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Genetic diversity and molecular differentiation among Javanese *Selaginella* revealed by SSR and ISSR markers

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Abstract. *Setyawan AD, Supriatna J, Darnaedi D, Rokhmatuloh, Sutarno, Sugiyarto, Sunarto, Kusumadewi Y. 2026. Genetic diversity and molecular differentiation among Javanese* Selaginella *revealed by SSR and ISSR markers. Biodiversitas 27 (5): d270520. https://doi.org/10.13057/biodiv/d270520. Selaginella* is a highly diverse lycophyte genus in tropical Asia, yet molecular information for Indonesian taxa remains limited, particularly for evaluating species differentiation across geographically separated populations. This study assessed genetic diversity and molecular relationships among 32 *Selaginella* accessions representing eight recognized species and three unidentified taxonomic entities, collected mainly from Java, Indonesia, with additional materials from Bali, Lombok, and Papua. Genetic variation was analyzed using four SSR primers and five ISSR primers, which together generated 122 scorable loci, all of them polymorphic. SSR markers produced 43 loci, whereas ISSR markers generated 79 loci and contributed higher discriminatory power through greater polymorphism information content, resolving power, and marker index. Genetic similarity coefficients ranged from 0.42 to 0.93, indicating moderate to high molecular affinity among accessions. Both UPGMA clustering and principal component analysis consistently separated the major taxa, although widespread species such as *S. plana* and *S. involvens* showed moderate internal dispersion across accessions from different localities. AMOVA revealed that 80.1% of molecular variation occurred within species and 19.9% among species, with significant species-level differentiation (PhiPT = 0.199; P = 0.001). Nei's genetic distance revealed moderate molecular affinity among most Javanese taxa, whereas the Lombok and Papuan accessions occupied more divergent molecular positions. Geographic origin had little influence on the overall molecular structure, suggesting that species identity was the primary determinant of genetic differentiation. These findings demonstrate that combining SSR and ISSR markers provides a reliable molecular framework for refining taxonomy and supporting future conservation efforts for tropical *Selaginella* in Indonesia.

Keywords: Genetic diversity, ISSR marker, molecular differentiation, *Selaginella*, SSR marker

INTRODUCTION

Selaginella is one of the oldest extant vascular plant lineages and represents a major component of tropical and subtropical lycophyte diversity. More than 700 species have been described worldwide, occupying diverse habitats ranging from forest floors and stream margins to rocky slopes and montane ecosystems. Owing to its evolutionary significance and ecological breadth, the genus has attracted considerable attention in studies of plant evolution, systematics, physiology, and bioactive compounds. Nevertheless, species identification remains challenging because morphologically similar taxa often exhibit overlapping vegetative characters, obscuring underlying biological differentiation (Weststrand and Korall 2016a, b; Zhou and Zhang 2023).

Indonesia is an important center of *Selaginella* diversity, supported by high humidity, complex topography, and diverse environmental conditions. Java, in particular, contains a mosaic of volcanic mountains, humid uplands, shaded valleys, and karst landscapes that support numerous species, including *S. ornata*, *S. remotifolia*, *S. plana*, *S. involvens*, and *S. opaca*. Although these taxa often occupy distinct ecological settings, field identification is frequently complicated by similarities in branching architecture, leaf morphology, and strobilus characteristics (Setyawan 2011; Setyawan et al. 2013). Furthermore, many diagnostic traits exhibit substantial plasticity in response to environmental conditions. Variations in light availability, moisture, and substrate characteristics may influence stem growth, branch density, leaf arrangement, and coloration, potentially obscuring taxonomic boundaries. Consequently, morphology alone may either underestimate or overestimate species diversity, particularly when reproductive structures are absent (Zhou et al. 2016; Zhang et al. 2021).

Molecular markers provide an important complementary approach for evaluating taxonomic

relationships and molecular differentiation among morphologically similar taxa. Among the available marker systems, Simple Sequence Repeats (SSR) and Inter-Simple Sequence Repeats (ISSR) remain practical tools for taxa with limited genomic resources. SSR markers provide locus-specific information, whereas ISSR markers generate multilocus profiles and often reveal substantial levels of polymorphism. The combined application of both marker systems enables assessment of molecular variation across complementary genomic scales while maintaining analytical efficiency in non-model plant groups (Bornet and Branchard 2001; Ng and Tan 2015; Jabari et al. 2023; Bidyananda et al. 2024).

Despite the ecological and taxonomic importance of *Selaginella*, molecular studies of Indonesian taxa remain limited compared with floristic, taxonomic, and phytochemical investigations (Setyawan 2009, 2011; Setyawan et al. 2013; Miftahudin et al. 2019). Recent AFLP- and ISSR-based studies have provided initial insights into genetic diversity and molecular structuring of Indonesian *Selaginella* (Setyawan et al. 2025; Jafron et al. 2025). However, comparative analyses integrating complementary marker systems across multiple species and geographically separated populations remain scarce. Accessions maintained in living collections provide valuable opportunities for such investigations because they preserve information on original geographic provenance while allowing standardized laboratory analysis (Xiao et al. 2021; Volis 2023).

Recent studies have demonstrated that clustering, ordination, and variance-partitioning approaches can complement morphological evidence in evaluating species relationships and internal diversity within lycophytes (Zhang et al. 2021; Shalimov et al. 2024; Zhou and Zhang 2023). Such approaches are particularly relevant for *Selaginella*, where accurate species recognition underpins ecological interpretation, evolutionary inference, and conservation planning in habitats increasingly affected by environmental change (Setyawan et al. 2018; Setyawan et al. 2020). Integrating molecular and morphological evidence is therefore essential for improving taxonomic resolution and understanding patterns of diversity within this ecologically important genus.

This study evaluated genetic diversity and molecular differentiation among Javanese *Selaginella* using combined SSR and ISSR markers across 32 accessions representing multiple species and geographic origins. Analyses included marker polymorphism, genetic similarity, hierarchical clustering, principal component analysis, and molecular variance partitioning. We hypothesized that widespread species would retain species-centered molecular cohesion despite moderate intraspecific variation among geographically separated populations and that ISSR markers would provide greater discriminatory power than SSR markers. The study further assessed the concordance between morphological species delimitation and molecular evidence to provide a stronger foundation for future taxonomic, evolutionary, and conservation studies of Indonesian *Selaginella*.

MATERIALS AND METHODS

Study materials and accession sources

A total of 32 *Selaginella* accessions were included in the molecular analysis, representing eight recognized species and three unidentified taxonomic entities. Most accessions originated from Java, Indonesia, while additional materials from Bali, Lombok, and Papua were incorporated to broaden geographic representation and evaluate molecular differentiation among geographically separated taxa (Table 1).

The recognized species comprised *S. plana, S. involvens, S. remotifolia, S. ornata, S. opaca, S. repanda, S. willdenowii,* and *S. rothertii*. Three accessions from Lombok were provisionally designated as *Selaginella* sp. "Grinjani", whereas two accessions from Papua were provisionally designated as *Selaginella* sp.01 "Gmeja" and *Selaginella* sp.02 "Gmeja". Prior to molecular analysis, all accessions were identified using vegetative characters, including branching architecture, microphyll arrangement, and strobilus morphology, following regional taxonomic references (Setyawan 2011; Weststrand and Korall 2016a, b).

