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Short Communication: Exploratory AFLP-based genetic structuring among selected *Selaginella* species from Indonesia

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Abstract. *Setyawan AD, Chikmawati T, Miftahudin, Sutarno, Sugiyarto, Sunarto. 2025. Short Communication: Exploratory AFLP-based genetic structuring among selected* Selaginella *species from Indonesia. Nusantara Bioscience 17 (2): 375-386. Selaginella* is one of the oldest groups of living vascular plants and helps us understand how early land plants evolved and diversified. Despite its ecological and morphological diversity, molecular data remain limited for many tropical species. This study presents an exploratory assessment of multilocus genetic structuring among selected *Selaginella* species using Amplified Fragment Length Polymorphism (AFLP) markers. AFLP profiles were generated using four selective primer combinations, producing a total of 248 polymorphic fragments. Genetic similarity among species was quantified using Jaccard's coefficient, and patterns of multilocus resemblance were examined through UPGMA clustering and principal coordinate analysis (PCoA). Pairwise similarity values ranged from 0.59 to 1.00, indicating a continuum of genetic differentiation rather than sharply delimited genetic groups. UPGMA clustering and PCoA revealed two broad species assemblages and substantial variation in multilocus genetic similarity among the analyzed taxa. The clustering pattern showed partial correspondence with major growth-form categories, with predominantly creeping to ascending species grouped separately from taxa exhibiting a cypress-like habit together with *S. ornata*. At the same time, several morphologically distinct species exhibited relatively high AFLP similarity, highlighting both congruence and incongruence between molecular and morphological patterns. Because the final comparative analyses were based on representative AFLP profiles for each species, the results should be regarded as an exploratory overview of interspecific multilocus differentiation rather than an assessment of intraspecific variation. Although AFLP markers do not permit direct phylogenetic inference, the findings provide a useful molecular baseline for future studies employing broader sampling and sequence-based or genomic approaches. Our findings demonstrate the value of integrating multilocus AFLP data with morphological evidence to better understand genetic structuring and species relationships in *Selaginella*. Although exploratory in nature, this study provides a useful molecular baseline for future sequence-based and genomic investigations.

Keywords: AFLP, genetic structuring, plant systematics, *Selaginella*, vascular plants

INTRODUCTION

Selaginella Pal. Beauv. (Selaginellaceae) represents one of the earliest-diverging lineages of extant vascular plants, occupying a pivotal phylogenetic position between bryophytes and euphyllophytes. *Selaginella* belongs to the lycophytes, a group that split off early from other vascular plants and still keeps many ancient features (Kenrick and Crane 1997; Pryer et al. 2001). These features include microphyllous leaves, unique strobilar organization, and specialized reproductive strategies, making *Selaginella* a key taxon for understanding the origin and early diversification of vascular plant body plans.

Beyond its evolutionary significance, *Selaginella* is also taxonomically diverse, comprising more than 700 described species distributed primarily in tropical and subtropical regions, with centers of diversity in Southeast Asia, the Neotropics, and Africa (Jermy 1990; Zhang et al. 2013). Species in this genus vary widely in shape, leaf arrangement, and habitat. Some creep in wet areas, while others tolerate drying out and live in seasonally dry places. This diversity has long attracted the attention of systematists, but it has also complicated species delimitation and infrageneric classification. Consequently, *Selaginella* provides an informative model for examining how early-diverging vascular plant lineages diversify and how morphological evolution relates to underlying genetic structure.

Traditional classification of *Selaginella* has relied heavily on morphological characters, particularly leaf morphology, strobilus structure, and branching patterns (Weststrand and Korall 2016a; Setyawan 2011). However, many of these traits are subject to ecological plasticity and convergent evolution, which can obscure phylogenetic signal and lead to taxonomic ambiguity. As a result, purely morphology-based approaches have proven insufficient to fully resolve relationships within the genus, especially among closely related or morphologically similar species.

Molecular data have increasingly been employed to address these limitations, primarily through chloroplast DNA sequences and, more recently, transcriptomic and genomic datasets (Pryer et al. 2004; Weststrand and Korall 2016b). While these studies have substantially improved our understanding of higher-level relationships within lycophytes, molecular sampling across *Selaginella* remains uneven and geographically biased. Many tropical species, particularly from Southeast Asia, remain underrepresented in molecular phylogenetic analyses, and comparative studies involving multiple species or populations are still limited. Moreover, most available molecular investigations rely on sequence-based markers, whereas multilocus fingerprinting datasets remain comparatively scarce. This gap restricts our ability to evaluate patterns of genetic differentiation and similarity across morphologically and ecologically diverse *Selaginella* taxa.

In this context, Amplified Fragment Length Polymorphism (AFLP) provides a useful exploratory tool for assessing genetic variation among multiple *Selaginella* species. AFLP markers generate multilocus fingerprints without prior sequence information and have been widely applied in studies of genetic diversity, species delimitation, and population structure, particularly in non-model and taxonomically challenging plant groups (Vos et al. 1995; Mueller and Wolfenbarger 1999). Although AFLP markers are dominant and do not permit direct estimation of allele frequencies, their high reproducibility and broad genomic representation make them suitable for detecting overall patterns of genetic similarity and differentiation.

The present study adopts an exploratory AFLP-based approach to examine genetic structuring among selected *Selaginella* species representing diverse morphological forms and geographic origins within Indonesia. Rather than reconstructing phylogenetic relationships, this study aims to provide baseline molecular information on how genetic variation is distributed among species and how these patterns relate to existing morphology-based classifications. By integrating AFLP-derived similarity analyses with clustering and multivariate approaches, this study seeks to (i) characterize multilocus genetic differentiation among selected *Selaginella* taxa and (ii) assess the relevance of observed genetic patterns to systematic studies of early-diverging vascular plants. The limitations of AFLP-based inference are explicitly acknowledged, and the results are interpreted as a foundation for future sequence-based and genomic investigations. Based on the ecological and morphological diversity of *Selaginella* and the limited availability of molecular data for many tropical species, we expected AFLP similarity patterns to be distributed along continuous gradients rather than forming sharply delimited clusters. We hypothesized that (i) AFLP similarity patterns would be distributed along continuous gradients rather than forming sharply delimited clusters, and (ii) AFLP-based genetic structuring would show partial congruence but occasional incongruence with morphology-based classifications.

MATERIALS AND METHODS

Plant material and sampling coverage

This study examined ten *Selaginella* species selected to represent a broad range of morphological traits and geographic origins within Indonesia (Table 1; Figure 1). The sampled taxa encompass diverse growth forms, leaf arrangements, and habitat preferences, reflecting the morphological heterogeneity characteristic of the genus. Species identification was based on morphological examination of living collections and herbarium references, following established taxonomic treatments (Weststrand and Korall 2016a; Setyawan 2011). Voucher specimens and living materials were curated to ensure taxonomic consistency throughout the molecular analyses.

Sampling coverage spanned five major Indonesian island groups, namely Java, Sumatra, the Lesser Sunda Islands, Sulawesi, and Papua, which collectively represent key biogeographic regions of the Indonesian archipelago. These islands differ markedly in geological history, climatic conditions, and ecological settings, providing a heterogeneous environmental backdrop for assessing interspecific genetic structuring among *Selaginella* species. Sampling was conducted across multiple islands, rather than from a single location, to capture a broader spectrum of genetic variation while maintaining practical and consistent sampling procedures.

