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Original Research

Genetic variations of *Adiantum incisum* Forssk. revealed by ISSR markers in the Western Ghats of Tamil Nadu, India

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ABSTRACT:

Inter Simple Sequence Repeats (ISSR) markers were used to measure the levels of genetic variation and patterns of the population structure within and among the five populations of *Adiantum incisum*, a terrestrial fern in India. For this purpose, a detailed study was conducted in three replicates at 2011-14 season in the collection points of Western Ghats, South India. Five wild *A. incisum* accessions (maiden hair) were evaluated for genotyping experiment. Results showed a significant variation among genotypes and were classified based on this variation in four groups by genetic cluster analysis. In the experiment, five ISSR primers amplified 63 polymorphic bands. The genetic identity data among genotypes were calculated and varied from 0.4603 to 0.7460. The percentage of polymorphism showed superior genotype that could be used for the conservation of species. ISSR proved to be a helpful marker for genotype identification prediction within a closed group of inter specific population in the study area.

Keywords:
ISSR analysis, *Adiantum incisum* Forssk, genetic variation, southern Western Ghats

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INTRODUCTION

The Indian subcontinent is endowed with an amazing array of herbal plants which constitute the main resource base of the health care system in the country. It's rich vegetation wealth and diversity is undoubtedly due to the immense variety of the climate and altitudinal variations coupled with the various ecological habitats.

The Western Ghats of peninsular India is of great phyto-geographical importance which constitutes one of the 34 global biodiversity hotspots along with Sri Lanka, on account of exceptional levels of plant endemism and higher levels of habitat loss.

The flora of Western Ghats comprises about 12,000 species ranging from unicellular cyanobacteria to angiosperms. In this spectrum, the flowering plants constitute about 27% of Indian flora with 4000 species of which, about 1500 species are endemic. Apart from harbouring a rich diversity of the angiosperm flora, the Western Ghats are also a rich repository of cryptogams such as pteridophytes, bryophytes, lichens, fungi and algae. The Western Ghats along with the Himalayas, Eastern Ghats and parts of Central India forms a major centre for the distribution of the ferns and fern-allies (MoEF, 2014).

Ferns in Western Ghats of South India, south of Palghat gap constitute about one third of the fern flora of India. Most of them occur on streams and stream banks in evergreen forests and shoals above 800m while some occur on exposed roadsides and clearings. (Manickam, 1995).

In spite of its relatively infrequent occurrence, long distance colonization is of disproportionate importance to species range expansions. Long-distance colonization requires plant species to possess a distinct set of capabilities, not only related to the dispersal of propagules, but also to plant and population establishment upon arrival. This involves di-spore characteristics, plant ontogenetic and morphological traits, as well as reproductive strategies. Genotypes possessing these capabilities will have a selective advantage over other genotypes when colonizing new and distant habitats. This advantage is becoming more important in a world increasingly under the pressure of climate change and fragmentation of natural habitats.

Various studies on plants and animals have shown that individuals with higher dispersal capacities tend to be found with greater frequency towards species range limits and that these enhanced capacities tend to have a genetic basis. Likewise, inbreeding rates often increase towards range margins.

Genetic diversity measurements are important for considering conservation of particular species. A decline in genetic variation can undermine the ability of an organism to respond to natural selection and consequently limits its evolutionary potential. Small populations are often subjected to the loss of alleles through genetic drift, or random fluctuations in allele frequency. Thus, any study in genetic diversity of *Adiantum incisum* has to address the above issues.

DNA markers have proved valuable in crop breeding, especially in studies on genetic diversity and gene mapping. The commonly used Polymerase Chain Reaction (PCR) based DNA marker system are Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphic (AFLP) and more recently Simple Sequences Repeats (SSRs) or microsatellite (Gupta and Varshney, 2000). The major limitations of these methods are low reproducibility of RAPD, high cost of AFLP and the need to know the flanking sequences to develop species specific primers for SSR polymorphism.