Most accessions originated from humid upland habitats, including volcanic slopes, forest margins, spring-associated habitats, and cultivated collections. Sampling covered Central Java, West Java, Yogyakarta, East Java, Bali, Lombok, and Papua. Natural populations were collected from several mountain and upland localities, while additional accessions were obtained from cultivated collections in Wonosobo, Depok, and Bogor Botanic Gardens (Table 1). Cultivated materials were included because vegetative propagation generally preserves genomic integrity in the absence of hybridization or prolonged artificial selection (Xiao et al. 2021; Sahin et al. 2024).

Selaginella plana was represented by the largest number of accessions (n = 11), followed by *S. involvens* (n = 6) and *S. remotifolia* (n = 4). Three accessions each were available for *S. ornata* and *Selaginella* sp. "Grinjani", while several taxa were represented by single accessions. All samples were assigned unique identification codes prior to molecular analysis to facilitate clustering, principal component analysis, and molecular variance calculations.

DNA extraction and sample preparation

Young vegetative tissues were selected for DNA extraction because recently developed shoots generally provide higher-quality nuclear DNA than older tissues (Aboul-Maaty and Oraby 2019; QIAGEN 2024). Fresh apical stems with attached microphylls were collected from each accession, cleaned to remove debris, and processed immediately whenever possible or stored temporarily at low temperature prior to extraction (Funk et al. 2017; QIAGEN 2024).

Approximately 100 mg of fresh tissue was used for each extraction. Stems and leaves were ground in liquid nitrogen to prevent DNA degradation, and genomic DNA was isolated using a modified CTAB protocol following Doyle and Doyle (1987). Ground tissue was incubated in

extraction buffer containing 2% CTAB, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1.4 M NaCl, and 0.2% β-mercaptoethanol at 65°C for 30 min. DNA was purified using chloroform:isoamyl alcohol (24:1), precipitated with cold isopropanol, washed with 70% ethanol, air-dried, and dissolved in sterile TE buffer.

DNA quality was evaluated on 1% agarose gels stained with ethidium bromide, and only samples showing clear high-molecular-weight bands were used for PCR (Green and Sambrook 2019). DNA concentration was standardized to approximately 50 ng per PCR reaction prior to amplification to ensure comparable template input among reactions (Weising et al. 2005). Samples exhibiting weak or inconsistent amplification were re-extracted and re-evaluated prior to analysis. All DNA preparations were stored at -20°C until use in subsequent SSR and ISSR amplifications.

SSR and ISSR primer selection and PCR amplification

Two molecular markers, SSR and ISSR, were employed to assess genetic variation in this study. SSR primers amplified specific genomic loci, whereas ISSR primers generated multilocus fragments from repeated regions distributed throughout the genome (Powell et al. 1996; Bornet and Branchard 2001).

Following preliminary screening, four SSR primers (FOR1, FOR2, FOR5, and FOR9) originally developed by Huang et al. (2007) and five ISSR primers (SBS807, SBS810, SBS811, SBS812, and SBS835) derived from Chen et al. (2005) were selected for final amplification. Primer selection was based on amplification success and the production of clear, reproducible banding patterns across multiple accessions. Primers showing weak amplification, overlapping fragments, or unstable profiles during repeated testing were excluded. Primer sequences, annealing temperatures, and amplification characteristics are presented in Table 2.

PCR amplification was performed in a final reaction volume of 25 µL containing 12 µL GoTaq Green Master Mix (Promega, USA), primer(s) at a working concentration of 2.5 µM (single primer for ISSR and primer pair for SSR), 50 ng template DNA, and nuclease-free water. The optimized amplification protocol for both SSR and ISSR markers consisted of an initial denaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 50 min, annealing at primer-specific temperatures for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. Annealing temperatures were optimized for each primer by gradient PCR and ranged from 50°C to 54°C.

Table 1. *Selaginella* accessions used in molecular analysis, sampling codes, and geographic origin in Indonesia

Species	Accession/ herbarium code	Geographic origin
Selaginella sp. "Grinjani"	ADS 451	Mount Rinjani, Lombok, West Nusa Tenggara
S. plana	ADS 453	Bogor Botanical Garden, Bogor, West Java
S. plana	ADS 470	Mount Lawu, Tawangmangu, Karanganyar, Central Java
S. plana	ADS 408	Southern Serayu Mountains, Kaliwiro, Wonosobo, Central Java
S. plana	ADS 318	Southern Serayu Mountains, Erorejo, Wonosobo, Central Java
S. plana	ADS 432	Mount Merapi, Sawangan, Magelang, Central Java
S. plana	ADS 361	Southern Serayu Mountains, Selomerto, Wonosobo, Central Java
S. plana	ADS 456	CIFOR Bogor, West Java
S. plana	ADS 313	Southern Serayu Mountains, Wadaslintang, Wonosobo, Central Java
S. plana	ADS 325	Southern Serayu Mountains, Padureso, Kebumen, Central Java
S. plana	ADS 422	Mount Merapi, Turgo Hill, Sleman, Yogyakarta
S. ornata	ADS 339	Mount Sumbing, Kalikajar, Wonosobo
S. ornata	ADS 336	Mounts Merapi-Merbabu complexes, Selo, Boyolali, Central Java
S. ornata	ADS 357	Mount Sindoro, Tlogojati, Wonosobo, Central Java
S. involvens	ADS 423	Mount Merapi, Plawangan, Sleman, Yogyakarta
S. involvens	ADS 420	Mount Merapi, Deles, Klaten, Central Java
S. involvens	ADS 490	Mount Salak, Gunung Malang, Bogor, West Java
S. involvens	ADS 415	Mount Sumbing, Candimulyo, Wonosobo, Central Java
S. involvens	ADS 457	Batukaru Mountain, Tabanan, Bali
S. remotifolia	ADS 276	Mount Lawu, Cemorosewu, Magetan, East Java
S. remotifolia	ADS 261	Dieng Highlands, Karangtengah, Banjarnegara, Central Java
S. remotifolia	ADS 300	Mount Sindoro, Tlogojati, Wonosobo, Central Java
S. remotifolia	ADS 338	Mounts Merapi-Merbabu complexes, Selo, Boyolali, Central Java
S. opaca	ADS 266	Mount Lawu, Tawangmangu, Karanganyar, Central Java
S. repanda	ADS 48	Wuryantoro, Wonogiri, Central Java
S. willdenowii	ADS 393	IPB Bogor, West Java
S. rothertii	ADS 106	CIFOR Bogor, West Java
S. involvens	ADS 386	Mount Lawu, Tawangmangu, Karanganyar, Central Java
Selaginella sp. "Grinjani"	ADS 461	Mount Rinjani, Lombok, West Nusa Tenggara
Selaginella sp. "Grinjani"	ADS 462	Mount Rinjani, Lombok, West Nusa Tenggara
Selaginella sp.01 "Gmeja"	ADS 144	Mount Meja, Manokwari, West Papua
Selaginella sp.02 "Gmeja"	ADS 145	Mount Meja, Manokwari, West Papua

Table 2. SSR and ISSR primers selected for amplification of *Selaginella* accessions used in this study

Marker system	Primer code	Primer sequence (5'-3')	Annealing temperature (°C)
SSR	FOR1	F: AGCTTGGTGTTGATGAGG / R: CCTTCTTGCTGCTTCTTC	52
SSR	FOR2	F: TGGTGCTTGTAGGTGATG / R: CAGCTTCTTCACCTTCCT	53
SSR	FOR5	F: GGTAGTGCTGGTGTTGTT / R: TCCACTCTTCCTCTTCTC	54
SSR	FOR9	F: TGTGGTGATGAGGTGAGG / R: CCTCTTGCTCTTCCTCTT	53
ISSR	SBS807	(AG)8T	50
ISSR	SBS810	(GA)8T	50
ISSR	SBS811	(GA)8C	50
ISSR	SBS812	(GA)8A	50
ISSR	SBS835	(AG)8YC	52