For each *Selaginella* species, two individuals were initially selected to provide biological replication and to evaluate the consistency of AFLP patterns within species. In addition, AFLP analyses were performed at least in duplicate for each sample to assess amplification reproducibility. Replicate individuals within species and duplicate AFLP runs produced highly similar banding patterns, indicating good consistency and reproducibility of the AFLP profiles.

The complete AFLP dataset therefore comprised 40 profiles, representing ten species, two biological replicates per species, and duplicate AFLP assays for each sample. Similarity-based clustering of the complete dataset revealed patterns broadly consistent with those observed in the species-level analyses, indicating high reproducibility of the AFLP fingerprints and limited variation among replicate profiles. Consequently, one representative AFLP profile was retained for each species and used in the comparative analyses presented in the main text.

The resulting analytical dataset therefore followed a single-representative-profile-per-species design, in which one AFLP profile represented each of the ten *Selaginella* species included in the final comparative analyses. This approach was intentionally adopted as an exploratory framework aimed at broad-scale assessment of multilocus genetic differentiation among species rather than detailed evaluation of intraspecific variation or population structure. Consequently, the analyses were not designed to quantify within-species genetic diversity, geographic structure, or population-level processes. Taxon selection was purposeful rather than exhaustive and was guided by the availability of well-documented specimens, clear morphological distinctiveness, and representation of different island groups.

DNA extraction and AFLP protocol

Freshly collected *Selaginella* plant materials were processed as soon as possible after harvesting to minimize DNA degradation. Young, healthy leaves were selected and immediately silica-dried in the field to preserve nucleic acid integrity prior to laboratory analysis. Approximately 0.5 g of silica-dried leaf tissue from each species was ground into a fine powder using liquid nitrogen and sterile mortars and pestles to ensure efficient cell disruption and homogenization.

Table 1. Sampling locations, morphological characteristics, and ecological context of *Selaginella* specimens used for AFLP analysis

Species	Herbarium code	Morphological characteristics (brief)	Locality	Island group / Province	Latitude (°S)	Longitude (°E)	Alt. (masl)	Habitat description
Selaginella ciliaris (Retz.) Spring	ADS 227	Small habit; leaves clustered around strobili	Karacak, Leuwiliang, Bogor	Java / West Java	6.596537	106.621324	342	Moist volcanic roadside cliff along drainage channel
Selaginella alligans Hieron.	ADS 142	Creeping habit; ascending branches; wiry rhizomes	Cycloops Mountains Nature Reserve, Jayapura	Papua / Papua	2.540623	140.515078	281	Forested mountain habitat within a nature reserve
Selaginella opaca Warb.	ADS 153	Moderate habit; creeping stems; sparse leaves	Batu Kahu Nature Reserve, Tabanan	Lesser Sunda / Bali	8.278795	115.148415	1,403	Montane forest on volcanic substrate
Selaginella ketra-ayam Alderw.	ADS 146	Very small habit; compact leaf arrangement	Mount Penumbing, Pangkal Pinang	Sumatra / Bangka Belitung	2.017728	105.180101	400	Plantation margin near the oil palm area
Selaginella sp. “Grinjani”	ADS 451	Moderate habit; resembling *S. plana* but smaller	Mount Rinjani, Sajang-Sembalun, East Lombok	Lesser Sunda / West Nusa Tenggara	8.356740	116.484974	1,225	Montane slope within a protected forest
Selaginella sp. “Gmeja”	ADS 144	Moderate habit; fleshy stems; sparse leaves	Mount Meja Protected Forest, Manokwari	Papua / West Papua	0.845200	134.068975	102	Shaded forest floor near protected forest margin
Selaginella involvens (Sw.) Spring	ADS 155	Moderate habit; leaves roll when dry; woody stem	Batu Kahu Nature Reserve, Tabanan	Lesser Sunda / Bali	8.278795	115.148415	1,403	Montane forest with seasonal moisture variation
Selaginella velutina Ces.	ADS 141	Moderate habit; fin-like structure; woody stem	Cycloops Mountains Nature Reserve, Jayapura	Papua / Papua	2.540623	140.515078	281	Forested mountain habitat within a nature reserve
Selaginella cupressina (Willd.) Spring	ADS 148	Small habit; apical strobili	Mount Soputan, Minahasa	Sulawesi / North Sulawesi	1.130195	124.768621	1,307	Volcanic mountain slope with montane vegetation
Selaginella ornata (Hook. & Grev.) Spring	ADS 124	Small habit; woody and fragile stem; brownish coloration	Cikaniki Research Camp, Halimun-Salak NP	Java / West Java	6.746725	106.537587	1,052	Shaded forest floor around the research station

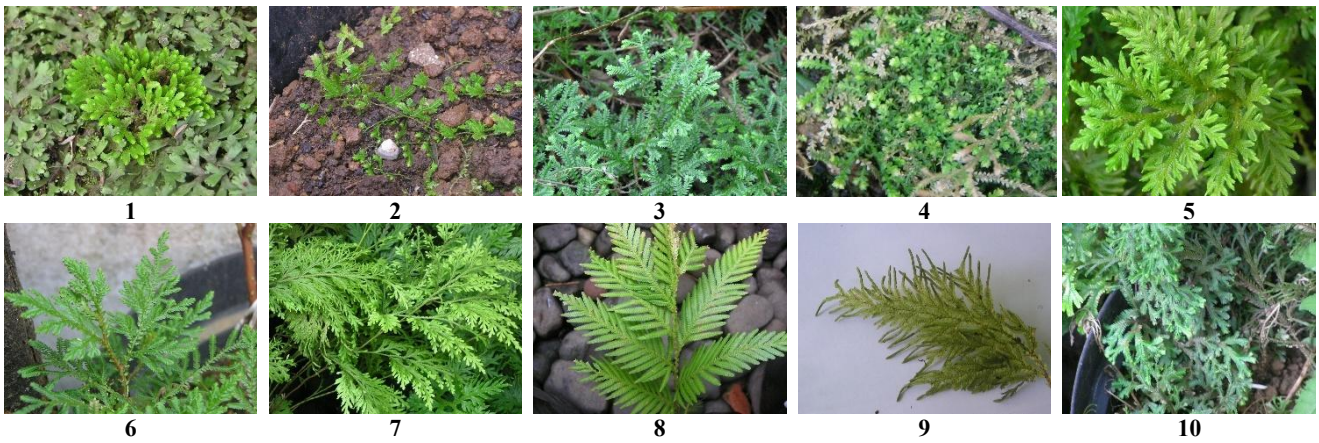

Figure 1. Morphological diversity of selected *Selaginella* species used in this study. Representative growth forms and leaf arrangements of the ten *Selaginella* species analyzed, illustrating variation in habit, stem structure, and leaf morphology across taxa sampled from different Indonesian island groups. Note: 1. *S. ciliaris*, 2. *S. alligans*, 3. *S. opaca*, 4. *S. ketra-ayam*, 5. *Selaginella* sp. “Grinjani”, 6. *Selaginella* sp.“Gmeja”, 7. *S. involvens*, 8. *S. velutina*, 9. *S. cupressina*, 10. *S. ornata*.

