



# Molecular Diversity of Fern Species of Agumbe Ghats of Karnataka using ISSR Markers

C. Suneetha, N. Nagesha<sup>1</sup>, S. Yuvaraj, A. Nataraja<sup>2</sup>, Kavita Kandpal, Vinayaka Muttu and Smitha Hegde<sup>3</sup>

Department of Horticulture, <sup>1</sup>Department of Biotechnology, UAS, GKVK, Bangalore-560 065, India

<sup>2</sup>Department of Horticulture, College of Agriculture, Karekere, Hassan-573 225, India

<sup>3</sup>Department of Biology, Center for Advanced Learning and Research, Akashbhavan, Kavoore, Mangalore-575 018, India  
E-mail: [suneethavsn@gmail.com](mailto:suneethavsn@gmail.com)

**Abstract:** Survey of the fern flora of the Agumbe Ghats in Karnataka was undertaken to assess the genetic diversity of ferns. Nine ISSR primers were selected to study the genetic diversity among 12 fern species. The annealing temperature for all ISSR primers was standardized using gradient PCR, where the annealing temperatures ranged from 42 °C to 56°C. ISSR fragments generated 32 to 66 bands per primer. A total of 389 polymorphic bands exhibited 100 per cent polymorphism per primer. The similarity coefficient between the species was within the range of 4 to 35 per cent. The dendrogram generated by ISSR markers revealed two major clusters, indicating that fern species have distributed based on frond shape, frond type, rhizome, habitat, stipe colour and texture. Based on molecular data, the highest genetic similarity of 35 % was observed between *Pteris confusa* and *Pteris biaurita*. In comparison, *Tectaria coadunata* and *Adiantum philippense* showed the least genetic similarity index of 4 per cent.

**Keywords:** Genetic diversity, Dendrogram, Similarity coefficient, ISSR markers, Fern species

India is one of the mega biodiversity countries among seventeen countries. It ranks 10<sup>th</sup> among the plant-rich countries of the world with the significant distribution of fern and fern allies observed in the Himalayas, western and eastern ghats (Myers et al., 2000). About 1289 pteridophytes are distributed in different geographical regions of India out of 12,000 species in the world. The eastern Himalayas and northeast India with about 845 taxa in 179 genera, representing approximately 67% of the pteridophytes known from the country. It is followed by South India, with the eastern and the Western Ghats, with 345 taxa in 117 genera and northern India, including the western Himalaya, with 340 taxa in 101 genera. About 17% of the total pteridophytic species are endemic to India (<https://bsi.gov.in>).

Pteridophyta (*Pteron*=Feather, *Phyton*=Plant) are the earliest vascular plants, often known as 'vascular-cryptogams'. They are the world's first vascular plants to emerge from the ground (Qiu and Palmer 1999). They are a critical evolutionary feature of the Earth's vegetation because they reflect the evolution of the vascular system and represent the development of seed habitat in the plants (Dixit 2000). The foliage of fern is highly valued in the international florist greenery market because of its long post-harvest life, low cost, year-round availability and versatile design qualities in form, texture and colour (Safeena 2013). Because of their delicate beauty and elegance, ferns are grown as ornamentals either indoors in households or outdoors in botanical gardens. Charaka Samhita describes *Adiantum*

*capillus-veneris* and *Marsilea minuta* as medicinal plants. At the same time, the rhizome of *Polystichum squamosum*, also known as 'Nirviri' in India, is used against scorpion and bug stings (Dixit 2000). Phytoecdysones are produced by ferns, which inhibit insect development and induce deformities (Balasubramanian et al., 2008). Ferns also play an essential part in wastewater bioremediation. Chinese bracken fern, *Pteris vittata* L., is hyper-accumulator of the hazardous arsenic metal (Ma et al., 2001).