Note: SSR primers followed Huang et al. (2007) and ISSR primers followed Chen et al. (2005). All primers were selected after preliminary screening for amplification clarity, reproducibility, and consistency. SSR markers generated locus-specific fragments, whereas ISSR markers produced multilocus dominant banding profiles. Marker performance statistics are presented in Table 3

Each amplification was performed in duplicate to verify reproducibility prior to scoring. Only stable and clearly resolved fragments that were consistently detected across duplicate amplifications were retained in the final binary matrix. Weak, diffuse, or inconsistently amplified bands were excluded from subsequent analyses. The resulting combined SSR-ISSR dataset was used for genetic similarity analysis, hierarchical clustering, principal component analysis, molecular variance analysis, and marker performance evaluation.

Electrophoresis and binary scoring of molecular loci

PCR products generated from SSR and ISSR markers were separated by agarose gel electrophoresis to visualize fragment patterns across all *Selaginella* accessions. Agarose concentration was adjusted according to the expected fragment size range, and electrophoresis was conducted under constant voltage until fragments were clearly resolved. Gels were stained and visualized under ultraviolet illumination prior to scoring (Green and Sambrook 2019).

A molecular size standard was included in each gel to ensure consistent fragment interpretation of fragment sizes across the samples amplified with the same primer. Fragment scoring emphasized positional consistency rather than precise fragment size, particularly for multilocus ISSR profiles (Powell et al. 1996; Weising et al. 2005).

Only clear, reproducible, and consistently amplified fragments were retained in the final binary matrix. Bands repeatedly detected at the same position were scored as present (1), whereas absent fragments were scored as absent (0). Weak, diffuse, or inconsistently amplified bands were excluded to avoid overestimating polymorphism due to amplification artifacts (Bornet and Branchard 2001).

Fragments generated by each primer were initially scored separately and subsequently combined into a single binary matrix. SSR fragments were treated as locus-specific characters, whereas ISSR fragments were analyzed as multilocus dominant markers. Because SSR fragments were scored as presence-absence bands rather than as allele-size variants, SSR loci were converted into binary data to enable direct integration with ISSR markers in subsequent analyses (Powell et al. 1996; Weising et al. 2005; Ng and Tan 2015). Each retained fragment was treated as a single binary variable and used for polymorphism analysis, genetic similarity estimation, hierarchical clustering, principal component analysis, and molecular variance partitioning (Ng and Tan 2015).

To minimize observer bias, gel images were examined repeatedly, and ambiguous loci were verified using original gel photographs and repeated amplification profiles. Only fragments that remained clear, reproducible, and positionally consistent across accessions were retained for analysis.

Molecular data analysis

Polymorphism and marker efficiency analysis

The combined SSR-ISSR binary matrix was used to evaluate molecular variation among the analyzed *Selaginella* accessions. Each clear and reproducible fragment was treated as a single locus, and only loci that consistently appeared across repeated amplifications were retained for further analysis. The total number of loci generated by each marker system, the number of polymorphic loci, and the proportional contribution of each marker type were subsequently determined. A locus was considered polymorphic when fragment presence at a given band position varied among accessions (Powell et al. 1996; Weising et al. 2005).

Marker productivity was additionally evaluated by calculating the average number of loci generated per primer. SSR and ISSR markers differ in amplification characteristics because SSR primers generally target a limited number of specific loci, whereas ISSR primers amplify multiple fragments distributed across repeated genomic regions (Bornet and Branchard 2001). Although a larger number of loci does not necessarily indicate higher analytical quality, greater locus production generally reflects broader genomic coverage. Marker informativeness was assessed using Polymorphism Information Content (PIC), calculated as $PIC = 2fi(1 - fi)$, where fi represents fragment frequency. Loci exhibiting intermediate fragment frequencies generally provide greater discriminatory power among accessions than loci that are either highly common or extremely rare (Ng and Tan 2015; Serrote et al. 2020; Abd-dada et al. 2023).

Resolving power (Rp) and Marker Index (MI) were also calculated to evaluate overall marker efficiency. Resolving power reflects the ability of a marker system to distinguish among accessions, whereas the marker index combines the level of polymorphisms and marker informativeness into a single estimate of analytical contribution (Akhtar et al. 2021; Abd-dada et al. 2023; Sahin et al. 2024). Summary parameters, including total loci, polymorphic loci, percentage polymorphism, mean loci per primer, PIC, Rp, and MI, were calculated separately for SSR and ISSR markers prior to clustering and multivariate analyses.

Genetic similarity and cluster analysis

Molecular similarity among *Selaginella* accessions was evaluated by comparing shared fragments within the combined SSR-ISSR binary matrix. All loci were scored as binary characters, with fragment presence recorded as 1 and fragment absence recorded as 0. Pairwise similarity coefficients were calculated using the Nei-Li coefficient, which is widely applied in dominant marker analyses because it gives greater weight to shared fragment presence than to shared absence, thereby reducing potential bias associated with fragment absence due to biological or technical factors (Nei and Li 1979). Similarity values ranged from 0 to 1, with higher values indicating stronger molecular resemblance among accessions (Powell et al. 1996).

The similarity matrix was initially examined to identify general molecular patterns among accessions, including levels of similarity within species, overlap among closely related taxa, and broader patterns of molecular separation. Hierarchical clustering analysis was subsequently performed using the Unweighted Pair-Group Method with Arithmetic mean (UPGMA), which is commonly employed for binary molecular marker datasets in plant studies. The resulting dendrogram was used to evaluate species-centered clustering patterns and to determine whether accessions originating from different geographic localities remained grouped within the same taxonomic units. Branch lengths were interpreted as indicators of relative molecular differentiation among accessions and species.

Principal component analysis

Principal Component Analysis (PCA) was used to visualize molecular relationships among *Selaginella* accessions without imposing a hierarchical branching structure. Unlike dendrogram analysis, PCA summarizes the main patterns of molecular variation in a reduced multivariate space. This approach allowed evaluation of whether the grouping patterns recovered in UPGMA remained consistent when no branching assumption was applied, which is why PCA is commonly used in marker-based plant studies (Ibrar et al. 2022; Bidyananda et al. 2024). The analysis was performed using the combined binary matrix, with each accession treated as a single unit and all loci contributing equally.

The analysis was based on covariance among loci. The first Principal Component (PC1) represented the largest source of molecular variation, whereas the Second Principal Component (PC2) represented the next largest component. Interpretation focused on these two axes because they summarized most of the molecular structure. The ordination pattern was evaluated by examining grouping compactness, species-centered distribution, and separation among taxa. Accessions positioned close together were interpreted as molecularly similar, whereas wider separation indicated stronger differentiation.

Particular attention was given to *S. plana* and *S. involvens* because both taxa were represented by multiple accessions from different geographic localities, allowing evaluation of internal dispersion. Compact clustering indicated consistent molecular composition despite geographic separation, whereas broader dispersion suggested detectable within-species variation.