Total genomic DNA was extracted following a modified cetyltrimethylammonium bromide (CTAB) protocol, which is widely used for plant tissues rich in secondary metabolites (Doyle and Doyle 1987; Cullings 1992). The powdered tissue was incubated in CTAB extraction buffer containing β-mercaptoethanol, followed by chloroform-isoamyl alcohol purification to remove proteins and polysaccharides. DNA was precipitated with cold isopropanol, washed with ethanol, and resuspended in TE buffer. DNA quality and concentration were assessed by agarose gel electrophoresis and spectrophotometric measurement, and only high-quality DNA samples were used for subsequent AFLP analyses.

The AFLP procedure followed a modified version of the protocol described by Vos et al. (1995). Approximately 250 ng of genomic DNA from each sample was digested with the restriction enzymes *PstI* and *MseI* (New England Biolabs, USA) in a total reaction volume of 20 µL, using the manufacturer's recommended buffers and incubation conditions. Digested fragments were subsequently ligated to double-stranded *PstI* and *MseI* adaptors using T4 DNA ligase (New England Biolabs, USA), generating adaptor-ligated fragments suitable for PCR amplification.

Pre-selective PCR amplification was performed using primers complementary to the *PstI* and *MseI* adaptor sequences with one selective nucleotide. Each 20 µL PCR reaction contained 1× PCR buffer, 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer, and 1 U Taq DNA polymerase (Thermo Fisher Scientific, USA). Thermal cycling conditions for pre-selective amplification consisted of an initial denaturation at 94 °C for 2 min, followed by 20 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 60 s, with a final extension at 72 °C for 5 min. Pre-selective PCR products were verified on agarose gels and diluted prior to selective amplification.

Selective PCR amplification was carried out using one fluorescently labeled *PstI* primer (P-11) in combination with four *MseI* primers (M-48, M-49, M-50, and M-51), each containing three selective nucleotides at the 3′ end. Reaction mixtures and reagent concentrations were identical to those used in the pre-selective PCR. Selective amplification employed a touchdown PCR profile consisting of an initial denaturation at 94 °C for 2 min, followed by 13 cycles of 94 °C for 30 s, annealing from 65 °C to 56 °C (−0.7 °C per cycle) for 30 s, and 72 °C for 60 s, followed by 23 additional cycles at a constant annealing temperature of 56 °C, and a final extension at 72 °C for 5 min.

Selective amplification products were separated and visualized using a Li-Cor DNA Analyzer Model 4300 (Li-Cor Biosciences, USA), with a 700 bp DNA ladder included for fragment size estimation. Only clear, reproducible bands were scored as present (1) or absent (0) for subsequent similarity, clustering, and ordination analyses. All AFLP reactions were conducted under identical conditions to ensure reproducibility and comparability across species and primer combinations.

This AFLP protocol was designed to generate reproducible multilocus fingerprint profiles suitable for presence-absence scoring across species. Although AFLP markers are dominant and anonymous, their high reproducibility and ability to sample multiple genomic regions make them suitable for exploratory assessments of genetic structuring, particularly in non-model plant taxa lacking extensive sequence resources (Mueller and Wolfenbarger 1999; Meudt and Clarke 2007).

AFLP band scoring and data matrix construction

AFLP profiles generated from selective amplification were scored manually based on the presence (1) or absence (0) of distinct DNA fragments across all samples. Only clear, reproducible, and well-resolved bands were included in the analysis to minimize scoring ambiguity. Faint, smeared, or inconsistently amplified fragments were excluded to reduce the risk of artefacts influencing the genetic similarity estimates. Band scoring was performed consistently across all species and primer combinations, using fragment size alignment relative to the internal DNA size ladder to ensure comparability among profiles. Scoring was conducted using standardized criteria and visually cross-checked to ensure consistency across gels and primer combinations.

The AFLP dataset was compiled into a binary presence-absence matrix, with rows representing individual species-primer combinations and columns representing scored AFLP fragments. Each fragment was treated as an independent locus, and fragments of identical electrophoretic mobility were assumed to be homologous across samples. Although inherent to AFLP analysis, this assumption enables broad multilocus comparisons among taxa lacking comprehensive sequence-based data (Vos et al. 1995; Mueller and Wolfenbarger 1999).

Given the dominant nature of AFLP markers, heterozygous and homozygous dominant genotypes cannot be distinguished, and allele frequencies cannot be directly estimated. Consequently, all subsequent analyses were conducted using similarity-based approaches that do not rely on Hardy-Weinberg equilibrium assumptions. The presence-absence matrix was therefore considered appropriate for calculating pairwise genetic similarity indices and for multivariate ordination and clustering analyses (Bonin et al. 2004).

To enhance data reliability, only bands that were consistently detectable across repeated inspections were retained in the final matrix. Scoring criteria and matrix construction were standardized across all primer combinations and species, ensuring that observed patterns of genetic structuring reflected genuine differences in multilocus AFLP profiles rather than methodological artefacts. The resulting binary matrix served as the primary input for genetic similarity calculations, UPGMA clustering, and principal coordinate analysis described in the subsequent section.

Genetic similarity and multivariate analyses

Genetic similarity among *Selaginella* samples was quantified using Jaccard's similarity coefficient, which is widely applied to binary presence-absence data derived from dominant molecular markers such as AFLP (Jaccard 1908). This coefficient considers shared presences of fragments while excluding joint absences, making it

particularly suitable for AFLP datasets where the absence of a band does not necessarily indicate homology. Pairwise similarity values were calculated from the binary AFLP matrix using the SIMQUAL module implemented in NTSYS-pc version 2.1 (Rohlf 2000), with the similarity option set to Jaccard and all bands equally weighted.

Hierarchical clustering was performed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) to visualize patterns of genetic structuring among species. The similarity matrix generated with Jaccard's coefficient was subjected to UPGMA clustering using the SAHN module of NTSYS-pc (method = UPGMA; arithmetic averaging). The resulting dendrogram represents phenetic relationships based on overall multilocus similarity rather than phylogenetic inference, and is therefore interpreted as a similarity-based clustering of AFLP profiles rather than a reconstructed evolutionary tree (Sokal and Michener 1958). The cophenetic correlation coefficient was calculated using the COPH and MXCOMP modules to evaluate the correspondence between the original similarity matrix and the UPGMA dendrogram (Sneath and Sokal 1973).

To further explore genetic relationships in a multivariate framework, Principal Coordinate Analysis (PCoA) was conducted based on the same genetic similarity matrix. PCoA was performed using the EIGEN module of NTSYS-pc 2.1, followed by projection of samples onto the principal coordinate axes using the PROJ function, with no dimensionality constraints imposed a priori. This ordination approach allows visualization of genetic differentiation among samples in reduced-dimensional space, facilitating comparison with clustering results and identification of major axes of variation without assuming hierarchical relationships (Legendre and Legendre 2012). Two- and three-dimensional ordination plots were used to assess consistency between PCoA patterns and UPGMA clustering.

RESULTS AND DISCUSSION

AFLP polymorphism and primer performance

Selective AFLP amplification using four primer combinations (P-11/M-48, P-11/M-49, P-11/M-50, and P-11/M-51) generated a total of 248 polymorphic fragments across the ten *Selaginella* species analyzed. Overall, AFLP band richness varied markedly among species, with some taxa producing fewer than 20 scorable fragments whereas others yielded more than 50 fragments, indicating substantial heterogeneity in AFLP polymorphism among taxa. Representative AFLP profiles illustrating the distribution and relative intensity of amplified fragments for each selective primer combination are shown in Figure 2.