Genetic diversity is the primary constituent that enables long-term survival and adaptation of a species, particularly under varying environmental conditions. The genetic structure of populations is often a reflection of various biological processes and interactions, including mutation, genetic drift, mating system, gene flow (Chung et al., 2023, Salgothra et al., 2023). Genetic diversity measurements are crucial when it comes to the conservation of some species. An organism's capacity to react to natural selection may be harmed by a loss in genetic variability, reducing its evolutionary potential.

Morphological characters which are used for the description are not highly reliable. Most morphological characters are controlled by the environment or change with the plant's growth, and isozymes are limited due to low polymorphism levels (Asha et al., 2006). As a result, DNA-based molecular marker techniques such as RAPD, ISSR and micro-satellites, or simple sequence repeats (SSRs) and inter-simple sequence repeats (ISSRs), are effective in

assessing plant genetic diversity because they provide an infinite number of potential markers to reveal differences at the molecular level (Ghafoor et al., 2007). The fundamental limitations of these approaches are the limited reproducibility of RAPD, the high cost of AFLP and the necessity to know the flanking sequences to generate species-specific primers for SSR polymorphism. The ISSR marker is such a DNA-based marker system that could screen genetic variability. Changes in DNA sequences and single-base substitutions, including DNA conformation changes, can be detected as shifts in electrophoretic mobility. The technique is useful in fields like genetic diversity, phylogenetic studies, gene tagging, genome mapping and evolutionary biology across a wide range of crop species. The primary objective of this study was to assess the genetic diversity and relationships among 12 fern species collected from the Agumbe Ghats in Karnataka. This evaluation was conducted using ISSR markers to generate molecular data for species identification, phylogenetic classification, and the development of conservation strategies.

#### MATERIAL AND METHODS

Survey of fern flora of Agumbe Ghats of Karnataka was undertaken to assess the genetic diversity of ferns, and 12 fern species were collected (Table 1). The experiment was conducted at the Horticulture Research Station, Department of Horticulture and Department of Plant Biotechnology, University of Agricultural Sciences, Gandhi Krishi Vigyan Kendra, Bengaluru-560065. The leaf samples were collected from the 12 different fern species and were stored at -80°C until DNA extraction. Genomic DNA was isolated from young leaves using the Cetyl Trimethyl-Ammonium Bromide (CTAB) method (Doyle and Doyle 1987) with some

modifications. The purity of DNA was analysed by running in 0.8 % agarose gel and the quantification of DNA was done using a UV spectrophotometer.

Twenty µl of PCR reaction mixture was prepared containing Takara mixture (master mix) 10.0µL, ISSR primer 2.0 µL from 5 pM stock, DNA template 1.0 µL, and Double distilled water 7.0 µL. PCR reaction was performed using 9 ISSR primers (Eurofins Genomics India Pvt. Ltd.). The list of ISSR markers primers used and annealing temperature used for the study is presented in Table 2. The PCR was performed by initial denaturation at 95°C for 4 min. Followed by 35 cycles of denaturation at 94°C for one minute, annealing at 42-56 °C for one minute (depending on primer), and extension at 72°C for 1.3 minutes and final elongation at 72°C for 20 min. The PCR products were resolved on 1.5 % Agarose gel prepared in 1x TAE buffer containing 0.5 ug/ml of the ethidium bromide at 85 V for 2 hours. PCR amplification was done for twelve fern species for different primers. Consistent and well-resolved amplified bands were manually

**Table 2.** ISSR primers used for diversity analysis with standardized annealing temperature

Primer	Sequence	T <sub>m</sub> (°C)
ISSR-1	GAGAGAGAGAGACC	44.0
ISSR-2	CTCTCTCTCTCTCTAC	48.0
ISSR-3	CACACACACACAAG	50.0
ISSR-4	CAGCACACACACACACA	42.0
ISSR-6	CTCTCTCTCTCTCTGTC	54.0
ISSR-807	AGAGAGAGAGAGAGAGTG	56.0
ISSR-835	AGAGAGAGAGAGAGAGCC	56.0
ISSR-844	CTCTCTCTCTCTGTC	42.0
ISSR-850	GTGTGTGTGTGTGTCC	56.0