Interpretation of single-accession taxa and materials from Lombok or Papua was conducted with greater caution because isolated ordination positions may reflect either genuine molecular divergence or limited sampling representation. PCA results were interpreted alongside the dendrogram, and accessions that were consistently grouped in both analyses were considered to exhibit stronger molecular coherence. Differences between PCA and clustering patterns were interpreted as indications that a single analytical method might not fully capture internal molecular variation.

Molecular variance and species differentiation

Analysis of Molecular Variance (AMOVA) was performed in GenAlEx using the combined SSR-ISSR dataset to evaluate the distribution of molecular variation within and among species. AMOVA is widely applied in dominant-marker studies because it partitions total molecular variation into hierarchical components based on predefined grouping factors (Abd-dada et al. 2023; Zhao et al. 2024). Each accession was assigned to its corresponding morphological species, and species identity was used as the grouping variable. The binary presence–absence matrix was converted into pairwise distance values in GenAlEx, after which total molecular variance was partitioned into among-species and within-species components.

Significance was tested using permutation procedures implemented in GenAlEx. PhiPT was used as the analog of fixation index for dominant binary markers. Values close to zero indicate weak differentiation, whereas larger values indicate stronger separation among groups (Peakall and Smouse 2012). Because the number of accessions differed among species, the interpretation focused on the overall variance structure rather than on direct population-level comparisons. Species represented by several accessions, *S. plana*, *S. involvens*, and *S. remotifolia*, contributed most strongly to within-species variance, whereas taxa represented by single accessions mainly influenced the among-species component.

Nei's genetic distance was also calculated in GenAlEx from the same binary matrix to estimate molecular divergence among taxa (Nei 1972). Pairwise accession-based distances were calculated and summarized at the species level to evaluate relative molecular affinity and differentiation among taxa. AMOVA and Nei distance were interpreted together because AMOVA quantified variance partitioning, whereas Nei distance provided direct measures of molecular divergence.

Geographic interpretation

A Mantel test was performed as a supplementary analysis to evaluate the relationship between geographic origin and molecular differentiation among *Selaginella* accessions (Mantel 1967; Peakall and Smouse 2012). Geographic origin was assigned according to documented source localities rather than cultivation sites because molecular identity is more closely associated with the source population than with the cultivation environment. For ex situ materials, original collection records were therefore retained as the geographic reference.

Geographic information was compiled from the original collection records of all accessions, including precise geographic coordinates recorded at the source localities. Sampling sites represented a range of environments, including mountain systems, spring-associated habitats, forest margins, and cultivated collections. Pairwise geographic distances among accessions were calculated from latitude and longitude coordinates and used to construct the geographic distance matrix for Mantel analysis. Accessions originating from nearby localities generally exhibited shorter geographic distances, whereas accessions from different islands or major biogeographic regions showed substantially greater spatial separation. Java served as the primary reference region, while Bali, Lombok, and Papua provided broader geographic contrasts for evaluating potential spatial influences on molecular differentiation (Ng and Tan 2015).

The significance of the association between geographic and molecular distance matrices was evaluated using permutation procedures implemented in GenAlEx. Interpretation of the Mantel results remained conservative because geographic origin and species identity were partially confounded in the dataset, such that molecular differentiation could reflect both taxonomic divergence and geographic separation. Consequently, Mantel results were interpreted in conjunction with UPGMA clustering, PCA ordination, and Nei genetic distance analyses, particularly for accessions from Lombok and Papua, which occupied more peripheral positions in the molecular analyses.

RESULTS AND DISCUSSION

Marker polymorphism and efficiency of SSR and ISSR systems

The combined SSR and ISSR analysis generated 122 scorable loci across 32 *Selaginella* accessions, all of which were polymorphic under the binary scoring criteria applied in this study. Four SSR primers generated 43 loci, whereas five ISSR primers produced 79 loci, indicating a greater multilocus contribution from the ISSR marker. Average locus productivity was also higher for ISSR (15.8 loci per primer) than for SSR (10.75 loci per primer), demonstrating broader fragment coverage under the amplification conditions employed. SSR and ISSR markers produced distinct amplification profiles. SSR markers generally generated fewer locus-specific fragments, whereas ISSR markers produced larger numbers of multilocus bands, resulting in broader genomic coverage and higher locus productivity.

ISSR markers contributed 64.8% of all polymorphic loci, compared with 35.2% for SSR markers (Table 3). All retained loci were polymorphic, resulting in 100% polymorphism for the selected primer set and demonstrating the effectiveness of the chosen primers for detecting molecular variation across both widespread and geographically restricted taxa.

A similar trend was observed for marker informativeness. Mean Polymorphism Information Content (PIC) values were 0.141 for SSR markers and 0.303 for ISSR markers, indicating higher discriminatory power for ISSR-derived fragments. Resolving power (Rp) also differed substantially, with values of 7.111 for SSR and 34.549 for ISSR markers. Likewise, Marker Index (MI) values were 6.063 and 23.937, respectively, indicating greater overall efficiency of ISSR markers in detecting molecular variation.

SSR markers provided stable locus-specific information, whereas ISSR markers contributed a larger proportion of multilocus variation and discriminatory power. Their combined application therefore provided complementary molecular information for assessing genetic similarity, clustering analysis, and species-level differentiation among the analyzed *Selaginella* taxa.

Genetic similarity and hierarchical clustering among *Selaginella* accessions

Pairwise genetic similarity coefficients calculated from the combined SSR and ISSR binary matrix ranged from 0.42 to 0.93, with a mean value of 0.68 (Table 4). These values indicate moderate to high levels of molecular similarity among the analyzed accessions. Although most accessions retained substantial molecular affinity, the observed variation in similarity coefficients suggests the presence of molecular differentiation associated with species boundaries and taxonomic identity.

Table 3. Polymorphism summary and marker efficiency generated by SSR and ISSR markers in Javanese *Selaginella*

Marker system	Number of primers	Number of retained loci	Polymorphic loci	Percentage of polymorphism (%)	Mean PIC	Resolving power (Rp)	Marker Index (MI)
SSR	4	43	43	100	0.141	7.111	6.063
ISSR	5	79	79	100	0.303	34.549	23.937
Combined SSR+ISSR	9	122	122	100	0.246	41.660	30.000

Note: PIC: Polymorphism Information Content, Rp: Resolving power, MI: Marker Index

Table 4. Range of genetic similarity among *Selaginella* accessions based on combined SSR and ISSR markers

Parameter	Value
Number of accessions analyzed	32
Total polymorphic loci	122
Minimum similarity coefficient	0.42
Maximum similarity coefficient	0.93
Mean similarity coefficient	0.68

Note: Similarity coefficients were calculated using combined binary data from SSR and ISSR markers based on Nei-Li similarity

Hierarchical clustering based on Nei-Li similarity coefficients separated the 32 accessions into two principal groups (A and B), each comprising two subordinate clusters (C-D and E-F, respectively) (Figure 1). Cluster C was dominated by *S. plana* accessions and also included one accession of *Selaginella* sp. "Grinjani" (ADS 451), whereas cluster D contained all *S. ornata* accessions together with several accessions of *S. involvens*. Cluster E comprised the four accessions of *S. remotifolia* together with *S. opaca*, *S. repanda*, *S. willdenowii*, *S. rothertii*, and one accession of *S. involvens* (ADS 386). Cluster F contained two additional accessions of *Selaginella* sp. "Grinjani" (ADS 461 and ADS 462) and the two Papuan accessions, *Selaginella* sp.01 "Gmeja" and *Selaginella* sp.02 "Gmeja".