The four selective primer combinations differed in their amplification performance and contribution to polymorphic information. All primer pairs successfully generated multiple fragments across species but varied in total band number, number of polymorphic fragments, and apparent fragment-size distribution. Some primer combinations consistently produced richer and more complex banding patterns, whereas others yielded fewer fragments across most species, reflecting differences in selective nucleotide composition and genomic target sites. A quantitative summary of primer performance, including total band number, number of polymorphic fragments, and fragment-size ranges, is presented in Table 2.

Marked interspecific differences were observed in AFLP polymorphism levels (Table 3). *Selaginella alligans*, *S. opaca*, *Selaginella* sp. "Grinjani", and *S. involvens* produced the highest numbers of polymorphic AFLP fragments, with 58, 56, 53, and 52 fragments, respectively. In contrast, the remaining species generated only 13-19 polymorphic fragments, resulting in comparatively simpler AFLP profiles. These results indicate substantial variation in multilocus AFLP patterns among the analyzed taxa.

The observed differences in AFLP polymorphism among species are unlikely to be attributable to methodological artefacts, as all samples were processed using identical laboratory procedures, primer combinations, and scoring criteria. Instead, the variation in AFLP profiles most likely reflects underlying differences in genome composition, restriction-site distribution, or sequence diversity among taxa.

Table 2. Performance summary of AFLP selective primer combinations used in this study

Selective primer combination	Total number of bands	Number of polymorphic bands	Fragment size range (bp)
P-11 + M-48	70	66	100-700
P-11 + M-49	67	63	100-700
P-11 + M-50	63	60	100-700
P-11 + M-51	62	59	100-700
Total	**262**	**248**	-

Note: A total of 248 polymorphic AFLP fragments were generated across four selective primer combinations. Values represent scored AFLP fragments used in subsequent similarity and multivariate analyses.

Table 3. Relative AFLP polymorphism levels among *Selaginella* species analyzed in this study

Species	Number of scored AFLP fragments	Polymorphism category
Selaginella ciliaris	17	Low
Selaginella alligans	58	High
Selaginella opaca	56	High
Selaginella ketra-ayam	14	Low
Selaginella sp. "Grinjani"	53	High
Selaginella sp. "Gmeja"	19	Low
Selaginella involvens	52	High
Selaginella velutina	16	Low
Selaginella cupressina	15	Low
Selaginella ornata	13	Low

Note**:** Values indicate the number of polymorphic AFLP fragments detected in each species across all four selective primer combinations. Because individual AFLP fragments may be shared among multiple species, values are not additive across taxa.

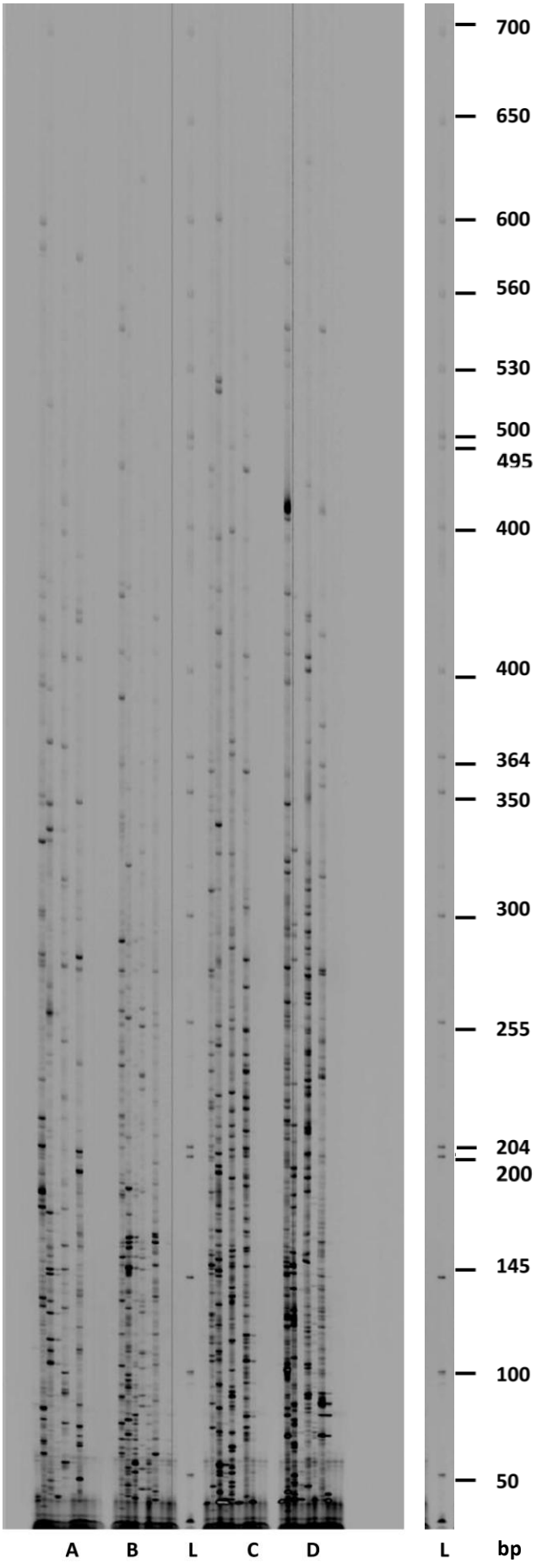

Figure 2. AFLP fingerprint profiles of selected *Selaginella* species generated using four selective primer combinations. A. P-11 + M-48; B. P-11 + M-49; C. P-11 + M-50; D. P-11 + M-51; L. DNA ladder. Species are arranged from left to right in the same order as in Figure 1. Numbers indicate species identity: 1, *S. ciliaris*; 2, *S. alligans*; 3, *S. opaca*; 4, *S. ketra-ayam*; 5, *Selaginella* sp. "Grinjani"; 6, *Selaginella* sp. "Gmeja"; 7, *S. involvens*; 8, *S. velutina*; 9, *S. cupressina*; 10, *S. ornata*. Only representative AFLP profiles are shown; duplicate runs produced highly similar banding patterns and are not presented.

Species exhibiting lower levels of AFLP polymorphism generally showed higher pairwise similarity values in subsequent analyses, whereas taxa with richer AFLP profiles contributed more strongly to overall genetic differentiation. Collectively, the four selective primer combinations generated sufficient polymorphic information to support similarity-based clustering and multivariate ordination analyses, demonstrating the utility of AFLP markers for exploratory assessments of genetic structuring in morphologically and genetically diverse plant groups.

Genetic similarity patterns among *Selaginella* species

Pairwise genetic similarity among the ten *Selaginella* species was calculated using Jaccard's coefficient based on the AFLP presence-absence matrix. Overall, similarity values spanned a broad range, from approximately 0.59 to 1.00, indicating substantial heterogeneity in AFLP profiles among the analyzed taxa. This wide range reflects pronounced differences in multilocus fragment composition and provides the quantitative basis for subsequent clustering and ordination analyses. A summary of the observed similarity ranges and their interpretation is presented in Table 4.

The highest similarity values were observed among species characterized by low AFLP polymorphism, including the pairs *Selaginella ciliaris-S. ketra-ayam* (similarity > 0.90) and *S. velutina-S. cupressina* (similarity > 0.90), indicating extensive sharing of AFLP fragments under the applied scoring criteria. In contrast, lower similarity values were typically associated with comparisons involving highly polymorphic taxa, such as *S. alligans* versus *S. ornata* and *S. opaca* versus *S. cupressina*, with similarity values approaching the lower end of the observed range (≈ 0.59-0.65).

