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Application of ISSR markers reveals extensive genetic variability in the tropical lycophyte *Selaginella ciliaris*

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Abstract. *Jafron, Sutarno, Pangastuti A, Solichatun, Sugiyarto, Sunarto, Setyawan AD. 2025. Application of ISSR markers reveals extensive genetic variability in the tropical lycophyte* Selaginella ciliaris. *Asian J Trop Biotechnol 22: 80-93.* Genetic information on early-diverging vascular plants remains limited, despite their ecological importance and emerging relevance in biotechnology. This study assessed genetic variability in the tropical lycophyte *Selaginella ciliaris* uses Inter-Simple Sequence Repeat (ISSR) markers as a cost-effective molecular approach for non-model plants. A total of 27 samples were analyzed using selected ISSR primers, generating clear and reproducible banding patterns. A total of 49 loci were scored, all of which were polymorphic (100% polymorphism), indicating a very high level of genetic variability. Dice genetic similarity coefficients were consistently low, ranging from 0.0000 to 0.0816, with the majority of pairwise comparisons falling below 0.05. Dice genetic similarity values were consistently low, and the frequency distribution of similarity classes was strongly skewed toward very low similarity ranges, reflecting pronounced multilocus differentiation. UPGMA cluster analysis further revealed the absence of dominant genetic groups, with samples forming small clusters and numerous singletons. These results demonstrate substantial genetic heterogeneity within *S. ciliaris* at a regional scale. The study confirms the effectiveness of ISSR markers for detecting genetic variability in non-model tropical plants lacking genomic resources. From an applied perspective, the high genetic diversity observed highlights the potential of *S. ciliaris* as a valuable genetic resource for early-stage germplasm screening, conservation planning, and future biotechnological research. This work provides baseline molecular insights into an understudied lycophyte and underscores the utility of ISSR markers as an accessible tool bridging biodiversity assessment and tropical plant biotechnology.

Keywords: Genetic diversity, ISSR markers, lycophyte, *Selaginella ciliaris*, tropical plant

INTRODUCTION

Lycophytes represent one of the earliest-diverging lineages of vascular plants and play an important role in understanding plant evolution, genetic diversity, and adaptation in terrestrial ecosystems (Kenrick and Crane 1997; Pryer et al. 2004; Wickett et al. 2014). Despite their evolutionary significance, lycophytes remain markedly underrepresented in molecular genetic studies compared with angiosperms and gymnosperms, particularly in tropical regions (Banks 2009, et al. 2011; Leebens-Mack et al. 2019). This imbalance is especially evident in Southeast Asia, where species richness is high but molecular data are scarce and unevenly distributed (Corlett 2014; Hughes 2017). Within this context, the genus *Selaginella* (Selaginellaceae) constitutes a key group of tropical lycophytes with broad ecological amplitude and notable biochemical potential (Jermy 1990; Banks 2009; Zheng et al. 2020), yet its genetic variability at local and regional scales remains insufficiently explored, particularly using population-level molecular approaches (Arrigo and Barker 2012; Weststrand and Korall 2016).

Molecular marker technologies have become essential tools in tropical plant biotechnology, particularly for assessing genetic variability in non-model species where genomic resources are limited. Information on genetic diversity is fundamental not only for evolutionary and ecological studies but also for applied purposes such as germplasm management, early-stage selection, and conservation-oriented biotechnology programs. In tropical plants, where many species are harvested from the wild or remain semi-domesticated, rapid and cost-effective molecular approaches are especially valuable for preliminary genetic screening and resource assessment (Mondini et al. 2009; Kalia et al. 2011).

Among available molecular markers, Inter-Simple Sequence Repeat (ISSR) markers have been widely recognized for their efficiency, reproducibility, and suitability for non-model plant species. ISSR markers target regions between microsatellite loci and do not require prior sequence information, making them particularly advantageous for taxa with limited genomic data. Compared with RAPD markers, ISSRs generally exhibit higher reproducibility, while offering a simpler and more economical alternative to SSR or SNP-based approaches (Zietkiewicz et al. 1994; Reddy et al. 2002). As a result, ISSR markers have been successfully applied in a wide range of tropical plants, including medicinal species, forest trees, and pteridophytes, to evaluate genetic variability and infer patterns of relatedness (Rakoczy-Trojanowska and Bolibok 2004).

Previous studies on *Selaginella* have mainly focused on taxonomy, ecology, and phytochemistry, particularly the presence of bioactive biflavonoids (Cao et al. 2010; Setyawan 2011; Chikmawati et al. 2012), while molecular genetic investigations remain limited and uneven across taxa and regions. Existing molecular studies largely address higher-level phylogenetic relationships or focus on a few model or medicinal species using sequence-based data rather than population-level markers (Pryer et al. 2004; Zhou et al. 2016). Consequently, intraspecific genetic variability in many tropical *Selaginella* species remains poorly understood. For *Selaginella ciliaris* (Retz.) Spring, a widespread tropical lycophyte inhabiting moist and heterogeneous microhabitats, molecular data on genetic variability are particularly scarce. Despite its ecological relevance, including its proposed role as a moisture bioindicator and use in traditional medicine, *S. ciliaris* has rarely been included in molecular diversity assessments, leaving a significant gap in population-level genetic information (Setyawan 2011; da Silva Almeida et al. 2013).

From a biotechnological perspective, genetic variability data are essential for germplasm screening, the selection of representative material, and the development of downstream applications such as in vitro culture and conservation-oriented biotechnology. However, baseline molecular information for *S. ciliaris* remains lacking, as no systematic ISSR-based assessment has yet been reported from tropical Southeast Asia. Most existing genetic studies on lycophytes focus on other genera or use alternative marker systems, limiting comparability and relevance for population-level analyses (Nybom and Bartish 2000; Ranker and Geiger 2009). Therefore, a focused evaluation using ISSR markers and multiple primers is needed to characterize genetic variability in *S. ciliaris* and to demonstrate the utility of ISSR markers for preliminary screening of underexplored tropical plant taxa.

Therefore, the present study aims to apply ISSR markers to assess the extent of genetic variability in *S. ciliaris* collected from a humid tropical landscape in Central Java, Indonesia. By generating ISSR profiles from multiple primers and analyzing genetic similarity patterns, this study seeks to provide baseline molecular data for *S. ciliaris* and to highlight the relevance of ISSR markers for genetic assessment in non-model tropical lycophytes. The findings are expected to contribute to both fundamental knowledge of lycophyte genetic diversity and applied perspectives in tropical biotechnology and germplasm evaluation. Based on these considerations, this study was conducted to test the following hypothesis: *S. ciliaris* exhibits high intraspecific genetic variability at a regional scale, which can be effectively detected using ISSR markers despite the absence of prior genomic resources.

MATERIALS AND METHODS

Study species and sampling design

Selaginella ciliaris is a tropical lycophyte commonly distributed in moist and shaded habitats, including forest understories, riverbanks, agricultural margins, and humid open areas. The species is characterized by a creeping growth form and microphyllous leaves, and it is frequently associated with environments exhibiting high soil moisture and relatively stable microclimatic conditions. Due to its ecological tolerance and wide local occurrence, *S. ciliaris* represents a suitable model for assessing genetic variability in non-model tropical lycophytes.

Sampling was conducted across a humid tropical landscape in Central Java, Indonesia, encompassing multiple districts that differ in elevation, land-use context, and microhabitat characteristics (Table 1). A total of 27 individual samples of *S. ciliaris* were collected from four administrative regions, representing lowland to highland environments. Sampling sites were selected to capture spatial heterogeneity while avoiding clonal repetition by maintaining a minimum distance between sampled individuals at each locality. Only healthy and morphologically typical individuals were collected to ensure DNA quality and taxonomic consistency.

Fresh leaf tissues were sampled in the field, cleaned of debris, and immediately stored under cold conditions prior to laboratory processing. Each sample was assigned a unique code corresponding to its collection site and elevation range. Geographic coordinates and elevation data were recorded to provide ecological context for subsequent genetic analyses. The sampling strategy was designed to provide a representative overview of intraspecific genetic variability across a moisture-rich tropical region, rather than to test explicit population genetic structure. This approach aligns with the objective of evaluating ISSR markers as an effective screening tool for detecting genetic variability in *S. ciliaris*.