The clustering pattern indicates that molecular relationships were associated primarily with taxonomic identity rather than geographic origin. Accessions of *S. plana*, *S. ornata*, and *S. remotifolia* consistently formed species-centered groups despite originating from different localities. In contrast, *S. involvens* exhibited broader molecular dispersion, with its accessions distributed across more than one cluster. Nevertheless, most *S. involvens* accessions remained associated within neighboring branches, indicating moderate intraspecific differentiation while retaining overall molecular affinity. Taxa represented by single accessions, including *S. opaca*, *S. repanda*, *S. willdenowii*, and *S. rothertii*, occupied relatively isolated positions within the dendrogram, whereas *Selaginella* sp.01 "Gmeja", Selaginella sp.02 "Gmeja", and two accessions of *Selaginella* sp. "Grinjani" formed the distinct cluster F. These patterns indicate greater molecular differentiation relative to the dominant multi-accession Javanese taxa.

Higher similarity values were generally associated with accessions belonging to the same widespread species, whereas lower similarity values occurred among taxonomically distinct or geographically isolated taxa. The combined SSR and ISSR dataset provided sufficient molecular resolution to distinguish the principal *Selaginella* taxa and to characterize hierarchical molecular relationships among accessions.

Principal component analysis and species-level molecular grouping

Principal Component Analysis (PCA) based on the combined SSR and ISSR binary matrix revealed clear multivariate separation among the major *Selaginella* taxa, although the degree of separation varied among species (Figure 2). The first two Principal Components (PC1 and PC2) explained 24.7% and 16.1% of the total molecular variation, respectively, accounting for 40.8% of the cumulative variation in the ordination space. PC1 primarily separated widespread taxa from more isolated accessions, whereas PC2 reflected additional variation associated with intraspecific dispersion. Overall, the ordination pattern was broadly consistent with the clustering structure recovered in the dendrogram.

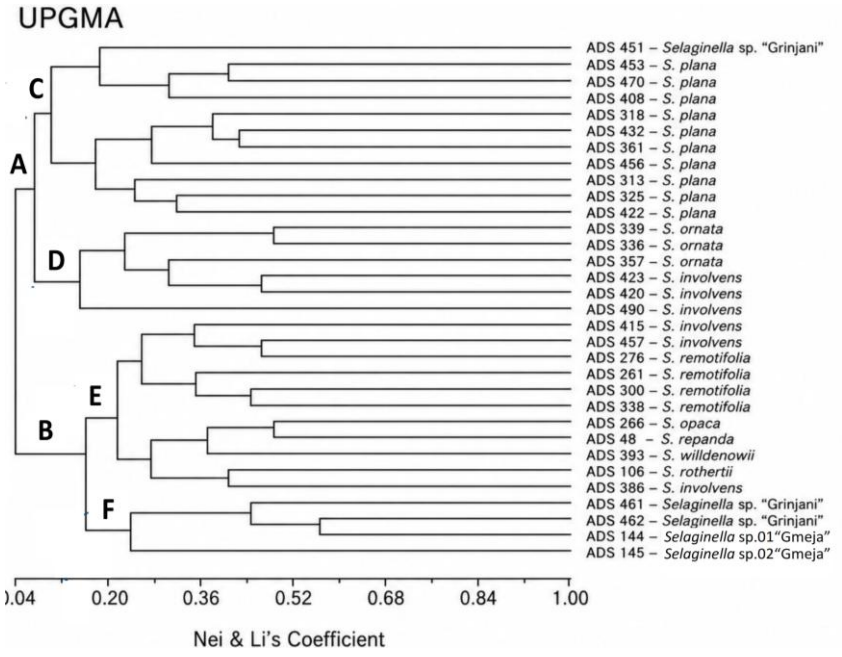

Figure 1. UPGMA dendrogram illustrating genetic relationships among 32 accessions of *Selaginella* inferred from combined SSR and ISSR markers. The dendrogram is divided into two major clusters (A and B), each containing two subordinate clusters (C-D and E-F, respectively)

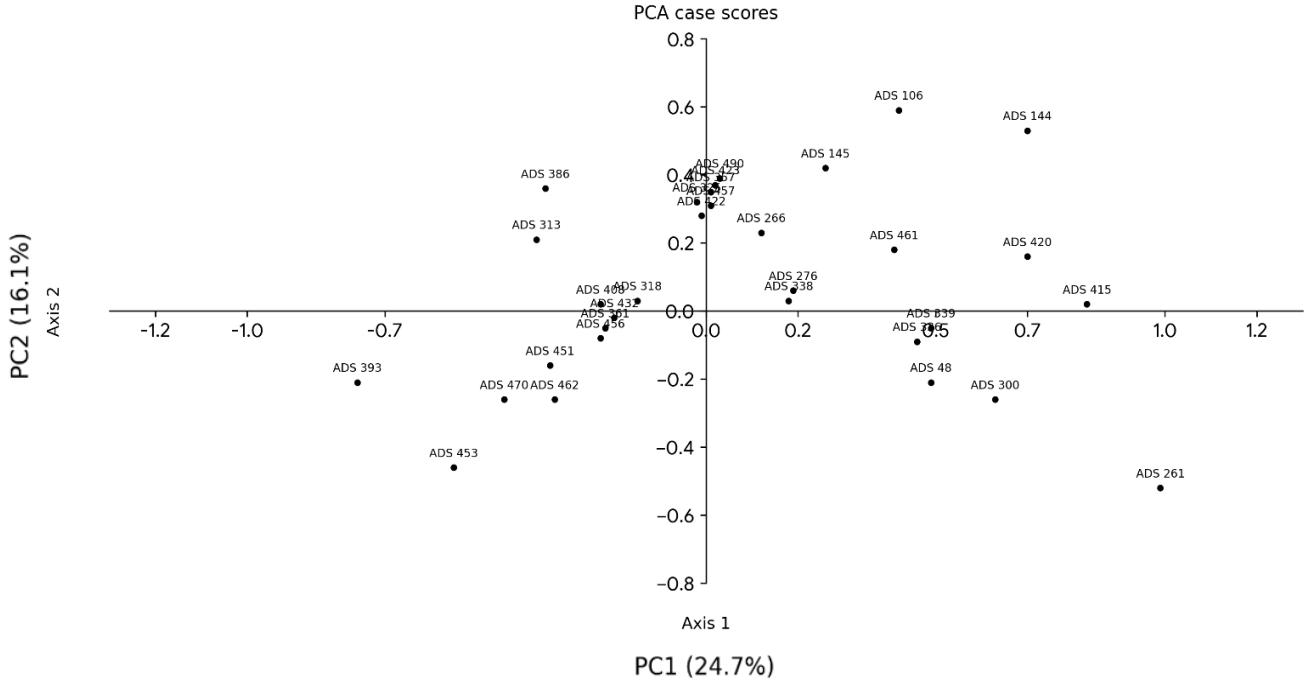

Figure 2. Principal Component Analysis (PCA) of 32 *Selaginella* accessions based on combined SSR and ISSR markers. The first two Principal Components (PC1 and PC2) explained 24.7% and 16.1% of the total molecular variation, respectively. Labels (e.g., ADS 451, ADS 453) represent accession/herbarium codes corresponding to the samples listed in Table 1

Selaginella plana occupied the broadest region of the ordination space. Most accessions formed a species-centered cluster, although moderate dispersion along both axes indicated detectable intraspecific molecular variation among accessions from Bogor, Tawangmangu, Klaten, Wonosobo, Kebumen, and Mount Merapi. Despite this variation, *S. plana* remained distinct from most other taxa and retained clear species-level cohesion.