ISSR is such a DNA based marker system which could be used for screening genetic variability. Changes in DNA sequences and single base substitutions including DNA conformation changes can be detected as shifts in electrophoretic mobility using these techniques.

In this present study, *A. incisum* Forssk, were collected from Western Ghats of Tamil Nadu. *A. incisum*

Forssk, is found in the plains and lower slopes of the hills of Punjab, Rajasthan, West Bengal, Tamil Nadu and Maharashtra. It is used to cure hemicrania, cough and fever; it is applied externally for skin diseases and used as a substitute for *A. capillus-veneris*. This fern yields adiantone, isoadiantone, fernene, hentriacontane, hentriacontanone-16 and beta-sitosterol on extraction using different solvents. The plant extract of *Adiantum incisum* Forssk. (Adiantaceae) is also used in the treatment of cough, diabetes, and skin diseases (Manickam, 1995).

The confusion prevailing at its species level is a great menace to the Pteridologists who try to identify it for its valuable usages. The morphology remains similar with that of the closely related species except minute characteristics with its chromosomal nature and chemistry stands unique. Thus, designing a specific strategy for its species identification in spite its variation stands as a credential task irrefutably. Also, the superior genotype of the species was identified so that the conservation of the species made easy with special initiative.

MATERIALS AND METHODS

Study area

Five Western Ghats regions viz., Kothayar, Gundar, Thirugarankudi, Kodaikanal and Kadana Dam are selected for the study due to the availability of these three species uniformly with no particular order.

DNA isolation

A. incisum samples were collected from the Western Ghats of Tamil Nadu, India. DNA was extracted from young leaves using the method described by Dellaporta *et al*. (1983). The DNA isolated was purified using Phenol- Chloroform method and the concentration of the DNA samples were determined using UV-Spectrophotometer at the optical density of 260 nm and 280 nm; the DNA samples were diluted to 25 ng μl^{-1} for PCR amplification.

Table 1. Place of collection of the plants and their accession ID

S.No	Species	Accession ID	Location
1.	*Adiantum incisum*	POP 1	Kothayar
2.		POP 2	Gundar
3.		POP 3	Thirugurankudi
4.		POP 4	Kodaikanal
5.		POP 5	Kadana dam

ISSR amplification

ISSR amplification reactions were carried out in 25-µl volume containing 50 ng template DNA, 0.5 U Taq DNA polymerase, 10 mM dNTP, 10 µM primer in 1× reaction buffer that contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, and 0.01% gelatine

Table 2. ISSR primers for study species and their sequences

S. No	Name of the primer	Primer Sequences
1	B07	$(CG)_5AG$
2	B09	$(ATG)_3CA$
3	G06	$(GC)_5CA$
4	G04	$(AT)_6GC$
5	L03	$(GC)_4AT$

(Williams *et al.,* 1990**)**. Amplifications were performed in an Eppendorf Master Cycler gradient. Amplification conditions were one cycle at 94°C for 4 min, and 94°C for 30 s, 55°C for 45 s, followed by stepwise reduction of 1°C for the first five cycles, and 72°C for 2 min. In subsequent 35 cycles, annealing temperature was maintained at 50°C, followed by one cycle of 7 min at 72°C. Amplified products were loaded on 2% agarose

Table 3. Overall genetic variation statistics for all loci in *Adiantum incisum*

S. No.	Parameters	Values
1.	Observed numbers of alleles	1.7778
2.	Effective numbers of alleles	1.5887
3.	Nei's (1973) gene diversity	0.3276
4.	Shannon's Information Index (Lewontin, 1972)	0.4741
5.	Overall percentage of polymorphism	77.78

gel and separated in 1× TBE buffer at 75 V. The gels were visualized under UV after staining with ethidium bromide and documented using a gel documentation and

Table 4. ISSR Profile of *A. incisum* using selected primers

S. No.	ISSR Primers	Total Number of Bands	Total Number of Polymorphic Bands	Percentage of Polymor-phism (%)
1	B07	63	20	31.74
2	B09	63	09	14.28
3	G04	63	12	19.04
4	G06	63	10	15.87
5	L03	63	12	19.04
		Total		99.97

image analysis system. The primers used for the ISSR analysis are listed out in Table 2.