DNA extraction protocol

Genomic DNA was extracted from fresh leaf tissue of *S. ciliaris* using a modified cetyltrimethylammonium bromide (CTAB) protocol originally described by Doyle and Doyle (1987), with adjustments to improve DNA quality from tissues rich in secondary metabolites. Approximately 1 g of young leaf material per sample was finely ground in liquid buffer containing CTAB extraction solution supplemented with β-mercaptoethanol to reduce oxidative interference from phenolic compounds. This step was particularly important given the known presence of biflavonoids and other phenolic constituents in *Selaginella* species (Setyawan 2011; Chikmawati et al. 2012).

The homogenized samples were incubated at 65°C to facilitate cell lysis and DNA release, followed by chloroform-isoamyl alcohol (24:1) extraction to remove proteins and other cellular debris. After centrifugation, the aqueous phase containing genomic DNA was carefully transferred to a new tube and subjected to DNA precipitation using sodium acetate and cold isopropanol. Precipitated DNA was pelleted by centrifugation, washed sequentially with absolute ethanol and 70% ethanol to remove residual salts and contaminants, and air-dried prior to resuspension in TE buffer, following standard plant DNA extraction procedures (Doyle and Doyle 1987; Sambrook and Russell 2001).

DNA quality and integrity were initially assessed by electrophoresis on 1.5% agarose gels stained with ethidium bromide, where high-molecular-weight DNA appeared as clear and intact bands with minimal smearing. DNA concentration and purity were further evaluated spectrophotometrically by measuring absorbance at 260 and 280 nm, and samples with acceptable A260/A280 ratios were selected for subsequent ISSR amplification (Sambrook and Russell 2001). Only DNA extracts exhibiting sufficient purity and concentration were used in PCR reactions to ensure reproducibility and consistency of ISSR banding patterns.

The modified CTAB method employed in this study has been widely applied for genomic DNA extraction in plants with high polysaccharide and polyphenol contents and is considered reliable for downstream PCR-based marker analyses (Porebski et al. 1997; Healey et al. 2014). This protocol provided DNA of adequate quality for ISSR analysis across all *S. ciliaris* samples included in the study.

Table 1. Sampling locations and ecological context of *Selaginella ciliaris* specimens used for ISSR analysis from Central Java, Indonesia

Sample code	Herbarium code	Locality	Sub-district / District	Lat (°S)	Lon (°E)	Elev (masl)	Habitat description
S1	ADS 320	Sidototo	Padureso, Kebumen	7.632096	109.787190	171	Rocky cliff of volcanic origin along the main road with spring seepage
S2	ADS 327	Merden	Padureso, Kebumen	7.655701	109.798297	41	Near a small culvert with water seepage from an irrigation channel
S3	ADS 527	Sumberejo	Wadaslintang, Wonosobo	7.592480	109.796584	211	Concrete foundation wall above drainage channel in front of the school
S4	ADS 529	Bonjok Lor	Bonorowo, Kebumen	7.735116	109.808111	13	Cemetery ground among gravestones, seasonally dry
S5	ADS 617	Bojongsari	Alian, Kebumen	7.665505	109.714523	38	Volcanic rock cliff near the roadside under an agroforestry canopy
S6	ADS 616	Lerep-kebumen	Poncowarno, Kebumen	7.673748	109.730892	76	Rocky volcanic cliff adjacent to dry farmland
S7	ADS 618	Tegalrejo	Poncowarno, Kebumen	7.663529	109.756191	36	Volcanic rock cliff within an agroforestry area near rice fields
S8	ADS 528	Sidototo	Padureso, Kebumen	7.633873	109.788195	156	A rocky cliff of volcanic origin along the main road
S9	ADS 620	Krakal	Alian, Kebumen	7.606684	109.705595	37	Volcanic rock cliff on a hill slope near a river
S10	ADS 621	Seliling	Alian, Kebumen	7.645456	109.702619	36	Rice-field embankment close to the settlement
S11	ADS 619	Kalirancang	Alian, Kebumen	7.619419	109.708571	50	Rocky volcanic cliff in dry mixed forest
S12	ADS 324	Padureso	Padureso, Kebumen	7.619299	109.791300	190	Roadside area beneath a bamboo canopy
S13	ADS 532	Loano	Loano, Purworejo	7.678617	110.036130	100	Shaded cemetery area among gravestones
S14	ADS 531	Sucen Jurutengah	Bayan, Purworejo	7.708872	109.973232	36	Irrigation channel margin, early dry-season condition
S15	ADS 530	Pasaranom	Grabag, Purworejo	7.823548	109.864862	15	Base of a coconut tree, drying during the dry season
S16	ADS 414	Candiyasan	Kertek, Wonosobo	7.355901	109.997890	1245	Eroded rainwater drainage cliff between the tea plantation and the vegetable fields
S17	ADS 574	Kalirejo	Salaman, Magelang	7.620467	110.130947	476	Cassava field margin, seasonally dry
S18	ADS 515	Kalikuto	Grabag, Magelang	7.382529	110.285728	584	Wet rice-field embankment near a large river
S19	ADS 514	Seloprojo	Ngablak, Magelang	7.369170	110.378618	1,094	Cemetery area
S20	ADS 549	Krinjing	Watumalang, Wonosobo	7.284581	109.876642	1,144	Steep vegetable field slope
S21	ADS 522	Kemiriombo	Kaliwiro, Wonosobo	7.446140	109.844412	605	Moist volcanic cliff near the roadside and a small spring
S22	ADS 525	Ngalian	Wadaslintang, Wonosobo	7.522216	109.822051	402	Pine forest, roadside, volcanic rock cliff
S23	ADS 537a	Kejiwan	Wonosobo, Wonosobo	7.340963	109.901510	791	Drainage channel beneath a steep volcanic cliff
S24	ADS 540	Derongisor	Mojotengah, Wonosobo	7.304136	109.883267	954	Very steep volcanic slope along the roadside vegetable field
S25	ADS 533	Burat	Kepil, Wonosobo	7.585051	110.002953	427	Pine forest understory along village road
S26	ADS 524	Tracap	Kaliwiro, Wonosobo	7.484343	109.836862	461	Volcanic rock cliff along the main road near mixed gardens
S27	ADS 622	Garung	Garung, Wonosobo	7.294078	109.921154	1,005	Building a wall near the river

Note: Elev: Elevation, masl: Meters above sea level

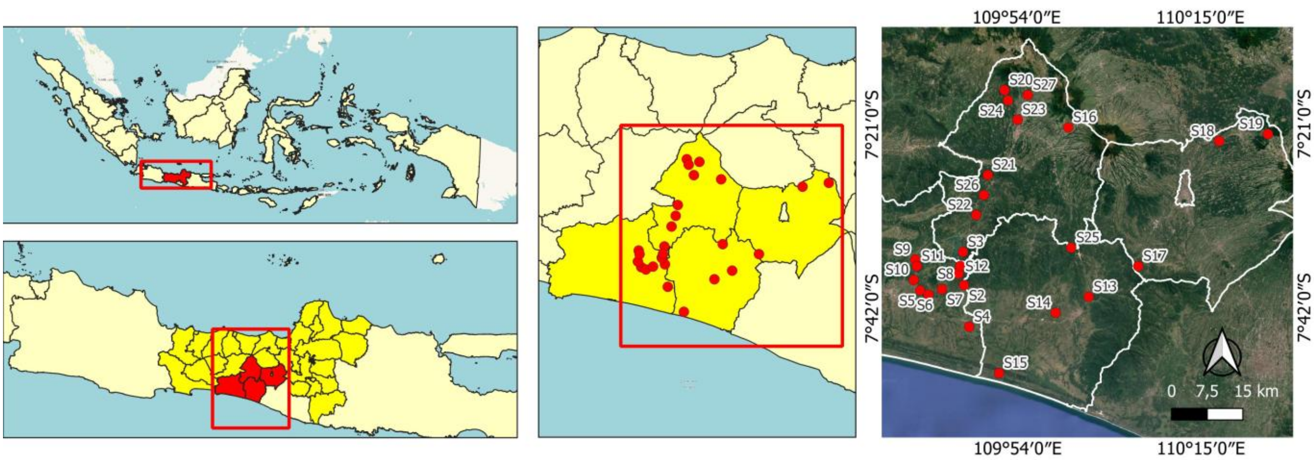

Figure 1. Geographic distribution of *Selaginella ciliaris* sampling sites across Central Java, Indonesia