A second major group corresponded to *S. involvens*. Its accessions occupied a neighboring but distinct region and remained relatively compact despite their broad geographic origins. Although positioned relatively close to *S. plana*, consistent with their comparatively low Nei genetic distance, complete overlap was not observed, indicating that both taxa retained distinct molecular identities.

Selaginella remotifolia and *S. ornata* occupied intermediate ordination positions and formed smaller species-centered clusters. Their accessions remained separated from the two dominant widespread taxa, suggesting moderate molecular affinity while maintaining clear species-level differentiation.

Taxa represented by fewer accessions, including *Selaginella* sp. "Grinjani", *S. opaca*, *S. repanda*, *S. willdenowii*, *S. rothertii*, *Selaginella* sp.01 "Gmeja", and *Selaginella* sp.02 "Gmeja", occupied more isolated positions in the ordination space. These placements corresponded to their longer external branches in the dendrogram and indicate greater molecular differentiation relative to the dominant Javanese taxa. Overall, species identity appeared to be the primary factor structuring the ordination pattern, whereas broader intraspecific dispersion was most evident in widespread taxa such as *S. plana* and *S. involvens*.

Molecular variance partitioning among species

Analysis of Molecular Variance (AMOVA) based on the combined SSR and ISSR dataset revealed that most molecular variation was distributed within species rather than among species (Table 5). Of the total molecular variation detected, 80.1% occurred within species, whereas 19.9% was attributable to differences among species. The observed PhiPT value (0.199; P = 0.001) indicated moderate but statistically significant genetic differentiation among the analyzed *Selaginella* taxa.

Table 5. Analysis of Molecular Variance (AMOVA) based on combined SSR and ISSR markers among *Selaginella* species

Source of variation	df	Sum of squares	Mean square	Estimated variance	Variation (%)
Among species	10	182.6	18.26	4.12	19.9
Within species	21	348.9	16.61	16.56	80.1
Total	31	531.5		20.68	100

Note: Additional statistics: PhiPT = 0.199, P = 0.001. AMOVA was performed using 122 polymorphic loci derived from combined SSR and ISSR binary data across 32 accessions representing eight recognized *Selaginella* species and three unidentified taxonomic entities

The predominance of within-species variation indicates that geographically separated accessions belonging to the same species retained substantial levels of molecular diversity. This pattern was particularly evident in species represented by multiple accessions, including *S. plana*, *S. involvens*, and *S. remotifolia*, which were sampled from different ecological regions across Java and adjacent islands. The observed intraspecific variation may reflect the influence of geographic separation, environmental heterogeneity, and historical population processes within these widespread taxa.

Although the proportion of variation among species was lower than that within species, the significant PhiPT value indicates that species identity consistently contributed to the overall molecular structure. In molecular marker studies, differentiation of this magnitude is generally interpreted as evidence that taxa remain genetically distinguishable despite substantial intraspecific variation. This interpretation is supported by both the UPGMA dendrogram and PCA ordination, which consistently recovered species-centered groupings among the analyzed accessions.

Nei's genetic distance analysis provided additional information on patterns of molecular divergence among taxa (Table 6). Several widespread Javanese species exhibited moderate genetic distance values, indicating partial molecular affinity while maintaining distinct species boundaries. For example, genetic distances were relatively low between *S. remotifolia* and *S. repanda* (D = 0.523), *S. remotifolia* and *S. opaca* (D = 0.591), *Selaginella* sp.02 "Gmeja" and *S. rothertii* (D = 0.625), and *Selaginella* sp.02 "Gmeja" and *Selaginella* sp.01 "Gmeja" (D = 0.625). These values suggest moderate molecular relatedness despite clear taxonomic separation. In contrast, higher genetic distance values were observed among more divergent taxa, particularly between *S. rothertii* and *S. plana* (D = 0.960), followed by *S. willdenowii* and *S. involvens* (D = 0.923), *S. willdenowii* and *S. rothertii* (D = 0.912), and *S. willdenowii* and *Selaginella* sp.01 "Gmeja" (D = 0.904). These elevated divergence values corresponded with their isolated positions in the dendrogram and their peripheral placement in PCA ordination space, indicating stronger molecular differentiation relative to the dominant Javanese taxa.

Taken together, the AMOVA and Nei genetic distance analyses indicate that the combined SSR and ISSR marker system effectively captured substantial intraspecific diversity while resolving significant species-level differentiation among the analyzed *Selaginella* taxa. The congruence among variance partitioning, genetic distance estimates, hierarchical clustering, and ordination analyses supports the molecular distinctiveness of the recognized species.

Geographic signal in molecular differentiation

A Mantel test was performed to evaluate the potential influence of geographic origin on molecular differentiation. The analysis revealed a weak but statistically significant positive association between geographic and molecular distances (r = 0.287, P = 0.041; Table 7), indicating that molecular divergence increased only slightly with geographic separation among accessions. Consequently, geographic origin alone did not adequately explain the observed molecular structure. Within the multispecies dataset analyzed, molecular variation was more strongly associated with species identity than with collection locality.

Table 6. Nei's genetic distance matrix among major *Selaginella* species based on combined SSR and ISSR markers

Species	***S. plana***	***S. involvens***	***S. remotifolia***	***S. ornata***	***S. opaca***	***S. repanda***	***S. willdenowii***	***S. rothertii***	***Selaginella* sp.01 " Gmeja"**	***Selaginella* sp. " Grinjani"**	***Selaginella* sp.02 " Gmeja"**
S. plana	0.000										
S. involvens	0.817	0.000									
S. remotifolia	0.686	0.658	0.000								
S. ornata	0.832	0.763	0.700	0.000							
S. opaca	0.723	0.661	0.591	0.731	0.000						
S. repanda	0.790	0.726	0.523	0.787	0.714	0.000					
S. willdenowii	0.878	**0.923**	0.846	0.818	0.895	0.692	0.000				
S. rothertii	**0.960**	0.734	0.797	0.814	0.667	0.784	0.912	0.000			
Selaginella sp.01 "Gmeja"	0.815	0.712	0.730	0.797	0.647	0.811	**0.904**	0.652	0.000		
Selaginella sp. "Grinjani"	0.774	0.768	0.724	0.756	0.698	0.722	0.806	0.749	0.702	0.000	
Selaginella sp.02 "Gmeja"	0.854	0.648	0.652	0.703	0.704	0.744	0.778	0.625	0.625	0.792	0.000

Note: Values represent average species-level Nei genetic distance calculated in GenAlEx from combined binary SSR and ISSR loci, using species means derived from accession-level pairwise comparisons. Bold values indicate the three highest genetic distances among species

Table 7. Mantel test summary between genetic distance and geographic origin of *Selaginella* accessions

Parameter	Value
Number of accessions analyzed	32
Genetic distance matrix	Nei-Li coefficient
Geographic grouping basis	Sampling locality
Mantel correlation coefficient (r)	0.287
Probability (P)	0.041

Note: Geographic categories were assigned according to original collection localities or ex situ source records when collection and cultivation origins differed

A similar pattern was observed in several widespread taxa. Accessions of *S. plana* from Bogor, Karanganyar, Klaten, Kebumen, Sleman, and Wonosobo remained grouped within a common molecular cluster despite substantial geographic separation. Likewise, accessions of *S. involvens* from Java, Sukabumi, and Bali occupied a relatively coherent species-centered position. These results suggest that species identity contributed more strongly to the observed molecular structure than geographic separation among localities.