Data Analysis

The gels from ISSR analysis were visualized at

Table 5. Distance between and population length in *Adiantum incisum* Forssk.

Between	And	Length
4	3	3.66294
3	pop1	26.61084
3	pop2	26.61084
4	2	7.54538
2	1	8.07904
1	pop3	14.64936
1	pop5	14.64936
2	pop4	22.72839

gel documentation system (Alpha Imager 1200). Based on the primary data (presence or absence of bands) and pair wise genetic distance between samples was calculated using NTSYS and POPGENE packages

RESULTS AND DISCUSSION

Analysis of five accessions of *A. incisum* Forssk**.** revealed 63 polymorphic loci. Ten primers were analyzed of which five primers (Table 2). generated reproducible, informative and easily scorable ISSR profiles. A total of 315 bands were scored, out of which 180 were polymorphic bands and the number of bands ranged from 09 to 20 per primer (Table 4). The genetic distance between the population ranged from 0.2930 to 0.7758 and the genetic identity ranged from 0.4603 to

Table 6. Nei's original measures of genetic identity and genetic distance in *Adiantum incisum*

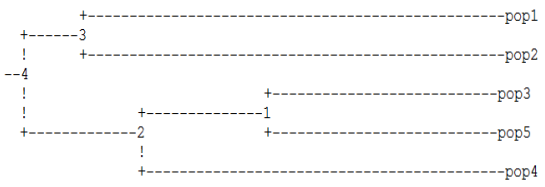
POP ID	1	2	3	4	5
1	*****	0.5873	0.6190	0.4921	0.6190
2	0.5322	*****	0.4603	0.6190	0.4921
3	0.4796	0.7758	*****	0.6190	0.7460
4	0.7091	0.4796	0.4796	*****	0.6508
5	0.4796	0.7091	0.2930	0.4296	*****

0.7460 (Table 06). The overall observed and effective numbers of alleles were 1.7778 and 1.5887 respectively and overall genetic diversity was 0.3276 (Table 3; Fig 2). The Shannan's information index was found to be 0.4741. The overall percentage of polymorphism was 77.78 (Table 3; Table 5).

The number of polymorphic loci and percentage of polymorphism was calculated by using the software POPGENE package version 1.3.2. Among these five populations, populations 1, 4, and 5 (Kothayar, Kodaikanal and Kadana Dam) showed high polymorphism. Considering these three populations, population 1 (Kothayar) showed highest polymorphism (Fig 1).

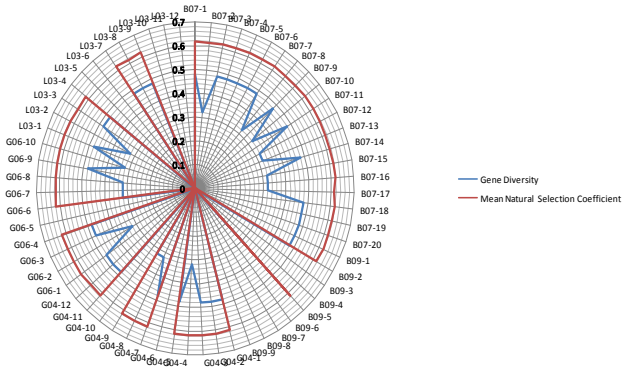
Hence, among the five accessions of *A. incisum* in the Kothayar accession (Pop 1) is considered as superior genotype, due to the high percentage of polymorphism in ISSR analysis.