**Table 1.** Ferns species collected from Agumbe Ghats of Karnataka

Botanical name	Latitude (E)	Longitude (N)	Elevation (m)
<i>Bolbitis semicordata</i>	75°05.283'	13°29.995'	663
<i>Cyathea gigantea</i>	75°05.283'	13°29.472'	670
<i>Christella parasitica</i>	75°05.270'	13°29.290'	666
<i>Angiopteris evecta</i>	75°05.279'	13°29.989'	673
<i>Pteris confusa</i>	75°05.279'	13°29.993'	667
<i>Tectaria coadunata</i>	75°04.931'	13°29.838'	615
<i>Athyrium falcatum</i>	75°04.875'	13°29.878'	619
<i>Adiantum philippense</i>	75°04.962'	13°29.864'	638
<i>Odontosoria chinensis</i>	75°04.947'	13°29.828'	625
<i>Pteris biaurita</i>	75°04.917'	13°29.861'	623
<i>Selaginella delicatula</i>	75°04.978'	13°29.840'	700
<i>Nephrolepis biserrata</i>	75°04.947'	13°29.852'	630

scored. Scoring of bands was done based on their presence ('1') or absence ('0') in the gel. The genetic associations were evaluated by calculating the Jaccard's Co-efficient (J) for pair-wise comparisons based on the proportion of shared bands produced by the primers. The pooled ISSR binary data were utilized for cluster analysis using Paleontological Statistics software (PAST; version 4.07) developed by Hammer *et al.* (2001).

### RESULTS AND DISCUSSION

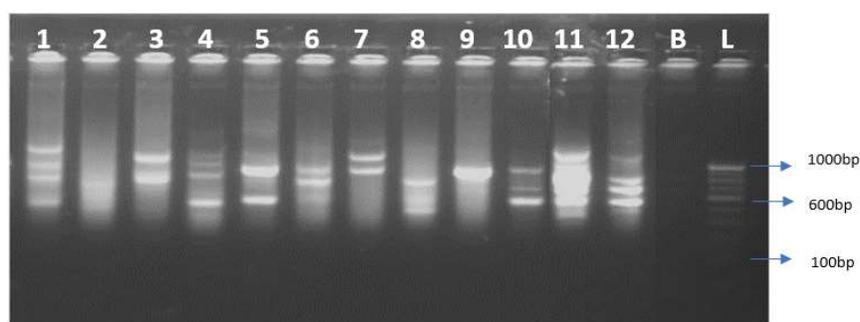
In the present study, 12 different fern species were collected from the Agumbe Ghats of Karnataka and their genetic diversity was studied using ISSR markers. Nine ISSR primers were used to study the relatedness within the species based on their geographical distribution. The ISSR fingerprint for 12 fern species using nine primers revealed 389 scorable bands. The number of bands scored for each

primer varied from 32 to 66 bands (Table 3), with an average of 43.22 bands per primer. Primer ISSR1 scored a maximum number of bands (66) followed by ISSR 850, ISSR 6, ISSR 3, and ISSR 844. The minimum number of bands was in ISSR 4 and ISSR 2 (32 and 33 bands, respectively). Total of 389 bands were observed with 100% polymorphism from all primers used in the study. For illustration, this vast range in percent polymorphism obtained in the present study proved the discriminatory power of ISSR molecular markers for distinguishing different cultivars reported in earlier literature Abirami *et al.* (2018) in *Asplenium nidus*, Chelliah *et al.* (2014) in *Adiantum incisum* Forssk, Vidyashree *et al.* (2019) in fern species and Dong *et al.* (2007) in *Ceratopteris pteridoides*.