ISSR primers and PCR amplification conditions

ISSR analysis was performed using three primers selected based on preliminary optimization for band clarity, reproducibility, and polymorphism detection in *S. ciliaris*. The primers used in this study were SBS 811, SBS 812, and SBS 835, each consisting of di- or tri-nucleotide repeat motifs with anchored sequences at the 3′ end. These primers have been previously applied in ISSR-based genetic diversity studies of various plant taxa and are known to generate multilocus amplification profiles suitable for assessing intraspecific variability (Zietkiewicz et al. 1994; Reddy et al. 2002).

PCR amplifications were carried out in a total reaction volume of 25 μL containing genomic DNA template, ISSR primer, nuclease-free water, and a commercially available PCR master mix comprising Taq DNA polymerase, dNTPs, $MgCl_2$, and reaction buffer. Approximately 20-50 ng of template DNA was used per reaction to ensure consistent amplification while minimizing nonspecific band formation. Primer concentration and annealing temperature were optimized prior to final amplification, and all primers were amplified using a uniform annealing temperature to maintain comparability across samples.

The PCR cycling program consisted of an initial denaturation step at 94°C, followed by 40 amplification cycles of denaturation at 94 °C, primer annealing at 55°C, and extension at 72°C. A final extension step at 72°C was included to complete DNA synthesis. Amplification reactions were performed using a programmable thermal cycler, and negative controls without template DNA were included in each PCR run to check for contamination.

ISSR markers were selected due to their high reproducibility and ability to detect polymorphisms without requiring prior genomic information. Compared with RAPD markers, ISSRs typically provide more stable banding patterns and higher discriminatory power, particularly in non-model plant species (Bornet and Branchard 2001; Reddy et al. 2002). The use of multiple primers in this study aimed to increase genome coverage and improve the robustness of genetic variability assessment in *S. ciliaris*. Only clear, reproducible bands consistently amplified across repeated reactions were considered for subsequent analysis.

Agarose gel electrophoresis and band visualization

PCR amplification products generated by ISSR primers were separated by horizontal agarose gel electrophoresis to visualize DNA banding patterns. Electrophoresis was performed using 1.5% (w/v) agarose gels prepared in 1× Tris-Acetate-EDTA (TAE) buffer. Agarose concentration was selected to allow adequate resolution of ISSR fragments ranging from approximately 100 to 3000 bp, which is typical for ISSR-based amplification profiles.

For each sample, amplified PCR products were mixed with loading dye and loaded into individual gel wells. A molecular size standard (100 bp or 1 kb DNA ladder) was included in each gel to estimate fragment sizes and to ensure consistency across runs. Electrophoresis was conducted at a constant voltage until sufficient separation of DNA fragments was achieved, allowing clear discrimination among bands of different molecular weights.

Following electrophoresis, gels were stained using ethidium bromide and subsequently visualized under ultraviolet (UV) transillumination. Digital images of the gels were captured using a gel documentation system to allow permanent record storage and accurate band scoring. Only well-resolved and distinct bands were considered suitable for further analysis. Faint or smeared bands, which may result from low DNA concentration or partial amplification, were excluded to minimize scoring errors.

Agarose gel electrophoresis remains a standard and reliable method for visualizing ISSR amplification products due to its simplicity, reproducibility, and compatibility with downstream data analysis (Sambrook and Russell 2001). The visualization procedure applied in this study ensured consistent detection of ISSR banding patterns across all *S. ciliaris* samples.

Band scoring and binary data matrix construction

ISSR banding patterns obtained from agarose gel electrophoresis were scored manually based on the presence or absence of distinct DNA fragments across all samples. Each clearly resolved band was treated as an independent locus, and only reproducible bands consistently amplified across repeated PCR reactions were included in the analysis. Bands of identical electrophoretic mobility were assumed to represent homologous loci, following standard practice in dominant marker analysis.

Band scoring was performed by visually inspecting gel images using a gel documentation system. To reduce subjectivity and scoring errors, only sharp and unambiguous bands were considered, whereas faint, smeared, or overlapping bands were excluded from the dataset. Fragment sizes were not measured as exact base-pair lengths but were visually estimated by comparison with a molecular weight marker (100 bp DNA ladder) included in each gel, and therefore should be interpreted as approximate values. Bands were ordered from the smallest to the largest estimated molecular weight to facilitate consistent scoring across samples.

The presence of a band at a given locus was coded as “1,” while its absence was coded as “0,” generating a binary data matrix for all *S. ciliaris* samples and ISSR loci. This binary matrix formed the basis for subsequent calculations of genetic similarity and cluster analysis. As ISSR markers are dominant, heterozygous and homozygous dominant states could not be distinguished, and band absence was interpreted as either the homozygous recessive state or the absence of the target amplification region.

Binary scoring of ISSR data is a widely accepted approach for assessing genetic variability in non-model plant species and has been successfully applied in numerous studies involving dominant molecular markers (Wolfe and Liston 1998; Roldán-Ruiz et al. 2000). By explicitly treating fragment sizes as approximate estimates and applying strict scoring criteria that exclude ambiguous bands, the resulting binary matrix provides a robust and methodologically sound representation of genetic variation suitable for downstream similarity analysis and dendrogram construction.

Genetic similarity analysis

Genetic similarity among *S. ciliaris* samples was quantified using the binary ISSR data matrix generated from band scoring. Pairwise genetic similarity coefficients were calculated using the Dice similarity index, which is particularly suitable for dominant molecular markers because it emphasizes shared band presence rather than shared absence. The Dice coefficient was calculated following the formula $S = \frac{2N_{ab}}{N_a+N_b}$, where N_{ab} is the number of bands shared by two samples, and N_a and N_b represent the total number of bands observed in each sample (Nei and Li 1979).

The similarity matrix provided a numerical representation of genetic relatedness among all sampled individuals and enabled direct comparison of ISSR profiles across different sampling sites. Similarity values theoretically range from 0 (no shared bands) to 1 (identical banding patterns), allowing assessment of relative genetic variability within the dataset. Given the dominant nature of ISSR markers, the analysis focused on overall genetic similarity rather than allele frequencies or heterozygosity estimates.

Genetic similarity analysis based on dominant markers has been widely applied in plant genetic diversity studies, particularly for non-model species lacking extensive genomic resources (Mohammadi and Prasanna 2003; Peakall and Smouse 2012). In the present study, the similarity matrix served as the primary input for hierarchical cluster analysis aimed at visualizing genetic relationships among *S. ciliaris* samples.

Cluster analysis and software used

Hierarchical cluster analysis was performed to visualize genetic relationships among *S. ciliaris* samples based on the ISSR-derived genetic similarity matrix. Clustering was conducted using the Unweighted Pair Group Method with Arithmetic mean (UPGMA), a commonly applied agglomerative algorithm in molecular marker studies. UPGMA constructs a dendrogram by iteratively grouping samples based on average pairwise similarity, providing an intuitive representation of genetic relatedness among individuals.

The genetic similarity matrix generated using the Dice coefficient was used as input for cluster construction. All clustering analyses were performed using the Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc) software package, version 2.0. The resulting dendrogram was examined to identify major groupings and patterns of genetic similarity among samples collected from different localities. No a priori grouping based on geographic origin was imposed, allowing clustering patterns to emerge solely from molecular data.

