of databases are being generated to preserve and popularize it. Among the various markers used to distinguish the taxonomically varied species, DNA markers are more suitable and ubiquitous to most of the living organisms.

Sayantani *et al*. (2012) developed the protocol for isolating genomic DNA from leaves of *Selaginella* species viz., *S. delicatula*, *S. repanda*, *S. bryopteris*, *S. plana* and *S. monospora* using a modified CTAB protocol of Rogers and Benedich (1994) and they observed that the DNA samples were suitable for genetic diversity analysis with RAPD markers. Similar to Sayantani *et al*. (2012) in the present study, the DNA of ten *Selaginella* species was extracted using a modified CTAB extraction method described by Murray and Thompson (1980). The extracted DNA samples were suitable for *rbcL* gene amplification and the amplified products were sequenced.

Many molecular markers with different advantage and disadvantage have been used in the population genetic study. Previous markers for genetic diversity analysis include RAPD, RFLP, AFLP, SSR, microsatellites, etc. Because of some disadvantages, in recent days, the use of sequence data from the DNA of chloroplasts (cp DNA) or nuclear ribosomal DNA (rDNA) and mitochondrial DNA have proven to be highly useful in elucidating both lower and higher level relationships. In that, *rbcL* data has been most valuable for inferring phylogenetic relationship between closely related species (Alvarez and Wendel, 2003).

Several studies have used DNA barcoding methods to identify pteridophytes (Ma *et al*., 2010; Ebihara *et al*., 2010; Li *et al*., 2011). Some researchers evaluated the potential to differentiate closely related sister species using DNA barcodes (Julian *et al*., 2009; Seberg and Petersen, 2009; Federici *et al*., 2013). In the previous studies, the plastid barcoding loci such as *rbcL*, *matK*, *trnH-psbA*, *trnL-F*, *rpoB* and *rpoC1* were thought to be suitable as DNA markers for species identification of medicinal pteridophytes. In the present study, the extent of genetic variations among the ten species of *Selaginella* collected from various localities of South India were assessed using *rbcL* gene. The results showed good universality with high inter-specific divergence among the studied *Selaginella* species and the results also supports for the precise identification of studied *Selaginella* species.

Azuma *et al*. (2000) performed phylogenetic analyses based on nucleotide sequences of the chloroplast encoded *rbcL* gene to examine phylogenetic relationships of the genus *Salix* together with other allied genera of the family Salicaceae. Phylogenetic analyses of *rbcL* sequences strongly suggest the monophyly of three commonly recognized genera (*Chosenia*, *Salix* and *Toisusu*). Two monophyletic groups are recognized within the larger monophyletic group. In the present study, the highly developed DNA barcoding studies were carried out in ten *Selaginella* species collected from various localities of South India using *rbcL* gene to explore the inter-specific relationships among them. The evolution of species

diversity in the genus *Selaginella* has been studied and their phylogenetic relationships obtained by MEGA 5.2 version. The phylogenetic tree of *Selaginella* species under study displayed both monophyletic and paraphyletic groups. Monophyletic taxon group includes *S. delicatula* with and without cones, *S. inaequalifolia*, *S. involvens*, *S. repanda*, *S. radicata S. tenera*, *S. brachystachya*, *S. wightii* and *S. bryopteris* while paraphyletic group includes *S. intermedia*.

Korall and Kenrick (2002) developed a phylogenetic framework for the club moss family Selaginellaceae based on maximum parsimony analyses using PAUP* software version 4.0. The chloroplast gene *rbcL* was sequenced for 62 species, which represent nearly 10% of living species diversity in the family. The analyses provide support for monophyly of subgenera *Selaginella* and *Tetragonostachys*. *Stachygynandrum* and *Heterostachys* are polyphyletic. Monophyly of *Ericetorum* is uncertain. Their results also indicate a large number of new grouping which was not recognized on morphological grounds. Gu *et al*. (2013) explored the use of DNA barcode ITS2 to identify medicinal plants of the Selaginellaceae family. A total of 103 samples were collected from the main distribution areas in China where those samples represented 34 species and contained almost all the medicinal plants of Selaginellaceae. The ITS2 region of the genome was amplified from those samples and sequenced using universal primers and reaction conditions. The success rates of the PCR amplification and sequencing were 100%. There was significant divergence between inter-specific and intra-specific genetic distances of the ITS2 regions, while the presence of a barcoding gap was obvious. Using the BLAST1 and nearest distance methods, their results proved that the ITS2 regions could successfully identify the species of all Selaginellaceae samples in which they examined. The ITS2 barcode can effectively identify medicinal plants of Selaginellaceae.