Table 4. Summary of AFLP-based genetic similarity patterns among *Selaginella* species

Similarity range (Jaccard)	General pattern	Interpretation
~0.90-1.00	Very high similarity	Species pairs with limited AFLP polymorphism; profiles dominated by shared fragments.
~0.70-0.89	Moderate similarity	Intermediate differentiation; partial sharing of AFLP fragments
~0.59-0.69	Lower similarity	Higher differentiation is associated with richer AFLP band profiles

General patterns of genetic similarity were closely associated with differences in AFLP polymorphism among species. Taxa producing relatively few polymorphic fragments tended to exhibit higher pairwise similarity values, whereas species with richer AFLP profiles showed lower similarity values and contributed more strongly to overall genetic differentiation. This pattern suggests that highly polymorphic taxa capture a broader spectrum of AFLP variation, thereby reducing apparent similarity with other species. Despite the broad similarity range, no species formed a uniformly distinct outlier across all pairwise comparisons. Instead, similarity values formed a continuum, with intermediate levels observed among several species pairs. This continuous distribution suggests gradual multilocus differentiation rather than sharply delimited genetic boundaries among the analyzed *Selaginella* taxa.

The observed similarity patterns should be interpreted as phenetic measures of multilocus resemblance rather than estimates of evolutionary distance. Because AFLP markers are dominant and anonymous, similarity values primarily reflect shared fragment presence rather than inferred ancestry. Nevertheless, these patterns provide an empirical foundation for identifying major groupings and trends in genetic structuring, which are further explored through hierarchical clustering and multivariate ordination analyses.

UPGMA-based genetic structuring

Similarity-based clustering using the UPGMA algorithm revealed clear patterns of genetic structuring among the analyzed *Selaginella* species based on AFLP presence-absence data. The resulting phenogram (Figure 3) shows that all species are connected within a single clustering framework, reflecting their shared generic affinity while still exhibiting substantial internal differentiation. Major groupings emerge progressively along the similarity scale rather than forming sharply separated clusters, consistent with the continuous similarity patterns observed in pairwise comparisons. The complete AFLP dataset originally comprised 40 profiles, including replicate individuals and duplicate AFLP runs. Similarity-based clustering of the complete dataset (Figure S1) produced a pattern broadly consistent with the species-level clustering results, supporting the use of one representative AFLP profile per species in the main analyses.

The cophenetic correlation coefficient was 0.91, indicating that the UPGMA phenogram provided a reliable representation of the AFLP similarity relationships among the analyzed species. Two major clusters were recognized in the species-level dendrogram. Cluster I comprised *Selaginella ciliaris*, *S. alligans*, *S. opaca*, *S. ketra-ayam*, *Selaginella* sp. "Grinjani", and *Selaginella* sp."Gmeja". These species were joined at relatively high similarity values and formed a cohesive assemblage across the upper portion of the dendrogram. Cluster II included *S. involvens*, *S. velutina*, *S. cupressina*, and *S. ornata*, which were separated from Cluster I at lower similarity values but exhibited relatively close relationships within the cluster.

The clustering pattern showed partial correspondence with overall morphological habit. Cluster I predominantly comprises creeping to ascending taxa, including *Selaginella* sp. "Grinjani", which shares several morphological characteristics with *S. plana* but differs in its smaller and more compact fronds, whereas Cluster II consists mainly of species exhibiting a cypress-like growth form together with *S. ornata*. Although *S. ornata* differs from the cypress-like taxa in several morphological characters, its erect habit and general branching architecture are more similar to this group than to the creeping or ascending species of Cluster I. The observed correspondence between AFLP-based clustering and growth form suggests that major morphological differences among the analyzed species may be associated with broader patterns of multilocus genetic similarity. However, this interpretation should be treated cautiously because AFLP markers reflect genome-wide genetic variation and are not necessarily linked directly to the morphological traits used in species delimitation.

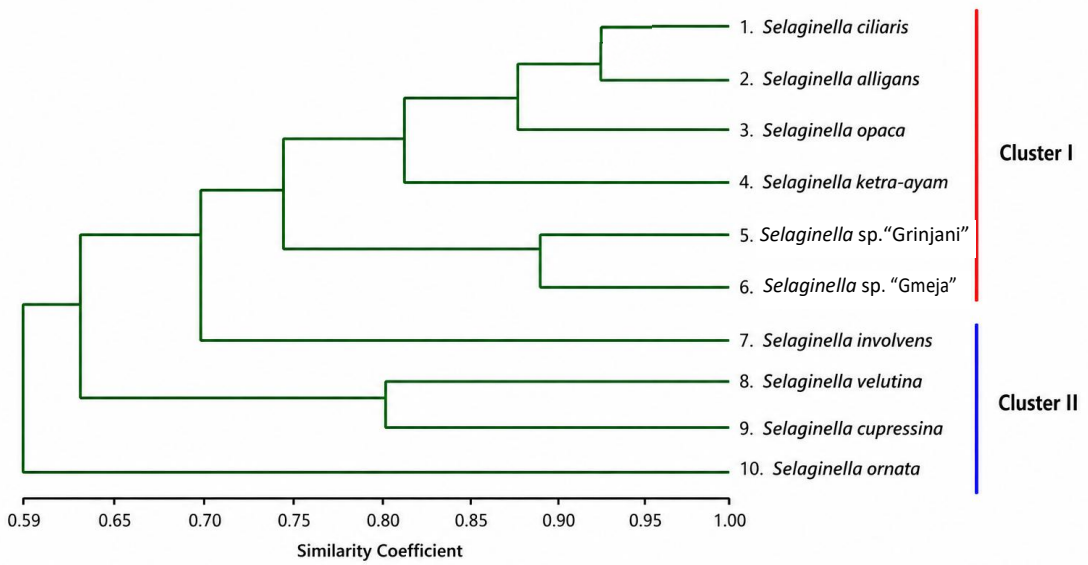

Figure 3. UPGMA dendrogram showing genetic relationships among ten *Selaginella* species based on AFLP markers. The phenogram was constructed using Jaccard's similarity coefficient and reveals two major species assemblages with similarity values ranging from 0.59 to 1.00.

Relationships inferred from the UPGMA phenogram should be interpreted as phenetic associations based on multilocus AFLP similarity rather than as hypotheses of evolutionary ancestry. The clustering reflects the degree of shared AFLP fragments among species and therefore provides a summary of relative genetic resemblance under the applied analytical framework. Consequently, close clustering does not necessarily imply recent common ancestry, nor does broader separation indicate deep evolutionary divergence.

The UPGMA analysis revealed substantial multilocus differentiation among the analyzed *Selaginella* species while also identifying coherent groups that correspond broadly to major morphological growth forms. The clustering results provide a useful framework for interpreting patterns of AFLP variation and are consistent with the multivariate relationships observed in the principal coordinate analysis.

PCoA ordination patterns

Multivariate ordination using principal coordinate analysis (PCoA) provided a complementary visualization of genetic relationships among *Selaginella* species based on AFLP-derived similarity data. The two-dimensional ordination plot (Figure 4) shows a structured distribution of samples along the first two coordinate axes, reflecting major gradients of genetic variation captured by the AFLP markers. The first and second principal coordinates explain approximately 33.2% and 20.1% of the total genetic variation, respectively, accounting for about 53.3% of the overall variation. Species are separated across ordination space rather than forming a single compact cluster, indicating appreciable multilocus differentiation within the genus. PCoA of the complete AFLP dataset (Figure S2) produced an ordination pattern broadly consistent with the species-level analysis shown in Figure 4, further supporting the use of one representative AFLP profile per species in the final comparative analyses.