UPGMA clustering has been extensively employed in ISSR-based genetic diversity studies and is considered appropriate for exploratory analysis of genetic relationships in non-model plant species (Sneath and Sokal 1973; Rohlf 2000). In this study, cluster analysis was used as a descriptive tool to summarize ISSR variation rather than to infer formal population genetic structure, in line with the screening-oriented objective of the research.

RESULTS AND DISCUSSION

Sampling coverage and amplification success

The ISSR analysis of *S. ciliaris* was based on 27 individual samples collected across a humid tropical landscape in Central Java, encompassing a wide range of elevations and microhabitat types. Sampling sites extended from lowland areas below 100 masl to highland environments exceeding 1,000 masl, and included agricultural margins, forest edges, riverbanks, plantation understories, montane zones, as well as anthropogenic microhabitats such as drainage channels, roadside embankments, and building walls. This spatial coverage was designed to capture environmental heterogeneity while maintaining a focus on intraspecific genetic variability rather than formal population subdivision (Table 2).

All genomic DNA samples extracted from the collected individuals were successfully amplified using the three selected ISSR primers (SBS 811, SBS 812, and SBS 835). Amplification success was consistent across samples, with each primer generating clear and scorable banding patterns for the majority of individuals. No amplification failure or complete primer-specific dropout was observed, indicating that the DNA extraction protocol and PCR conditions were suitable for *S. ciliaris* across different habitat contexts.

ISSR amplification profiles showed multilocus banding patterns across samples, with variability in fragment presence and size (Figure 2). Limited smearing and the absence of non-specific amplification indicate that the PCR conditions and primer selection were appropriate. Negative controls consistently showed no amplification, suggesting the absence of contamination.

Table 2. Summary of sampling coverage and ISSR amplification success in *Selaginella ciliaris*

Parameter	Description
Total number of samples	27 individuals
Geographic coverage	Central Java, Indonesia
Elevation range	<100 to >1,000 masl
Number of sampling regions	5 districts/cities
Habitat types	Agricultural margins, forest edges, riverbanks, plantations, montane humid zones
ISSR primers used	SBS 811, SBS 812, SBS 835
Amplification success rate	100% of samples amplified
Primer dropout	Not observed
Contamination (negative controls)	Not detected

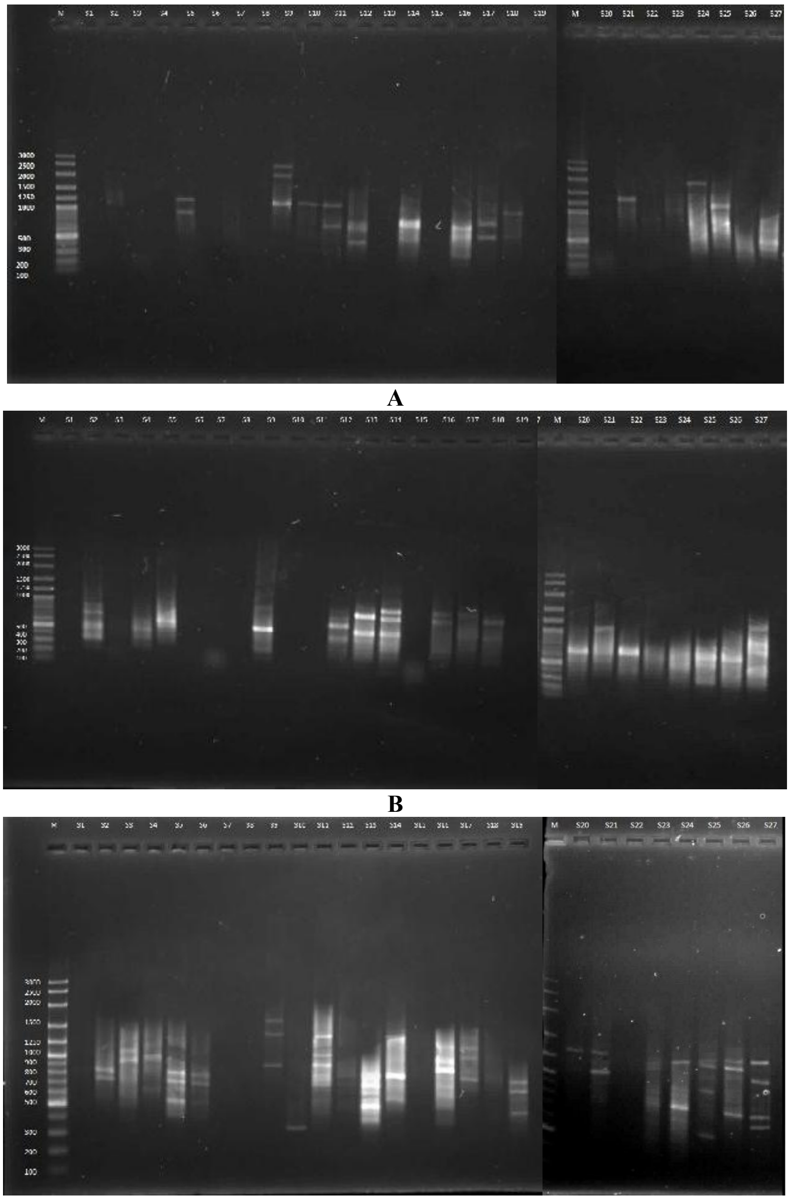

Figure 2. ISSR amplification profiles of *Selaginella ciliaris* generated using primers SBS 811 (A), SBS 812 (B), and SBS 835 (C), showing polymorphic banding patterns across samples S1-S27 resolved with a 100 bp DNA ladder

The combination of broad sampling coverage and high amplification success provided a robust dataset for subsequent genetic variability analysis. The consistent performance of all three ISSR primers across geographically and ecologically diverse samples supports their suitability for assessing genetic variability in *S. ciliaris* and other non-model tropical lycophytes.

ISSR band profiles and polymorphism levels

ISSR amplification using three primers (SBS 811, SBS 812, and SBS 835) generated clear and reproducible multilocus banding patterns across all *S. ciliaris* samples. A total of 49 distinct ISSR bands were scored across the 27 individuals, with fragment sizes ranging approximately from 140 bp to over 2200 bp. All scored bands were polymorphic, resulting in an overall polymorphism level of 100% across the dataset (Table 3).

The number of bands produced per primer varied substantially. Primer SBS 811 generated 12 bands, primer SBS 812 produced the lowest number with 9 bands, whereas primer SBS 835 yielded the highest number of bands, totaling 28. Despite these differences in band number, all primers consistently amplified polymorphic loci, indicating their effectiveness in detecting genetic variability within *S. ciliaris*. No monomorphic bands were observed for any primer, underscoring the high level of genetic heterogeneity detected by the ISSR marker system.

Banding profiles differed markedly among individuals, with no two samples exhibiting identical ISSR patterns across all loci. Variations were observed both in the presence or absence of specific bands and in the overall distribution of fragment sizes (Figure 2). Several primers produced bands that were widely shared among multiple samples, while others generated bands restricted to a small subset of individuals, contributing to the overall polymorphic signal.

Primer SBS 835 not only produced the highest number of bands but also covered the widest fragment size range, suggesting broader genome coverage relative to the other primers. In contrast, primers SBS 811 and SBS 812 produced fewer bands but still contributed unique polymorphic loci not detected by SBS 835. The combined use of three primers, therefore, increased the resolution of genetic variability detection by expanding the number of loci surveyed.

The ISSR band profiles revealed extensive polymorphism across *S. ciliaris* samples, providing a robust molecular dataset for subsequent similarity analysis and clustering. The complete absence of monomorphic loci highlights the suitability of ISSR markers for capturing genetic variability in this non-model tropical lycophyte and supports their use as an effective screening tool for preliminary genetic assessment.

Primer performance and fragment size distribution

The three ISSR primers used in this study differed markedly in their amplification performance and fragment size distribution, providing complementary multilocus information for assessing genetic variability in *S. ciliaris*. Primer performance was evaluated based on the total number of amplified fragments, fragment size range, and the contribution of each primer to overall polymorphism (Table 4).