Similar to the previous findings, in the present study the genomic DNA of selected ten species of *Selaginella* collected from various localities of South India, were isolated and amplified using *rbcL* primer. The phylogenetic analysis of ten *Selaginella* species was carried out using four methods viz., minimum evolution, neighbor joining, UPGMA and maximum parsimony. The phylogenetic analysis of *Selaginella* species was studied using minimum evolution, neighbor joining and UPGMA methods showed similar trees whereas maximum parsimony tree displayed varied tree. The evolutionary tree using minimum evolution, neighbor joining and UPGMA methods represents two main clusters C_1 and C_2. The cluster C_1 is made of monophyletic taxon group viz., *S. delicatula* with and without cones, *S. inaequalifolia*, *S. involvens*, *S. repanda*, *S. radicata* and a paraphyletic taxon *S. intermedia* with the distance value 0.11. The cluster C_2 consist of monophyletic taxon group viz., *S. brachystachya*, *S. wightii* and *S. bryopteris* and a paraphyletic taxon *S. tenera* with highest distance range 0.12. The phylogenetic tree of maximum parsimony analysis represents two main clusters C_1 and C_2. The cluster C_1 is made of monophyletic taxon group viz., *S. delicatula* with and without cones, *S. inaequalifolia*, *S. involvens*, *S. repanda*, *S. radicata* and a paraphyletic taxon *S. intermedia* with the distance value 24.33. The cluster C_2 involves monophyletic taxon groups namely *S. brachystachya*, *S. wightii* and *S. bryopteris* and a paraphyletic taxon *S. tenera* with highest distance range 41.00. The *rbcL* barcode can effectively identify medicinal plants of Selaginellaceae. The results provide a scientific basis for the particular identification of plants of the family Selaginellaceae. This study may broaden the application of DNA barcoding in the medicinal plant field and benefit phylogenetic investigations. In addition *Selaginella* species are often traded internationally for their medical and ornamental importance, the present research provides a convenient tool to validate the studied *Selaginella* species.

The present investigation was undertaken to identify the molecular taxonomical variation among the ten *Selaginella* species viz., *S. intermedia* (Bl.) Spring, *S. inaequalifolia* (Hook. et Grev.) Spring, *S. involvens* (Sw.) Spring, *S. tenera* (Hook & Grev.) Spring, *S. wightii* Hieron., *S. brachystachya* (Hook. & Grev.) Spring, *S. repanda* (Desv.) Spring, *S. radicata* (Hook. & Grev.) Spring, *S. bryopteris* (L.) Bak, *S. delicatula* (Desv.) Alston with and without cones (Desv.) Alston using biochemical and molecular analysis. To know the molecular relationships among the selected species of *Selaginella* following biochemical viz., SDS-PAGE for protein separation, MALDI-TOF MS analysis for protein identification, PAGE for enzyme separation and molecular characterization includes DNA sequencing using *rbcL* gene was carried out. To fulfill the objectives of the present study the *Selaginella* species were collected from their natural habitats of South India. For protein analysis, the samples were isolated and separated by the method described by Anbalagan (1999) and the gels were stained using the method described by Mortz *et al*. (2001) and Sorensen *et al*. (2002). MALDI-TOF MS analysis was carried out using Applied Biosystems MALDI-TOF Voyager De-Pro spectrometer. For isozymic analysis, the enzymes isoperoxidase, isoesterase, acid phosphatase, alkaline phosphatase and polyphenol oxidase were isolated, separated and stained by the method explained by Sadasivam and Manickam (1991). For genomic analysis, the DNA was isolated by the modified CTAB method described by Murray and Thomson (1980).

To calculate the similarity indices the presence and absence of the MW-Rf values/ spectral peak values (m/z) were calculated as “1” and “0”. The results obtained through biochemical and molecular analyses were documented and the similarity indices among the studied species were calculated. The similarity indices values were plotted in NTSys software

and the cladograms were constructed using UPGMA. The cladograms were used to reveal phylogenetic relationship among the studied *Selaginella* species.

SDS-PAGE protein profiling was carried out to know inter-specific relationship among the studied *Selaginella* species collected from South India. SDS-PAGE demonstrated the inter-specific variability among the studied *Selaginella* species in which, a total of 190 bands with different molecular weight were displayed in the SDS-PAGE gel system of *Selaginella*. Each region expressed different proteins which act as representative of the expression of a particular gene of the studied *Selaginella* species. The protein profile of *S. repanda* showed high number (25) of protein bands and *S. delicatula* with cones showed low number (12) of protein bands. Distinct protein bands were observed among the ten *Selaginella* species which acts as biochemical markers of the studied *Selaginella* species. In addition, these protein bands will make possible to identify the individual *Selaginella* species and act as fingerprint in plant systematic studies.