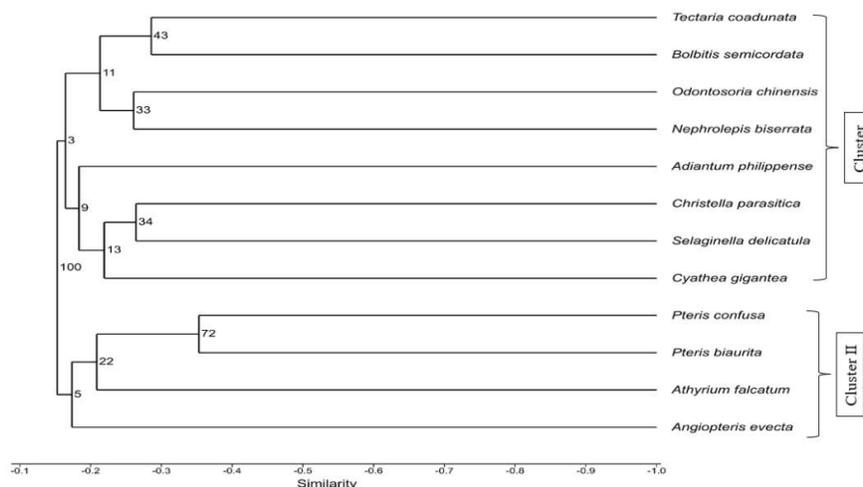
Using the UPGMA cluster analysis method, a dendrogram was generated based on Jaccard's similarity coefficient differentiated all the 12 fern species. All species were grouped into two major clusters. These were further divided into sub-clusters (Fig. 2). The first significant cluster constituted eight species. The second cluster constituted four species. The major clusters I in the dendrogram (Fig. 2) is further divided into two sub-clusters. Sub-cluster I consist of two groups; Group I and Group II. Group I consist of *Tectaria coadunata* and *Bolbitis semicordata*. Group II includes *Odontosoria chinensis* and *Nephrolepis biserrata*. Subcluster II includes two groups: group I and group II. Group I include single specie *Adiantum philippense*. Group II is further divided into two subgroups: subgroup I include two species, *Christella parasitica*, *Selaginella delicatula*, and subgroup II consists of single species *Cyathea gigantea*. The major cluster II is subdivided into two sub-clusters. Subcluster I includes two groups, group I consists of two species, *Pteris confusa* and *Pteris biaurita*, group II includes a single specie *Athyrium falcatum*, Sub-cluster II two consists of single species *Angiopteris evecta*.

**Table 3.** Polymorphism scoring of twelve fern species in relation with nine ISSR primers

Name of the primer	No. of polymorphic bands	Total No. of bands produced	Percentage polymorphism
ISSR 1	66	66	100
ISSR 2	33	33	100
ISSR 3	42	42	100
ISSR 4	32	32	100
ISSR 6	49	49	100
ISSR 807	38	38	100
ISSR 835	39	39	100
ISSR 844	40	40	100
ISSR 850	50	50	100
Total	389	389	100
Mean	43.22	43.22	



**Fig. 1.** Agarose gel electrophoresis separation of PCR products using ISSR 4 primer for 12 fern species from 1 to 12 1) *Bolbitis semicordata* 2) *Cyathea gigantea* 3) *Christella parasitica* 4) *Angiopteris evecta* 5) *Pteris confusa* 6) *Tectaria coadunata* 7) *Athyrium falcatum* 8) *Adiantum philippense* 9) *Odontosoria chinensis* 10) *Pteris biaurita* 11) *Selaginella delicatula* 12) *Nephrolepis biserrata*, L- ladder, B- blank