Primer SBS 835 showed the highest amplification performance, generating 28 distinct fragments across a wide size range from approximately 140 bp to over 2200 bp. This primer contributed more than half of the total ISSR loci scored in the study and exhibited a broad distribution of fragment sizes, indicating extensive genome coverage. Bands amplified by SBS 835 were distributed across low-, medium-, and high-molecular-weight regions, allowing discrimination among individuals based on multiple loci.

Table 3. ISSR primers, band profiles, and polymorphism levels detected in *Selaginella ciliaris*

Primer code	Primer sequence (5′-3′)	Annealing temp. (°C)	Total bands	Polymorphic bands	Monomorphic bands	Polymorphism (%)	Fragment size range (bp)*
SBS 811	ACA CAC ACA CAC ACA CC	55	12	12	0	100	~150-1800
SBS 812	ACA CAC ACA CAC ACA CG	55	9	9	0	100	~150-1100
SBS 835	AGA GAG AGA GAG AAG GCC	55	28	28	0	100	~150-2200
Total	-	-	49	49	0	100	~150-2200

Note: *: Fragment sizes were visually estimated by comparison with a 100 bp DNA ladder and are therefore approximate; values are presented as rounded ranges for clarity and consistency

Table 4. Performance of ISSR primers and fragment size distribution in *Selaginella ciliaris*

Primer code	Total fragments	Contribution to total loci (%)	Minimum fragment size (bp)*	Maximum fragment size (bp)*	Dominant fragment size range (bp)
SBS 811	12	24.49	~150	~1800	~400-1500
SBS 812	9	18.37	~150	~1100	~300-900
SBS 835	28	57.14	~150	~2200	~200-2000
Total	49	100	~150	~2200	-

Note: *: Fragment sizes were visually estimated by comparison with a 100 bp DNA ladder and are therefore approximate; values are presented as rounded ranges for clarity and consistency

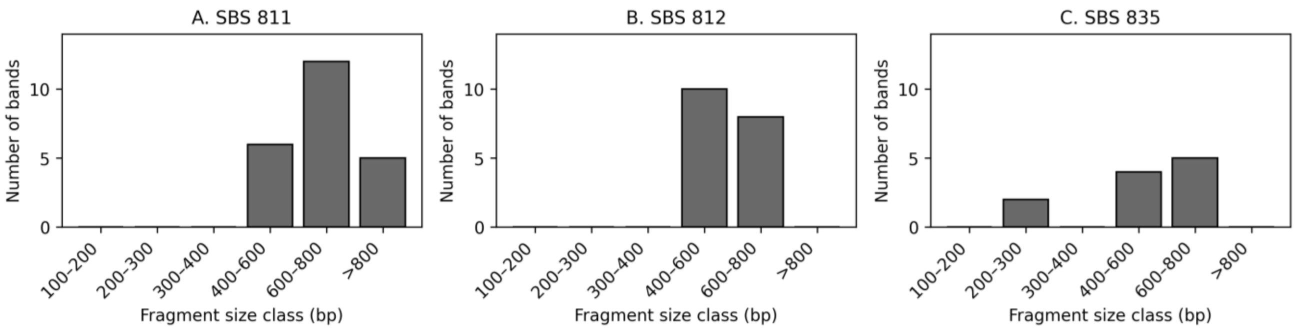

Figure 3. Fragment size distribution of ISSR loci amplified by primers SBS 811 (A), SBS 812 (B), and SBS 835 (C) in *Selaginella ciliaris*. Fragment sizes were visually estimated by comparison with a 100 bp DNA ladder and are therefore approximate. For descriptive purposes, fragments were grouped into size classes to summarize overall amplification patterns across samples (S1-S27), whereas Tables 3 and 4 present rounded fragment size ranges (bp) for clarity and consistency

Primer SBS 811 produced 12 fragments with sizes ranging from approximately 150 bp to 1800 bp. Although the number of fragments was lower than that of SBS 835, SBS 811 amplified several mid- to high-molecular-weight bands that were not detected by the other primers. These fragments contributed additional polymorphic information and enhanced the overall resolution of the ISSR dataset.

Primer SBS 812 generated the smallest number of fragments, totaling nine bands, with fragment sizes concentrated in the lower to mid-molecular-weight range (approximately 155-1100 bp). Despite its lower fragment yield, SBS 812 consistently amplified clear and scorable bands across all samples and contributed unique loci that complemented those produced by SBS 811 and SBS 835.

The combined fragment size distribution of the three primers covered a wide molecular range, from approximately 140 bp to more than 2200 bp (Figure 3). This broad distribution indicates that ISSR amplification targeted multiple genomic regions and repeat motifs within *S. ciliaris*. No primer produced excessive clustering of bands within a narrow size interval, reducing the risk of redundancy among loci.

Differences in primer performance and fragment size distribution demonstrate the advantage of using multiple ISSR primers to maximize genome coverage and polymorphic locus detection. The complementary amplification profiles generated by SBS 811, SBS 812, and SBS 835 provided a balanced and informative dataset for subsequent genetic similarity and clustering analyses.

Genetic similarity indices among samples

Genetic similarity among the 27 *S. ciliaris* samples was quantified using the Dice similarity coefficient calculated from the binary ISSR data matrix. Pairwise similarity values revealed a wide range of genetic relationships among individuals, indicating substantial genetic variability within the sampled dataset (Table 5). Similarity coefficients ranged from complete dissimilarity (0.00) to low similarity values not exceeding 0.0816, demonstrating that no two samples shared highly similar ISSR profiles across all loci.

Most pairwise comparisons showed similarity values close to zero, reflecting the presence of numerous unique or rarely shared ISSR bands among samples. A limited number of sample pairs exhibited relatively higher similarity values compared with the rest of the dataset, although these values remained low in absolute terms. Such patterns indicate heterogeneous distribution of ISSR loci among individuals rather than the presence of closely related genetic groups.

The overall distribution of similarity values was skewed toward low similarity classes, with the majority of pairwise coefficients falling below 0.05 (Figure 4). This distribution suggests that genetic variation within *S. ciliaris* is largely structured at the individual level rather than being dominated by shared multilocus profiles. The absence of high similarity values further supports the observation that identical or near-identical genotypes were not detected among the sampled individuals.

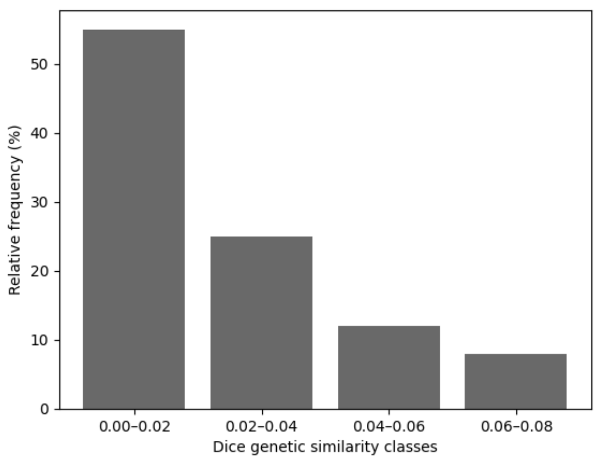

Figure 4. Frequency distribution of Dice genetic similarity values among *Selaginella ciliaris* samples based on ISSR markers

Summary statistics of the similarity matrix confirmed this pattern, with low mean similarity and a narrow upper range of values (Table 5). These results are consistent with the high proportion of polymorphic ISSR loci detected across all primers and reflect extensive multilocus variability within the species. The similarity matrix generated in this analysis provided the quantitative basis for subsequent hierarchical clustering aimed at visualizing genetic relationships among samples.

The genetic similarity indices derived from ISSR data demonstrate pronounced genetic differentiation among *S. ciliaris* individuals sampled across Central Java. The low similarity values across most sample pairs highlight the discriminatory power of ISSR markers in resolving genetic variability in this tropical lycophyte.