Species characterized by low AFLP polymorphism tend to occupy relatively proximate positions in ordination space, forming a loosely grouped assemblage with limited dispersion along the principal axes. This pattern is consistent with their high pairwise similarity values and tight clustering observed in the similarity-based analysis. In contrast, species with richer AFLP band profiles are distributed more broadly across the ordination space, contributing to greater separation along the primary axes of variation. These taxa, therefore, exert a stronger influence on the overall structure of the PCoA configuration.

Three-dimensional ordination of the complete AFLP dataset (Figure S2B) further clarifies these relationships. The third principal coordinate accounts for an additional ~12.4% of the total genetic variation, bringing the cumulative variation explained by the first three axes to approximately 65-66%. The remaining variation was distributed among additional coordinate axes, reflecting the multidimensional nature of AFLP-derived multilocus data. In three-dimensional space, the relative positions of species remain largely consistent with the two-dimensional representation, indicating that the major patterns of genetic structuring are robust across multiple axes of variation. No abrupt outliers are detected, supporting the interpretation that genetic differentiation among the analyzed taxa occurs along continuous gradients rather than discrete boundaries.The ordination patterns show strong qualitative agreement with the UPGMA phenogram. Species grouped closely in the dendrogram tend to plot near one another in PCoA space, whereas more distantly clustered taxa occupy more separated positions. This concordance between hierarchical clustering and multivariate ordination reinforces the internal consistency of the AFLP dataset and supports the reliability of the inferred genetic structuring. As with the clustering analysis, the PCoA results are interpreted as phenetic representations of multilocus similarity rather than as estimates of evolutionary distance.

Discussion

Interpretation of AFLP-based genetic structuring in Selaginella

The genetic patterns we saw among *Selaginella* species are real biological differences, not artifacts from the AFLP method or data analysis. The wide range of detected polymorphism levels indicates substantial heterogeneity in multilocus genetic variation within the genus. Similar variation has been reported in other early-diverging vascular plant lineages, where differences in genome organization and restriction-site distribution influence multilocus fingerprint profiles (Mueller and Wolfenbarger 1999; Meudt and Clarke 2007).

Species with low AFLP polymorphism generally exhibited high pairwise similarity values and compact clustering, whereas taxa with richer AFLP profiles contributed more strongly to overall genetic structuring. These patterns suggest differences in the amount of detectable multilocus variation among species and may reflect variation in genome composition, ecological adaptation, or historical diversification. Comparable associations between AFLP polymorphism and genetic differentiation have been reported in other lycophytes and ferns (Vos et al. 1995; Schneider et al. 2009).

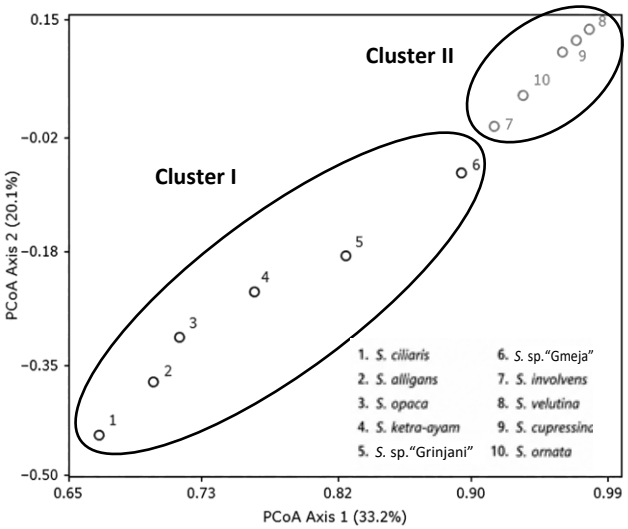

Figure 4. Two-dimensional PCoA ordination of ten *Selaginella* species based on AFLP-derived similarity. Axis 1 and Axis 2 explain 33.2% and 20.1% of the total variation, respectively.

The species-level clustering also showed partial correspondence with overall morphological habit. Cluster I was composed predominantly of creeping to ascending taxa, including members of the *S. plana* affinity group, whereas Cluster II consisted mainly of species exhibiting a cypress-like growth form together with *S. ornata*. Although AFLP markers are not directly linked to diagnostic morphological traits, this pattern suggests that major differences in growth form and branching architecture may coincide with broader patterns of multilocus genetic similarity. The placement of *S. ornata* within the cypress-like assemblage is notable because its erect habit more closely resembles members of this group than the creeping or ascending taxa assigned to Cluster I.

The absence of sharply delimited genetic groups suggests that differentiation within *Selaginella* is better viewed as a continuum rather than a set of discrete genetic clusters. Similar patterns of gradual divergence have been reported in other lycophyte lineages, even when morphological differentiation appears pronounced (Pryer et al. 2004; Weststrand and Korall 2016b). Such partial incongruence is not unexpected because morphological diversification and genome-wide genetic differentiation may proceed at different rates.

Importantly, the AFLP-based structuring observed here should be interpreted as phenetic genetic resemblance rather than explicit evolutionary relationships. Nevertheless, AFLP markers provide a useful overview of how genetic variation is distributed among taxa, particularly in groups where sequence-based data remain limited. Taken together, the results reveal substantial multilocus differentiation among *Selaginella* species while highlighting a partial correspondence between genetic similarity and major growth-form categories. These findings demonstrate the value of integrating molecular and morphological evidence and provide a foundation for future sequence-based and genomic studies.

Molecular-morphological congruence and incongruence

Comparisons between AFLP-based genetic structuring and traditional morphology-based classifications in *Selaginella* reveal a complex pattern of both congruence and incongruence. In several cases, morphologically distinct species also exhibit clear genetic differentiation, supporting the taxonomic value of certain morphological characters. Such congruence suggests that some external traits, particularly those related to growth habit and branching architecture, may reflect underlying multilocus genetic differentiation accumulated over evolutionary time. Similar correspondence between molecular markers and morphology has been reported in lycophytes and ferns, where distinct life forms often coincide with broad patterns of genetic differentiation (Pryer et al. 2004; Schneider et al. 2009).

However, the AFLP results also highlight instances in which pronounced morphological differences are not accompanied by strong genetic separation. A clear example is provided by *S. ciliaris* and *S. ketra-ayam*, which differ markedly in overall habit and leaf arrangement (Figure 1), yet exhibit very high AFLP similarity and cluster closely in both the UPGMA phenogram and PCoA ordination (Figures 3 and 4). Despite their contrasting morphology, these species share a substantial proportion of AFLP fragments, indicating limited multilocus differentiation under the applied marker system. This pattern suggests that morphological differentiation and AFLP-based genetic differentiation may not always proceed at comparable rates.

The high AFLP similarity between morphologically similar creeping species such as *S. ciliaris* and *S. ketra-ayam* (similarity > 0.90) suggests that minor morphological differences in leaf arrangement and size may not reflect substantial genome-wide differentiation. This pattern is consistent with the hypothesis that morphological diversification in *Selaginella* may involve regulatory changes in a limited number of genes rather than extensive genomic divergence. Conversely, the clear separation between creeping (Cluster I) and cypress-like (Cluster II) species in both UPGMA and PCoA analyses indicates that major shifts in growth form are associated with broader multilocus genetic differentiation.