Figure 1. UPGMA dendrogram of *A. incisum* based on Nei's Genetic distance derived using ISSR markers



Dendrogram was drawn based on Nei's (1972) with the consideration of genetic distance. The methodology followed is UPGMA based on neighbour joining method of PHYLIP Version 3.5.

Fig 2. Summary of genetic variation statistics for all loci in *Adiantum incisum*



The dendrogram of *A. incisum* Forssk. (Fig 1) produced 2 clusters. Cluster 2 was larger, containing population 3, 5 and 4. Here, population 3 and 5 were closely related together than population 4. In the first cluster, population 1 and 2 formed a separate clade. It was understood that there was considerable amount of genetic variability between the populations 1, 2 and 3, 4, 5 of *A. incisum* Forssk.

The morphological variations were counter confirmed by the genetic variations present in the plants through ISSR markers. Specific primers that mediate differentiation between the species were identified successfully. The genetic relationship exemplified by the molecular markers via DNA fingerprinting shows their closeness and relativity. Out of ten primers, five revealed consistent banding pattern and thus revealed variability within the species.

The identification of a clear systematic relationship thus paves a way for better understanding of the species in their position.

ISSR markers were used to compare genetic differentiation within and between the selected species. Analysis of genetic variability indicated all the five populations examined, sexual recombination had been the predominant source of genetic variation than asexual reproduction (Mes, 1998; Van Der Hulst, 2000; Kjolner *et al.,* 2006). This finding is in good agreement with other previous studies, which have nearly all used allozyme markers to infer the mating systems operating in natural populations of ferns.

Allozyme studies showed that populations of diploid homosporous ferns are usually dominated by sexual random mating or outcrossing (Soltis and Soltis, 1987; Ranker and Geiger, 2008). Secondly, gene flow from the neighboring populations would slow the diversification but only via sexual reproduction. Similar patterns have been identified in a number of other fern species **(**Ranker, 1992; Ranker and Geiger, 2008) Rumsey, 1999; Chen *et al.,* 2010).

The reason for this genetic variation is most likely arisen from differences in the DNA contents of the progenitor species. This suggestion is consistent with the occurrence of diploid apomicts in other ferns, including taxa of the *Adiantum incisum* (Pravin, 2005). The clades are constructed through Popgene 2.1 and related lineages show strong patterns of reticulate evolution and higher the genetic diversity of these species, greater is their viability in the environment.

However, when evolutionary relationships were considered using Phylogenetically Independent Contrasts (PICs), no significant correlation was found. The discrepancy between analyses is interesting, and although the significance of the raw data should not be discounted, it does highlight the importance of using PICs to determine the evolutionary association of statistically non-independent traits **(**Garland, 1992**).**

Previous authors have pointed out that this inference should be restricted to very close relatives, and the distinction between diploids and their autoploid offspring (Moran, 1982; Barrington, 1986).

Our findings are consistent with suggestions that the high chromosome numbers and conserved chromosome sizes reported for many homosporous ferns

has contributed to the hypothesis that the evolution of fern genomes is less dynamic than the evolution of angiosperm genomes. This has been suggested to be due to a higher retention rate of chromosomes and the possible suppression of Transposable Elements (TEs) in homosporous ferns **(**Barker, 2011; Bainard *et al.,* 2011**).** The inferred constancy of chromosome size is based on physical measurements (Wagner and Wagner, 1980) and the reported correlation between chromosome number and genome size (Bainard *et al.,* 2011).

It also confirms with the hypothesis that genome size variation in homosporous ferns are driven by poly-ploidisation. Our study provides evidence that the genome evolution is occurring in these study plants. Indeed, given the extent of hybridization and reticulate evolution reported in homosporous ferns in general, it seems likely that changes in genome size are probably more widespread across ferns but may have been largely overlooked due to the low level of sampling.

CONCLUSION

Genetic diversity between *Adiantum incisum* species found on the Western Ghats region is identified. ISSR markers proved amplification in the selected species thus validates its genetic variation strategy

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