Cluster patterns revealed by the UPGMA dendrogram

Hierarchical cluster analysis based on the Dice genetic similarity matrix resulted in a UPGMA dendrogram that illustrated genetic relationships among the 27 *S. ciliaris* samples (Figure 5). The dendrogram revealed a clear hierarchical structure characterized by multiple small clusters and long branch lengths, reflecting the low genetic similarity values observed among most sample pairs. No single dominant cluster encompassing a large proportion of samples was detected.

At a low similarity threshold, the dendrogram resolved the samples into several primary clusters, each composed of a limited number of individuals (Table 6). Most clusters consisted of two to four samples, while a number of individuals appeared as single-member branches, indicating pronounced genetic distinctiveness. The separation of samples occurred early in the clustering process, consistent with the low maximum similarity value observed in the similarity matrix.

Samples originating from geographically proximate localities were not consistently grouped in the dendrogram. Individuals collected from the same district or similar habitat types were frequently assigned to different clusters, whereas some samples from geographically distant sites appeared within the same cluster. This pattern indicates that clustering was driven primarily by ISSR banding profiles rather than by geographic proximity or habitat similarity.

Several clusters were supported by relatively shorter branch lengths, indicating slightly higher genetic similarity among their member samples compared with the overall dataset. However, even within these clusters, similarity values remained low, and no cluster exhibited near-identical ISSR profiles. The presence of numerous singleton branches further emphasized the high level of multilocus variability detected across samples.

The UPGMA dendrogram provided a visual summary of genetic relationships among *S. ciliaris* individuals and complemented the quantitative similarity analysis. By integrating multilocus ISSR data, the clustering analysis effectively captured patterns of genetic heterogeneity within the sampled population. The dendrogram served as a descriptive tool to illustrate the extent of genetic variability rather than to infer formal population structure or evolutionary relationships.

Discussion

Extent of genetic variability detected by ISSR

The ISSR analysis revealed an exceptionally high level of genetic variability in *S. ciliaris*, as evidenced by the detection of 100% polymorphic loci across all primers and consistently low Dice similarity values among sampled individuals. Such extensive multilocus variability indicates pronounced genetic heterogeneity within the species, even at a regional spatial scale. In ISSR-based studies, complete polymorphism is generally interpreted as a strong signal of genetic differentiation and high discriminatory power of the marker system, particularly in non-model plant taxa where genome-wide data are unavailable (Agarwal et al. 2008; Kalia et al. 2011).

Comparable levels of high ISSR polymorphism have been widely reported in plant genetic studies, particularly in species characterized by broad ecological tolerance, clonal growth strategies, or fragmented habitats. High levels of polymorphism are generally interpreted as evidence of substantial intraspecific genetic variability and strong discriminatory power of ISSR markers in non-model plant taxa (Agarwal et al. 2008; Kalia et al. 2011). In lycophytes and pteridophytes, however, molecular data remain relatively scarce, limiting direct comparisons across taxa. Nevertheless, available studies on ferns and other early-diverging vascular plants suggest that pronounced genetic variability is not uncommon, especially in species occupying heterogeneous microhabitats (Ranker and Geiger 2009).

Table 5. Summary statistics of Dice genetic similarity indices among *Selaginella ciliaris* samples based on ISSR data

Parameter	Value
Number of samples	27
Total pairwise comparisons	351
Minimum similarity	0.0000
Maximum similarity	0.0816
Mean similarity	0.031
Median similarity	0.028
Standard deviation	0.018
Similarity coefficient used	Dice

Table 6. Cluster membership of *Selaginella ciliaris* samples based on UPGMA analysis of ISSR data at a cut-off of genetic distance (1 − Dice) = 0.05

Cluster	Sample codes	Number of samples
Cluster I	S27, S7, S8, S15	4
Cluster II	S2, S23	2
Cluster III	S20, S16, S17, S25	4
Cluster IV	S5, S12, S6, S10, S4, S13, S22	7
Cluster V	S3, S26	2
Cluster VI	S19, S21	2
Cluster VII	S1, S18	2
Singleton A	S11	1
Singleton B	S24	1
Singleton C	S9	1
Singleton D	S14	1

Note: Clusters were defined by cutting the UPGMA dendrogram at a genetic distance (1 − Dice) = 0.05; samples merging above this threshold were treated as separate clusters/singletons

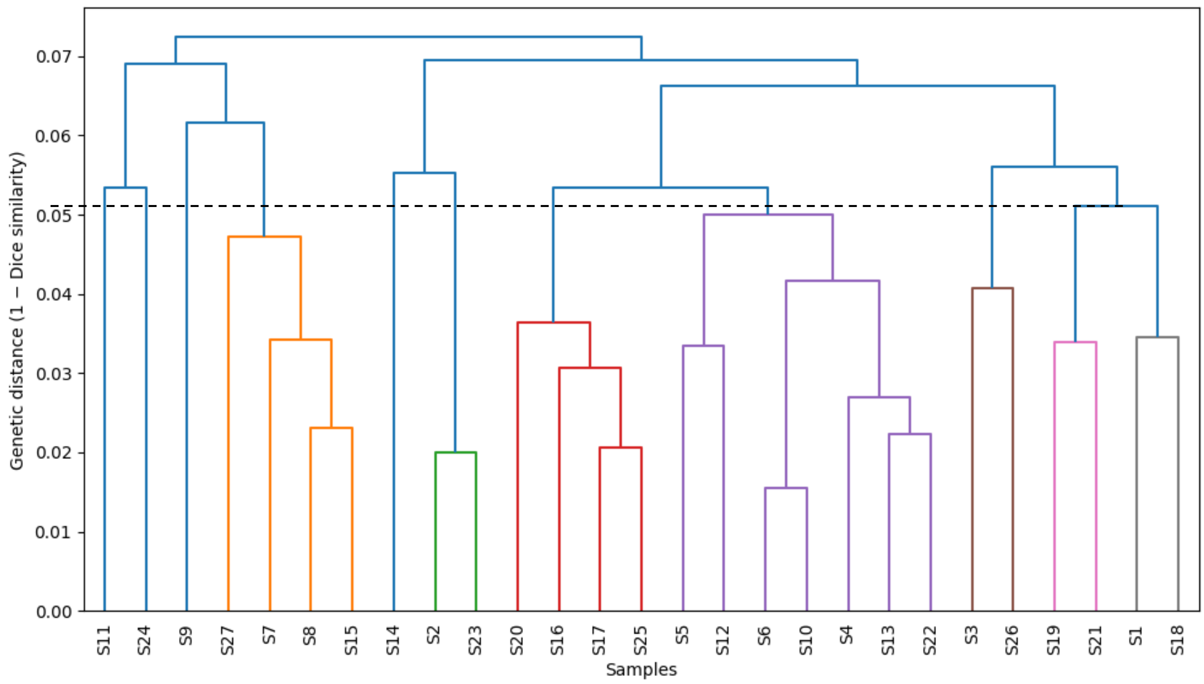

Figure 5. UPGMA dendrogram showing genetic relationships among *Selaginella ciliaris* samples based on Dice similarity coefficients derived from ISSR markers

The low range of Dice similarity values observed in this study further underscores the extent of genetic differentiation among *S. ciliaris* individuals. In ISSR-based analyses, low pairwise similarity is commonly associated with multilocus profiles dominated by rare or individual-specific bands, particularly in non-model plant species lacking genome-wide resources (Reddy et al. 2002; Agarwal et al. 2008). Such patterns indicate that genetic variation is distributed primarily at the individual level rather than concentrated within a limited number of closely related genotypes, a characteristic frequently reported for dominant marker systems (Wolfe and Liston 1998). This interpretation is consistent with the absence of identical ISSR profiles among samples and the prevalence of singleton or weakly connected branches in the UPGMA dendrogram.

Several biological and ecological factors may contribute to the high genetic variability detected in *S. ciliaris*. Lycophytes often exhibit mixed reproductive strategies, including vegetative propagation and sexual reproduction via spores, which can promote both local persistence and genetic diversification. Additionally, the occupation of moisture-rich but spatially heterogeneous habitats may facilitate genetic differentiation through microhabitat-driven selection or limited gene flow among local patches (Page 2002; Pryer et al. 2004). Although such mechanisms were not explicitly tested in the present study, the observed ISSR patterns are consistent with expectations for species inhabiting fragmented tropical landscapes.