The PCA ordination (Figure 2) further supported this interpretation. Accessions belonging to the same species occupied similar regions of the ordination space regardless of geographic origin. Although broader dispersion was evident in some widespread taxa, such variation remained within species boundaries and did not result in the formation of distinct molecular groups. Geographic influence was detectable but relatively weak, becoming most evident among accessions originating outside Java. *Selaginella* sp. "Grinjani" from Lombok occupied a distinct ordination position, whereas *Selaginella* sp.01 "Gmeja" and *Selaginella* sp.02 "Gmeja" from Manokwari occurred in more peripheral positions characterized by longer dendrogram branches and higher Nei genetic distances relative to the dominant Javanese taxa.

However, interpretation of these patterns requires caution because the non-Javanese accessions also represent distinct taxonomic entities. Consequently, the relative contributions of geographic isolation and taxonomic differentiation cannot be fully disentangled within the present sampling design. Although the Mantel analysis detected a weak but statistically significant association between geographic and molecular distances ($r = 0.287$, $P = 0.041$), the overall molecular structure appeared to be influenced more strongly by species identity than by geographic origin. Within Java, geographically proximate localities did not consistently exhibit the highest similarity values, whereas geographically distant populations frequently remained molecularly similar when assigned to the same species. These findings suggest that habitat continuity, dispersal history, and species-level genomic cohesion contributed substantially to the observed molecular structure. Geographic origin appears to represent a secondary source of molecular variation, whereas species identity remains the primary determinant of molecular differentiation among the analyzed *Selaginella* taxa.

Discussion

Comparative performance of SSR and ISSR markers in resolving Selaginella diversity

In this dataset, ISSR markers contributed most of the polymorphic information recovered across the analyzed *Selaginella* accessions. Of the 122 polymorphic loci scored from the combined matrix, 79 were generated by ISSR primers, whereas SSR primers contributed 43 loci (Table 3). ISSR markers also showed higher values of polymorphism information content, resolving power, and marker index, indicating that multilocus amplification captured broader variation than the locus-specific SSR set used in this study.

This difference reflects the contrasting genomic targets of the two marker systems. ISSR primers anneal to repeated microsatellite motifs distributed throughout the genome and can therefore amplify multiple loci simultaneously, whereas SSR primers depend on conserved flanking regions surrounding specific microsatellite loci (Bornet and Branchard 2001; Powell et al. 1996). Consequently, ISSR generated more polymorphic fragments, while SSR produced fewer but highly reproducible loci that improved confidence in fragment scoring.

Similar trends have been reported in other pteridophytes, where ISSR markers often reveal broader polymorphism and provide efficient discrimination among accessions, particularly when species-specific marker development is unavailable (Animasaun et al. 2018; Vidyashree et al. 2019; Mathur et al. 2021). A recent ISSR study of *Selaginella ciliaris* from Indonesia likewise demonstrated complete polymorphism and substantial multilocus genetic variability, further supporting the effectiveness of ISSR markers for molecular characterization in tropical *Selaginella* (Jafron et al. 2025). The pattern observed here is therefore consistent with the broader performance of ISSR markers in non-model plant groups.

The distinction is particularly relevant in *Selaginella*, where most previous molecular studies relied primarily on plastid markers such as *rbcL*, *trnL-F*, and *matK* (Weststrand and Korall 2016a, b). Although these loci are valuable for reconstructing deeper evolutionary relationships, they often provide limited resolution for recent divergence among closely related accessions. In contrast, the combined nuclear marker dataset used here detected variation even among accessions assigned to the same morphological species.

Despite their lower polymorphism, SSR markers played an important complementary role. Many SSR fragments were consistently shared within major species groups, and their congruence with ISSR-based clustering and PCA patterns strengthened confidence in species-level differentiation. Together, ISSR and SSR markers provided a practical and reliable framework for assessing genetic diversity and molecular relationships in tropical *Selaginella*, combining broad genomic coverage with stable locus-specific information.

Species-level molecular cohesion and internal diversity across widespread taxa

The combined molecular dataset indicates that several widespread *Selaginella* species maintained clear species-centered molecular cohesion while exhibiting measurable internal diversity. AMOVA showed that 80.1% of total molecular variation occurred within species (Table 5), indicating that most molecular differences were distributed among conspecific accessions. This pattern was consistent in both clustering and ordination analyses, where widespread taxa remained grouped despite moderate internal dispersion. Similar coexistence of species cohesion and internal diversity has been reported in other widespread pteridophytes, where ecological flexibility promotes gradual differentiation without obscuring taxonomic boundaries (Mossion et al. 2022; Chao et al. 2024; Wei and Zhang 2022).

Among the analyzed taxa, *S. plana* exhibited the broadest internal molecular dispersion. Accessions from Bogor, Karanganyar, Klaten, Wonosobo, Kebumen, and Mount Merapi remained associated within a single major cluster but displayed evident internal substructure. This pattern suggests that geographically separated populations accumulated multilocus differences while retaining overall molecular cohesion, a trend commonly associated with environmental heterogeneity among habitats.

Selaginella involvens showed a similar but less pronounced pattern. Accessions from Java and Bali remained grouped within a distinct molecular cluster despite moderate internal variation. Such patterns are common in widespread spore-dispersed plants, where local differentiation occurs while long-distance dispersal maintains broader genetic connectivity (Mathur et al. 2021; Pelosi and Sessa 2021).

The SSR-ISSR dataset also revealed levels of internal molecular structure that may be less apparent in plastid-based studies of *Selaginella*. Previous analyses using *rbcL* and *trnL-F* frequently reported broad intraspecific stability because plastid loci generally evolve more slowly (Weststrand and Korall 2016a, b; Zhou et al. 2016). In contrast, the multilocus nuclear markers used here appeared more sensitive to recent differentiation among geographically separated accessions.

The pattern was less evident in taxa represented by single accessions, including *S. opaca, S. repanda*, and *S. willdenowii*, because internal variation could not be evaluated directly. Nevertheless, the similarity matrix (Table 4) indicates that widespread taxa contributed disproportionately to the within-species molecular variation detected in this study, consistent with observations from other ecologically widespread fern lineages (Mathur et al. 2021; Pelosi and Sessa 2021).

Molecular differentiation and taxonomic implications among Javanese Selaginella

The combined SSR and ISSR matrix generally supports the morphological delimitation of the principal *Selaginella* taxa. Most taxa formed species-centered clusters in both the UPGMA dendrogram and PCA ordination, despite substantial within-species variation (80.1%) relative to among-species variation (19.9%). This concordance between molecular and morphological evidence is consistent with recent AFLP analyses of Indonesian *Selaginella*, which likewise showed that multilocus markers generally support species recognition despite partial incongruence among some taxa (Setyawan et al. 2025). Such agreement is particularly important in *Selaginella*, where phenotypic plasticity often complicates species identification (Setyawan 2011; Weststrand and Korall 2016a, b).

The among-species component is noteworthy because several characters traditionally used in *Selaginella* taxonomy are influenced by environmental conditions. Branch orientation, leaf overlap, dorsal–ventral asymmetry, and strobilus development may vary with moisture, canopy cover, and substrate characteristics, making identification difficult when reproductive structures are absent. Nevertheless, morphologically distinct taxa generally retained distinguishable molecular positions even when ecological settings overlapped. Similar observations have been reported in other lycophytes and ferns, where multilocus markers provide complementary evidence for species recognition when vegetative morphology alone is insufficient (Zhang et al. 2021; Zhou and Zhang 2023; Shalimov et al. 2024).