**Fig. 2.** Dendrogram of genetic relationship among 12 ferns based on ISSR marker

**Table 4.** Similarity coefficient matrix of 12 fern species by using ISSR marker

Botanical name	1	2	3	4	5	6	7	8	9	10	11	12
<i>Bolbitis semicordata</i>	1											
<i>Cyathea gigantea</i>	0.21	1										
<i>Christella parasitica</i>	0.25	0.23	1									
<i>Angiopteris evecta</i>	0.23	0.10	0.18	1								
<i>Pteris confusa</i>	0.20	0.20	0.21	0.19	1							
<i>Tectaria coadunata</i>	0.29	0.10	0.20	0.08	0.15	1						
<i>Athyrium falcatum</i>	0.16	0.10	0.15	0.20	0.19	0.21	1					
<i>Adiantum philippense</i>	0.12	0.20	0.18	0.12	0.16	0.04	0.09	1				
<i>Odontosoria chinensis</i>	0.23	0.20	0.17	0.12	0.22	0.24	0.23	0.16	1			
<i>Pteris biaurita</i>	0.13	0.07	0.15	0.13	0.35	0.09	0.23	0.14	0.16	1		
<i>Selaginella delicatula</i>	0.23	0.21	0.26	0.25	0.17	0.09	0.09	0.17	0.17	0.09	1	
<i>Nephrolepis biserrata</i>	0.22	0.18	0.16	0.16	0.28	0.16	0.10	0.19	0.26	0.12	0.16	1

The similarity matrix coefficient ranged from 4 to 35 per cent suggesting a higher to low genetic variation within fern species (Table 4). The highest genetic similarity of 35 per cent was observed between *Pteris confusa* and *Pteris biaurita*, followed by *Christella parasitica* and *Selaginella delicatula*, *Odontosoria chinensis*, *Nephrolepis biserrata* had 26 per cent genetic similarity. In comparison, *Tectaria coadunata* and *Adiantum philippense* showed the least genetic similarity index of 4 per cent. The variation in similarity among the species may be attributed to species diversity and their inherent genetic makeup. Chelliah et al. (2014) reported that genetic identity among five *Adiantum incisum* Forssk. individuals ranged from 0.46 to 0.75, while genetic distances varied from 0.29 to 0.78, as measured by the ISSR marker system. Similar findings were reported by Perrie et al. (2003) in New Zealand fern species, Lalitha et al. (2014a) in chrysanthemums, Lalitha et al. (2014b) in

tuberose, and Vidyashree et al. (2019) in other fern species. The present molecular characterization study using ISSR markers would be an effective tool for genetic diversity analysis and its relationships within species and the conservation of this potential fern species.

## CONCLUSION

The study assessed the genetic diversity of 12 fern species from the Agumbe Ghats of Karnataka using ISSR markers. Nine primers generated 389 scorable bands, with an average of 43.22 bands per primer, and revealed 100% polymorphism, confirming the high discriminatory potential of ISSRs. Among the primers, ISSR1 produced the maximum bands (66), while ISSR4 yielded the least (32). Cluster analysis based on UPGMA grouped all the species into two major clusters with several sub-clusters, clearly reflecting their genetic relatedness and divergence. The similarity

coefficient ranged from 4 to 35%, indicating substantial variation among the species. The closest genetic relationship was observed between *Pteris confusa* and *Pteris biaurita* while the lowest similarity was between *Tectaria coadunata* and *Adiantum philippense*. These findings highlight considerable genetic variation, likely due to inherent genetic makeup and ecological adaptations. The study establishes ISSR markers as a reliable and robust tool for evaluating genetic diversity, species identification, and conservation planning in ferns of the Western Ghats biodiversity hotspot.

#### AUTHORS CONTRIBUTION

Suneetha C was responsible for the initiation and conceptualization of the study. Nagesha N supervised the molecular work and designed the ISSR primers. Yuvaraj S wrote and edited the manuscript. Nataraj A, Kavita Kandpal, and Vinayaka Muttu contributed to the collection, identification, herbarium preparation of ferns, and laboratory work. Sunitha Hegde carried out the identification and classification of the collected ferns and critically reviewed the manuscript.

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