From a biotechnological perspective, the detection of extensive genetic variability has important implications. High genetic heterogeneity provides a broad genetic base for germplasm screening and selection, particularly in early-stage studies aimed at identifying genetically distinct material for further investigation. Dominant molecular markers such as ISSR have been widely advocated as effective tools for preliminary genetic assessment in non-model and tropical plant species, especially prior to the application of more resource-intensive genomic approaches (Reddy et al. 2002; Nybom 2004). In this context, the present results demonstrate that ISSR markers are well-suited for capturing genetic variability in *S. ciliaris* and support their application in future studies focusing on conservation biotechnology, metabolite diversity, or in vitro culture development.

The high level of ISSR-detected genetic variability observed in *S. ciliaris* highlights both the biological complexity of this tropical lycophyte and the effectiveness of ISSR markers as a screening tool. These findings contribute baseline molecular information for an understudied lineage and provide a foundation for subsequent applied and comparative studies in tropical plant biotechnology.

Comparison with lycophytes and other pteridophytes

When compared with available molecular studies on lycophytes and other pteridophytes, the level of genetic variability detected in *S. ciliaris* appears notably high. Although molecular investigations in lycophytes remain limited relative to seed plants, existing syntheses and comparative studies indicate that moderate to high genetic differentiation is common within and among populations of early-diverging vascular plants, particularly those occupying heterogeneous or fragmented habitats (Pryer et al. 2004; Ranker and Geiger 2009). Such patterns are consistent with the evolutionary history and ecological breadth of lycophytes.

In fern lineages, which share key aspects of reproductive biology with lycophytes, comparable levels of high intraspecific genetic variability have been widely documented. Studies employing dominant and codominant molecular markers in ferns frequently report extensive polymorphism and low genetic similarity among individuals, even within relatively restricted geographic regions. These patterns have been attributed to large effective population sizes, long evolutionary histories, and complex life cycles involving independent gametophyte and sporophyte stages (Page 2002; Ranker and Geiger 2009).

Compared with many fern species, however, the genetic similarity values observed in *S. ciliaris* are particularly low, indicating pronounced multilocus differentiation at the individual level. This contrast may reflect differences in growth form and ecological strategy. Lycophytes such as *Selaginella* often exhibit creeping or mat-forming habits and occupy microhabitats characterized by sharp gradients in moisture, light availability, and substrate conditions. Fine-scale environmental heterogeneity may therefore promote localized selection and restricted gene flow, contributing to elevated genetic differentiation among neighboring individuals (Page 2002; Pryer et al. 2004).

From a methodological perspective, the complete polymorphism observed in *S. ciliaris* is consistent with expectations for ISSR-based analyses of non-model vascular plants. Dominant multilocus markers such as ISSR are known to reveal high levels of individual-specific variation, particularly in taxa lacking genomic resources and occupying complex environments (Reddy et al. 2002; Agarwal et al. 2008). While many previous studies of lycophytes and pteridophytes have emphasized population-level differentiation, the present results highlight the importance of individual-level genetic variability as a component of overall diversity.

The genetic patterns documented in *S. ciliaris* are consistent with, and in some respects exceed, levels of variability reported for other lycophytes and pteridophytes. This comparison underscores the value of *S. ciliaris* as a representative system for exploring genetic diversity in early-diverging vascular plants and highlights the need for broader comparative molecular studies within this underrepresented group. From a broader perspective, the findings reinforce the view that lycophytes harbor substantial yet largely unexplored genetic diversity, with important implications for tropical plant conservation and biotechnology.

Effectiveness of ISSR markers for non-model tropical plants

The results of this study demonstrate that ISSR markers are highly effective for detecting genetic variability in *S. ciliaris*, a non-model tropical plant for which genomic resources remain unavailable. The complete polymorphism observed across all primers, and the clear differentiation among individuals, confirm the suitability of ISSR markers for exploratory genetic analyses in taxa where sequence-based approaches are still impractical or cost-prohibitive. Such performance is consistent with methodological assessments emphasizing the high discriminatory power of ISSR markers in non-model plant species, particularly during the early phases of genetic evaluation (Reddy et al. 2002; Agarwal et al. 2008).

One of the primary advantages of ISSR markers lies in their independence from prior genomic information. Unlike microsatellites or SNP-based methods, ISSR primers target conserved simple sequence repeat motifs distributed throughout the genome, enabling rapid multilocus screening with minimal technical requirements (Zietkiewicz et al. 1994). This characteristic is especially valuable for tropical plant groups such as lycophytes, ferns, and medicinal plants, which remain underrepresented in genomic databases. In the present study, the consistent amplification success across all samples further highlights the robustness of ISSR markers when applied to plant material collected from heterogeneous tropical environments (Kalia et al. 2011).

ISSR markers have been widely adopted as a practical tool for assessing genetic diversity and germplasm differentiation in non-model plant taxa. Their multilocus nature often reveals substantial individual-level variation, producing clear and reproducible banding patterns suitable for similarity-based analyses (Wolfe and Liston 1998; Agarwal et al. 2008). The distinct ISSR profiles obtained for *S. ciliaris* support this general pattern and demonstrate that reliable genetic fingerprints can be generated even in early-diverging vascular plants with complex genomic architectures.

Despite these advantages, certain limitations of ISSR markers should be acknowledged. As a dominant marker system, ISSR data do not allow direct estimation of heterozygosity or allele frequencies, and interpretations are therefore typically restricted to measures of genetic similarity or diversity rather than formal population genetic parameters. Nevertheless, for non-model species at an exploratory stage, ISSR markers provide an effective balance between analytical resolution and feasibility, particularly when the primary objective is to document genetic variability rather than to model evolutionary processes in detail (Reddy et al. 2002; Peakall and Smouse 2012).

From a biotechnological perspective, the effectiveness of ISSR markers in *S. ciliaris* underscores their value as a preliminary molecular screening tool prior to downstream applications. Information on genetic variability derived from ISSR analysis can guide the selection of genetically distinct material for conservation planning, in vitro propagation, and subsequent phytochemical or metabolomic investigations. In tropical plant biotechnology, where many taxa remain genetically underexplored, ISSR markers continue to offer a cost-effective and informative approach for generating baseline molecular data and prioritizing future analytical efforts (Zietkiewicz et al. 1994; Reddy et al. 2002).

Overall, the successful application of ISSR markers in this study reinforces their continued relevance for non-model tropical plants. While next-generation sequencing approaches are becoming increasingly accessible, ISSR markers remain a valuable methodological option for rapid genetic assessment, particularly in under-studied plant lineages such as lycophytes. Their strategic use can facilitate broader inclusion of tropical biodiversity in molecular, conservation, and biotechnological research.

Implications for germplasm screening and early-stage selection

The extensive genetic variability detected in *S. ciliaris* has direct implications for germplasm screening and early-stage selection in tropical plant biotechnology. High levels of multilocus polymorphism indicate the presence of a broad genetic base, which is a critical prerequisite for effective selection, conservation, and utilization of plant genetic resources. In non-model species, where genomic information remains limited, dominant molecular markers such as ISSR provide an efficient means of identifying genetically distinct material at an early stage of research and development (Reddy et al. 2002; Agarwal et al. 2008).

ISSR-based screening allows rapid differentiation among individuals without the need for detailed phenotypic or biochemical characterization. In the context of germplasm management, this approach facilitates the prioritization of genetically diverse accessions for further evaluation, thereby reducing redundancy and optimizing the allocation of limited research resources. Such strategies have been widely advocated in plant genetic resource studies, where molecular markers are used as an initial filter to guide ex situ conservation efforts and subsequent experimental work (Karp et al. 1997; Nybom 2004). The low genetic similarity values observed among *S. ciliaris* samples further suggest that even limited sampling can capture substantial genetic diversity, which is advantageous during early phases of germplasm collection.