Similar patterns were observed among several species occupying adjacent positions within Cluster I, where recognizable differences in habit, branching architecture, and leaf morphology were accompanied by relatively high AFLP similarity values. These observations indicate that morphological divergence in *Selaginella* can occur without extensive multilocus differentiation detectable by AFLP markers. Such patterns are not unexpected in early-diverging vascular plants, where morphological traits may respond to ecological conditions and developmental processes independently of broader patterns of genetic differentiation (Kenrick and Crane 1997; Niklas and Kutschera 2010).

This phenomenon, often referred to as the decoupling of morphological and genetic evolution, has been widely documented in plants, where phenotypic traits may evolve under ecological or developmental influences without corresponding changes in genome-wide molecular markers (Cronk 2001; Schneider et al. 2009). In the present study, the observed incongruence suggests that certain morphological characters, including leaf size, orientation, and branching architecture, may be evolutionarily more labile than the multilocus AFLP patterns detected across the genome. Because AFLP markers provide a genome-wide fingerprint of multilocus variation rather than direct measures of adaptive or functional evolution, discrepancies between molecular similarity and external morphology are not unexpected. Consequently, molecular and morphological datasets should be regarded as complementary rather than competing sources of systematic evidence. Integrating both approaches provides a more comprehensive understanding of species diversification and evolutionary relationships in *Selaginella* and other early-diverging vascular plant lineages.

Morphological plasticity has long been recognized as a challenge in *Selaginella* taxonomy. Environmental factors such as moisture availability, light intensity, and substrate type can influence leaf size, orientation, and branching architecture, potentially producing similar morphologies in genetically distinct taxa or contrasting morphologies in genetically similar taxa (Weststrand and Korall 2016a; Setyawan 2011). In this context, AFLP-based analyses,

which capture multilocus genetic variation largely independent of phenotype, provide an important complement to morphology-driven interpretations.

Recent plastome-scale analyses have likewise highlighted the complexity of evolutionary relationships within *Selaginella*, showing that even extensive plastome datasets cannot fully resolve its phylogeny (Zhou et al. 2022). Although AFLP markers are not intended for phylogenetic reconstruction, the partial congruence and incongruence observed in the present study further support the value of integrating molecular and morphological evidence to better understand species relationships in *Selaginella*.

Methodological scope and limitations of AFLP-based inference

The use of Amplified Fragment Length Polymorphism (AFLP) in this study should be understood within its intended exploratory scope. AFLP markers are dominant, anonymous, and multilocus in nature, generating genetic fingerprints without prior sequence information. These characteristics make AFLP particularly useful for preliminary assessments of genetic differentiation in non-model plant groups, including early-diverging vascular plants for which molecular resources remain limited (Vos et al. 1995; Mueller and Wolfenbarger 1999).

A major advantage of AFLP is its ability to generate large numbers of polymorphic loci in a cost-effective and reproducible manner. In *Selaginella*, where molecular data remain scarce for many tropical species, AFLP provides a practical approach for detecting broad patterns of genetic structuring among taxa. Similar applications in ferns and lycophytes have demonstrated the utility of AFLP for revealing relative levels of genetic differentiation in exploratory studies (Esselman et al. 2000; Schneider et al. 2009).

Nevertheless, several limitations should be acknowledged. As a dominant marker system, AFLP does not distinguish homozygous from heterozygous states, preventing direct estimation of allele frequencies and other population-genetic parameters (Bonin et al. 2004; Holsinger and Wallace 2004). In addition, AFLP analyses assume homology among comigrating fragments, although size homoplasy may occur, particularly in taxa with repetitive genomes (Meudt and Clarke 2007).

The sampling design also constrains the scope of inference. Although each species was initially represented by replicate individuals and duplicate AFLP assays to evaluate profile consistency, the final comparative analyses were conducted using one representative AFLP profile per species. Consequently, the results primarily reflect interspecific multilocus similarity and do not address intraspecific variation, population structure, or geographic differentiation. These limitations are consistent with the exploratory objectives of the study but should be considered when interpreting the observed clustering patterns.

Recent genomic approaches, including plastome phylogenomics and reduced-representation sequencing methods, offer substantially higher resolution for investigating evolutionary relationships and population structure. However, AFLP remains useful as a preliminary screening tool for identifying genetically informative taxa, generating hypotheses, and guiding future molecular investigations in groups where genomic resources are still limited.

Despite these limitations, the present study provides a useful molecular baseline for understanding genetic structuring in *Selaginella* and identifying taxa that merit further investigation using sequence-based and genomic approaches.

Implications for early vascular plant systematics

The AFLP-based genetic structuring observed in this study provides a useful molecular perspective on species relationships within *Selaginella*. Although the analysis was exploratory and not intended for phylogenetic reconstruction, the observed patterns reveal how multilocus genetic variation is distributed among morphologically distinct taxa and highlight areas of both correspondence and incongruence between molecular and morphological evidence.

A notable result is the partial correspondence between AFLP-based clustering and major growth-form categories. Species assigned to Cluster I were predominantly creeping to ascending taxa, including members of the *S. plana* affinity group, whereas Cluster II consisted mainly of cypress-like species together with *S. ornata*. The placement of *S. ornata* within the cypress-like assemblage is particularly interesting because, despite several morphological differences, its erect habit and branching architecture resemble those of the cypress-like taxa more closely than those of the creeping or ascending species. This pattern suggests that broad morphological organization may coincide with multilocus genetic similarity at the species level.

At the same time, the AFLP results demonstrate that morphological distinctness does not necessarily correspond to strong multilocus genetic differentiation. Conversely, some morphologically similar species occupied relatively distinct positions in the AFLP-based analyses. These observations indicate that external morphology alone may either underestimate or overestimate underlying genetic differentiation, highlighting the value of integrative systematics in which molecular and morphological evidence are evaluated jointly rather than independently.

The relatively close association of *S. alligans* and *S. opaca* also warrants further investigation. Although these species occur in different geographic regions, their AFLP profiles suggest a degree of genetic affinity that may reflect shared ancestry, retention of ancestral polymorphism, or limitations of currently recognized diagnostic characters. Distinguishing among these alternatives will require broader geographic sampling and sequence-based analyses.

The results suggest that morphological diversification and multilocus genetic differentiation may proceed at different rates within *Selaginella*. Consequently, AFLP-derived genetic structuring should be regarded as complementary to morphology-based taxonomy rather than as a substitute for it. Despite its limitations, AFLP provides a useful baseline for identifying taxa and species groups

that merit further investigation and for guiding future studies using expanded sampling, DNA sequence data, and genomic approaches.