Nei's genetic distance further demonstrated that molecular differentiation was not uniform across taxa. Several Javanese species exhibited moderate genetic distances (Table 6), indicating partial affinity while remaining separated in both clustering and ordination analyses. *S. plana* and *S. involvens* occupied neighboring regions in PCA space but remained associated with separate species-centered clusters in the dendrogram. A comparable pattern was observed between *S. ornata* and *S. remotifolia*, indicating that ecological similarity and geographic overlap did not necessarily eliminate molecular differentiation among morphologically recognized taxa.

The case of *S. ornata* is particularly informative because this species may exhibit intermediate vegetative morphology when reproductive structures are absent. Nevertheless, it retained a distinct molecular position in both clustering and ordination analyses, suggesting that species-level differentiation was maintained despite vegetative plasticity. Likewise, *S. remotifolia* remained associated within a distinct molecular group despite moderate variation among accessions from different mountain systems.

A stronger contrast was observed in *Selaginella* sp. “Grinjani”. The Lombok accessions occupied positions outside the principal Javanese groups and exhibited relatively high Nei distance values from most taxa (Table 6). Their separation in both dendrogram and PCA analyses suggests substantial molecular differentiation that may reflect both taxonomic distinctiveness and geographic isolation. Other taxa represented by single accessions, including *S. opaca, S. repanda, S. willdenowii*, and *S. rothertii*, also occupied distinct molecular positions, although their interpretation remains limited by restricted sampling.

These findings demonstrate that multilocus molecular markers provide valuable complementary evidence for evaluating species boundaries in Indonesian *Selaginella*. Although molecular variation within species remained substantial, the overall congruence among morphology, clustering patterns, ordination results, and genetic distance estimates supports the recognition of the principal taxa included in this study. Additional sampling from underrepresented regions and taxa will be necessary to evaluate broader patterns of molecular diversity and clarify potentially distinct lineages outside Java.

Geographic influence and limits of spatial genetic interpretation

The Mantel analysis indicated a weak but statistically significant positive association between geographic origin and molecular distance among the analyzed *Selaginella* accessions, with a correlation coefficient of 0.287 ($P = 0.041$). This suggests that spatial separation explained only a limited portion of the molecular variation recovered in the combined marker matrix. Several accessions from distant localities occupied adjacent positions in PCA space, whereas some samples from relatively similar regions remained separated because they belonged to different taxa. Consequently, the ordination pattern followed species identity more consistently than geographic proximity.

Part of this weak signal reflects the structure of the dataset. Mantel analysis is generally more informative when applied within a single species represented by multiple populations, allowing direct comparison between geographic distance and intraspecific molecular variation (Peakall and Smouse 2012). In the present study, multiple species were analyzed simultaneously, and interspecific divergence contributed more strongly than locality-based differences, thereby obscuring potential geographic trends.

The dispersal biology of *Selaginella* may also contribute to the limited geographic structure observed. Like many lycophytes and ferns, *Selaginella* produces minute spores capable of long-distance dispersal through air currents, reducing strict isolation-by-distance patterns (Barrington 1993). This was evident in *S. plana* and *S. involvens*, whose geographically dispersed accessions remained grouped within coherent molecular clusters despite moderate internal variation. Similar patterns have been reported in other widespread fern species, where long-distance dispersal maintains genetic cohesion across broad habitat ranges (Vidyashree et al. 2019; Mathur et al. 2021).

Geographic contrast became more apparent outside the principal Javanese sampling range. *Selaginella* sp. "Grinjani" from Lombok, together with *Selaginella* sp.01 "Gmeja" and *Selaginella* sp.02 "Gmeja" from Manokwari, occupied more isolated positions in both ordination and clustering analyses and exhibited relatively high Nei distance values. However, because these accessions also represent distinct taxa, their molecular separation cannot be attributed solely to geography. Taxonomic distinctiveness and regional origin are therefore partially confounded in the present dataset.

Interpretation of spatial influence is further constrained because some materials originated from cultivated collections rather than directly from natural populations. Such accessions retain the genomic identity of their source populations, whereas cultivation locality does not necessarily reflect evolutionary origin. Overall, geographic influence appears to represent a secondary source of molecular variation, while species identity, dispersal history, and source lineage exerted stronger effects on the observed molecular structure (Xiao et al. 2021; Volis 2023).

Methodological implications and future molecular directions for tropical Selaginella research

In the present dataset, the combined SSR and ISSR markers effectively separated most analyzed *Selaginella* taxa while capturing internal variation within widespread species. SSR fragments remained stable across repeated amplifications and supported species-level grouping, whereas ISSR markers generated most polymorphic loci and provided greater resolution of within-species variation. This complementary performance makes the marker combination practical for exploratory molecular studies of tropical lycophytes, particularly when genomic resources are limited and multiple taxa are analyzed simultaneously. Together with recent AFLP- and ISSR-based studies on Indonesian *Selaginella* (Setyawan et al. 2025; Jafron et al. 2025), the present findings demonstrate that multilocus fingerprinting approaches provide an effective molecular framework for assessing genetic diversity and species differentiation in tropical lycophytes prior to more comprehensive sequence-based or genomic investigations.

Future studies would benefit from integrating plastid regions such as *rbcL*, *trnL-F*, and *matK*, which remain useful for evaluating deeper evolutionary relationships and lineage continuity across islands and mountain systems. Higher-resolution approaches, including SNP-based analyses and targeted low-copy nuclear sequencing, could further improve the detection of recent divergence, hidden lineage structure, and cryptic taxa that may remain unresolved using dominant multilocus markers alone (Breinholt et al. 2021; Pezzini et al. 2023; Zhao et al. 2024).

Several limitations should also be acknowledged. Some taxa were represented by only a single accession, restricting assessment of within-species variation and geographic structure. In addition, the use of dominant multilocus markers without sequence-based validation limits phylogenetic inference and fine-scale lineage resolution. Addressing these limitations through broader sampling and higher-resolution molecular approaches will strengthen future taxonomic, phylogeographic, and conservation studies of Indonesian *Selaginella*.

In conclusion, the combined SSR and ISSR marker system effectively characterized molecular diversity and differentiation among Javanese *Selaginella*. A total of 122 polymorphic loci were generated, with ISSR markers providing greater polymorphism and marker efficiency, while SSR markers contributed stable locus-specific information. Clustering, PCA, and AMOVA consistently indicated that most morphologically recognized taxa occupied distinct molecular positions despite substantial

within-species variation. The predominance of molecular variation within species (80.1%) demonstrates considerable internal diversity, particularly in widespread taxa such as *S. plana* and *S. involvens*, whereas significant among-species differentiation (19.9%) supports their overall molecular distinctiveness. Accessions from Lombok and Papua occupied more isolated molecular positions and exhibited greater genetic divergence relative to the dominant Javanese taxa, suggesting the presence of broader regional differentiation within Indonesian *Selaginella*. Overall, the concordance between molecular and morphological evidence supports the current recognition of the principal taxa examined in this study. These findings provide a useful foundation for future taxonomic, phylogeographic, and conservation research, particularly through expanded sampling across the Indonesian archipelago and the application of higher-resolution molecular markers.

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