Early-stage selection based on molecular diversity is particularly relevant for taxa with recognized biotechnological potential but limited agronomic or breeding history. Species of *Selaginella* are increasingly noted for their unique secondary metabolites and physiological adaptations, yet systematic selection frameworks remain under-developed. By identifying genetically distinct individuals through ISSR screening, subsequent studies can be more effectively directed toward linking genetic variation with functional traits such as metabolite composition, growth performance, or stress tolerance (Karp et al. 1997; Nybom 2004). Although the present study did not directly evaluate phenotypic or biochemical variation, the observed genetic patterns provide a rational foundation for such integrative approaches.

From a practical standpoint, ISSR-based germplasm screening is cost-effective and readily scalable, making it particularly suitable for research environments with limited infrastructure, which are common in many tropical regions. Compared with high-throughput sequencing approaches, ISSR markers require minimal laboratory investment while still generating informative multilocus data suitable for preliminary genetic assessment (Reddy et al. 2002; Agarwal et al. 2008). This balance is especially valuable during exploratory stages of research, when the primary objective is to establish baseline genetic information rather than to conduct fine-scale genomic analyses.

Furthermore, the application of ISSR markers in early-stage selection can contribute to longer-term conservation and utilization strategies. By identifying genetically distinct accessions at the outset, germplasm collections can be designed to maximize genetic representation while minimizing sampling bias. Such collections provide an essential foundation for future conservation biotechnology, breeding initiatives, and metabolite-oriented research. In this context, the present findings highlight the strategic value of ISSR-based screening as an entry point for advancing applied research on *S. ciliaris* and other non-model tropical plants.

Relevance to conservation and future biotechnological studies

The pronounced genetic variability detected in *S. ciliaris* has important implications for both conservation planning and the direction of future biotechnological studies. From a conservation perspective, high levels of multilocus genetic diversity are widely associated with enhanced adaptive potential and increased resilience to environmental change. In tropical ecosystems characterized by strong microhabitat heterogeneity and rapid land-use transformation, such genetic heterogeneity may improve the capacity of *S. ciliaris* populations to persist under shifting environmental conditions (Frankham et al. 2010; Hoban et al. 2020).

The absence of highly similar genotypes and the prevalence of small clusters or individual-specific lineages indicate that genetic variation in *S. ciliaris* is broadly distributed among individuals rather than concentrated within a limited number of dominant genetic lineages. This pattern supports conservation strategies that emphasize the protection of multiple sites and diverse microhabitats, rather than focusing exclusively on a small number of populations. For non-model tropical plants, where comprehensive population genetic data are often unavailable, ISSR-based assessments provide a practical first step toward identifying genetically representative units for conservation planning and management (Frankham et al. 2010; Laikre et al. 2020).

Beyond conservation relevance, the genetic patterns observed in this study also provide a valuable foundation for future biotechnological research. Species of *Selaginella* have been increasingly recognized for their diverse secondary metabolites and physiological adaptations, yet systematic investigation of genotype-phenotype relationships remains limited. High genetic variability, as revealed by ISSR markers, suggests the potential presence of substantial biochemical and functional diversity within *S. ciliaris*, which may be explored further through targeted phytochemical, metabolomic, or in vitro culture studies (Karp et al. 1997; da Silva Almeida et al. 2013).

ISSR-based genetic screening offers a rational approach for selecting plant material for downstream applications by enabling the identification of genetically distinct accessions for focused analyses. This strategy reduces redundancy and increases the likelihood of capturing functionally relevant variation, particularly in species lacking established breeding or improvement programs. In tropical plant biotechnology, where many taxa remain genetically underexplored, early integration of molecular diversity data can improve research efficiency while supporting conservation-oriented utilization strategies (Reddy et al. 2002; Nybom 2004).

Looking forward, the findings of this study highlight several priorities for future research. Integrating ISSR-based genetic information with ecological, biochemical, and phenotypic data would allow a more comprehensive assessment of adaptive and functional diversity in *S. ciliaris*. Although next-generation sequencing approaches provide higher resolution, ISSR markers remain a valuable and accessible tool for guiding initial exploration and prioritization in under-studied tropical plant lineages (Agarwal et al. 2008; Kalia et al. 2011).

The genetic variability documented here reinforces the relevance of *S. ciliaris* for both conservation and biotechnological research. By providing baseline molecular data and demonstrating the effectiveness of ISSR markers, this study helps bridge the gap between biodiversity assessment and applied tropical biotechnology, underscoring the importance of incorporating genetic information into future conservation and utilization efforts.

Methodological limitations and future research directions

Despite the effectiveness of ISSR markers in revealing extensive genetic variability in *S. ciliaris*, several methodological limitations should be acknowledged. First, ISSR markers are dominant in nature, which precludes direct estimation of allele frequencies, heterozygosity, and population genetic parameters under Hardy-Weinberg assumptions. As a result, interpretations are necessarily restricted to measures of genetic similarity and overall variability, rather than detailed inference of population structure, gene flow, or evolutionary processes (Reddy et al. 2002; Peakall and Smouse 2012). This limitation is inherent to dominant marker systems and should be considered when interpreting the genetic patterns reported in this study.

Second, the genetic patterns identified in this study are derived from a regional sampling framework and a limited set of ISSR primers. While this approach is appropriate for exploratory assessment, broader geographic coverage and the inclusion of additional marker systems would be required to evaluate large-scale population structure, phylogeographic patterns, or fine-scale spatial genetic processes. Multivariate ordination analyses (e.g., PCoA) or hierarchical variance partitioning methods such as AMOVA were not applied because the sampling design was not based on clearly predefined populations, and applying such analyses under these conditions could lead to overinterpretation of population-level structure. Moreover, environmental and ecological variables were not explicitly incorporated into the analysis, limiting inference regarding the potential drivers of genetic differentiation observed among individuals.

Future research should therefore aim to integrate ISSR-based screening with higher-resolution molecular approaches. The use of codominant markers such as microsatellites (SSR) or single-nucleotide polymorphism (SNP) datasets generated through next-generation sequencing or reduced-representation sequencing techniques is strongly recommended to enable robust estimation of genetic structure, gene flow, and adaptive variation in *S. ciliaris*. In addition, coupling genetic information with metabolomic, physiological, or ecological data would facilitate the investigation of genotype-phenotype relationships relevant to both conservation and biotechnological applications (Andrews et al. 2016; Hoban et al. 2020).

Nevertheless, within the context of early-stage research on non-model tropical plants, the methodological framework employed here remains appropriate. ISSR markers offer a cost-effective and accessible entry point for genetic assessment, generating essential baseline molecular data that can guide the design of more targeted and resource-intensive studies in the future. Accordingly, the present study should be viewed as a foundational screening effort rather than a comprehensive population genetic analysis.

In conclusion, this study demonstrates that *S. ciliaris* exhibits exceptionally high intraspecific genetic variability at a regional scale in tropical Central Java. ISSR analysis of 27 individuals using three primers generated 49 loci, all of which were polymorphic (100%), indicating extensive multilocus variation. Pairwise Dice genetic similarity values were uniformly low (0.0000-0.0816), with most comparisons below 0.05, reflecting pronounced genetic differentiation among individuals. UPGMA cluster analysis revealed no dominant genetic group; instead, samples formed several small clusters and multiple singletons, indicating that genetic variation is broadly distributed at the individual level rather than concentrated within a few closely related lineages. The consistent amplification success and clear banding patterns confirm the effectiveness of ISSR markers as a reliable and practical tool for genetic assessment in non-model tropical plants. From an applied perspective, ISSR analysis provides an efficient approach for generating baseline genetic information in taxa lacking genomic resources, particularly during early exploratory stages. The high genetic heterogeneity observed highlights the value of ISSR markers for early-stage germplasm screening, supporting the selection of genetically distinct material while minimizing redundancy. Overall, the documented genetic diversity underscores the importance of conserving multiple populations and microhabitats to maintain the evolutionary potential of *S. ciliaris* and provides a foundation for future applied and conservation-oriented studies.

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