In conclusion, this study provides an exploratory assessment of multilocus genetic structuring among selected *Selaginella* species using AFLP markers. Four selective primer combinations generated 248 polymorphic fragments, revealing substantial heterogeneity in AFLP profiles among species. Pairwise genetic similarity values ranged from 0.59 to 1.00, indicating a continuum of genetic differentiation rather than sharply delimited genetic groups. UPGMA clustering and principal coordinate analysis (PCoA) identified two broad species assemblages and revealed substantial variation in multilocus genetic similarity among the analyzed taxa. The clustering pattern showed partial correspondence with major growth-form categories, with predominantly creeping to ascending species grouped separately from taxa exhibiting a cypress-like habit together with *S. ornata*. At the same time, several morphologically distinct species exhibited relatively high AFLP similarity, highlighting both congruence and incongruence between molecular and morphological patterns. Although constrained by the dominant nature of AFLP markers and the use of representative AFLP profiles in the final comparative analyses, the results provide a useful molecular baseline for future studies incorporating broader taxonomic sampling, multiple populations per species, and sequence-based or genomic approaches. Our findings demonstrate the value of integrating multilocus AFLP data with morphological evidence to better understand genetic structuring and species relationships in *Selaginella* while providing a useful molecular baseline for future sequence-based and genomic investigations.

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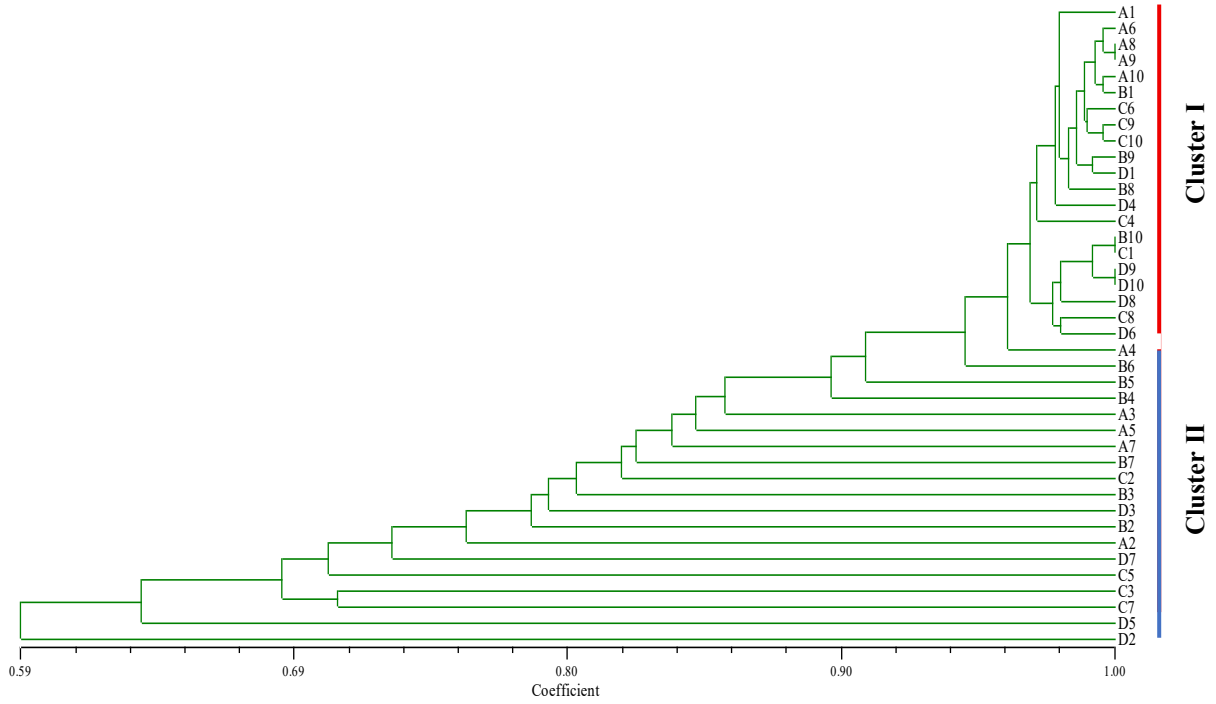

Figure S1. UPGMA clustering of the complete AFLP dataset comprising 40 profiles from ten *Selaginella* species. The dendrogram was constructed using Jaccard's similarity coefficient based on AFLP presence-absence data. Each species is represented by four AFLP profiles corresponding to the four selective primer combinations used in this study. The overall clustering pattern is broadly consistent with the species-level relationships presented in Figure 3 and illustrates the reproducibility of AFLP profiles across primer combinations. Note**:** A-D denote AFLP primer combinations (A = P-11/M-48, B = P-11/M-49, C = P-11/M-50, D = P-11/M-51); numbers (1-10) denote species identities as defined in Figure 2. This figure presents the complete AFLP dataset used during preliminary analyses, whereas Figure 3 summarizes species-level relationships using one representative AFLP profile per species.

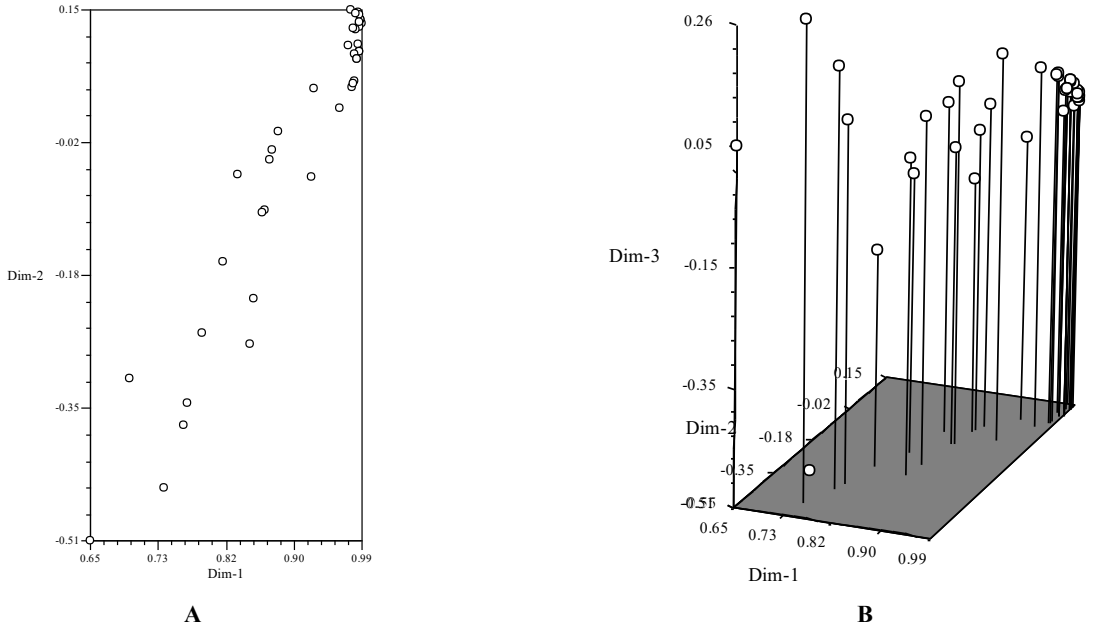

Figure S2. Principal coordinate analysis (PCoA) of the complete AFLP dataset comprising 40 profiles from ten *Selaginella* species. A. Two-dimensional ordination based on the first two principal coordinate axes; B. Three-dimensional ordination based on the first three principal coordinate axes. The overall ordination pattern is broadly consistent with the species-level relationships summarized in Figure 4 and illustrates the distribution of AFLP profiles generated from different primer combinations.Note: A-D denote AFLP primer combinations (A = P-11/M-48, B = P-11/M-49, C = P-11/M-50, D = P-11/M-51); numbers (1-10) denote species identities as defined in Figure 2. This figure presents the complete AFLP dataset used during preliminary analyses, whereas Figure 4 shows the species-level ordination based on one representative AFLP profile per species.