MALDI-TOF MS analysis expressed different ion peaks ranged from 0 - 1, 00,000 Da among ten *Selaginella* species collected from different localities of South India. The results showed total of 483 spectral peak values. *S. involvens* displayed maximum number (86) of spectral peaks ranged from 527 to 99215 m/z values. *S. radicata* depicted minimum number (21) of spectral peaks varied from 694 to 91754 m/z values. There were 71 unique spectral peaks are observed that explains the genetic similarities and variation of studied *Selaginella* species. Evolutionary tree was constructed by submitting the spectral peak values of individual *Selaginella* species to NTSYS software. The morphologically distinguished character of *S. tenera*, *S. involvens*, *S. wightii*, *S. brachystachya* and *S. repanda* is confirmed in the taxonomic tree by the occurrence of distinct spectral values. Thus, in the present study, the occurred spectral peaks of MALDI-TOF MS analysis acted as a spectroscopic tool to distinguish the studied *Selaginella* species.

In the present study, five kinds of enzyme systems viz., esterase, peroxidase, acid phosphatase, alkaline phosphatase and polyphenol oxidase were characterized to identify inter-specific relationship among the studied ten species of *Selaginella*. In the PAGE system of studied *Selaginella* species, each enzyme was characterized with distinct enzymatic bands which are representative of the appearance of a particular gene locus coding for that specific isozyme. The cladogram of studied *Selaginella* species based on the esterase profile showed the distinct nature of *S. intermedia*, *S. involvens*, *S. radicata*, *S. tenera* and *S. brachystachya* which is a supplementary evident to distinguish the morphologically unique species. The cladistic tree based on the peroxidase profile of studied *Selaginella* species explained the uniqueness of *S. involvens*, *S. brachystachya* and *S. tenera*. The evolutionary tree based on the acid phosphatase profile displayed the separated character of *S. tenera*, *S. delicatula* with cones and *S. bryopteris*. Addition to that, the acid phosphatase system revealed the developmental stages variation in *S. delicatuala* with and without cones. Thus, the result supports to identify the medicinally important *S. delicatula* without cones.

The phylogenetic tree of alkaline phosphatase profile displayed the restricted character of *S. intermedia*, *S. tenera* and *S. wightii* where the cladogram based on polyphenol oxidase profile of *Selaginella* species displayed the uniqueness of *S. brachystachya*, *S. radicata* and *S. bryopteris*. The present study shows that the ten *Selaginella* species were easily separated isozymically and also revealed the similarities and variation among them. In addition, the result obtained in the present study explained that the PAGE analysis can provide an easy, low cost and quick way for the identification of *Selaginella* species and also have a better knowledge of the genetic affinity of germplasm.

DNA barcoding is an advanced molecular tool applied for species identification and to study the taxonomic variation among the closely related species. In the present study, DNA barcoding using *rbcL* gene was applied to distinguish the inter-specific variation among the

selected *Selaginella* species viz., *S. intermedia*, *S. inaequalifolia*, *S. involvens*, *S. tenera*, *S. wightii*, *S. brachystachya*, *S. repanda*, *S. radicata*, *S. bryopteris*, *S. delicatula* with and without cones. Multiple sequence alignment was executed for the studied ten *Selaginella* species using MULTALIN 5.4.1 version and Clustal W tool. The sequence alignment results illustrated that the sequence of *S. involvens*, *S. intermedia*, *S. inaequalifolia*, *S. wightii* and *S. tenera* were distinct from the other studied *Selaginella* species. Thus, the sequence alignment results displayed inter-specific variation among the studied *Selaginella* species.

The phylogenetic relationship of studied *Selaginella* species was analyzed using MEGA 5.2 version software. The cladistic tree explains the close relationship between *S. delicatula* with and without cones. This proves that *rbcL* data is most valuable for inferring phylogenetic relationship between closely related species. The phylogenetic tree of the ten *Selaginella* species yielded two main groups such as monophyly and paraphyly. Monophyletic taxon group includes *S. delicatula* with and without cones, *S. inaequalifolia*, *S. involvens*, *S. repanda*, *S. radicata*, *S. brachystachya*, *S. wightii* and *S. bryopteris*. Paraphyletic taxon group includes only *S. intermedia* and *S. tenera*. This mono and paraphyly expression was a reflection of genetic as well as geographical variation. Thus the results of phylogenetic analysis using MEGA 5.2 version software showed inter-specific similarity and variation among the studied *Selaginella* species. DNA barcoding using *rbcL* gene in particular may be applied to resolve the disputes of taxonomic identities, affairs and authentication of the particular *Selaginella* species in plant systematics.

Finally, it can be concluded from the present research that the synergistic application of biochemical and molecular markers can be used as a prominent tool to identify the taxonomic relationship among the studied *Selaginella* species.

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