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Comparative HPLC fingerprinting of flavonoid profiles in *Selaginella* species

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Abstract. *Setyawan AD, Chikmawati T, Miftahudin, Lotulung PDN, Sutarno, Sugiyarto, Sunarto. 2025. Comparative HPLC fingerprinting of flavonoid profiles in* Selaginella *species. Asian J Nat Prod Biochem 23: 131-145.* Flavonoids are among the most prominent secondary metabolites in *Selaginella*, yet comparative information on flavonoid composition across multiple species remains limited. Most existing phytochemical studies focus on individual species or isolated compounds, providing little insight into interspecific chemical relationships within the genus. This study applies RT-based HPLC fingerprinting to comparatively assess flavonoid profile variation among 20 samples representing 17 *Selaginella* species collected from diverse ecological and geographical settings in Indonesia. Methanolic extracts were analyzed under standardized chromatographic conditions, and flavonoid-related peaks were aligned using a retention time tolerance of ±0.2 min to construct a binary presence-absence fingerprint matrix comprising 11 RT-defined features. Interspecific similarity was evaluated using Sørensen and Jaccard coefficients, followed by hierarchical clustering (UPGMA) and complementary ordination to visualize chemical relationships among samples. The results reveal pronounced qualitative differences in flavonoid fingerprints among species, characterized by both shared and species-specific chromatographic features. Conspecific samples collected from different localities exhibited stable core flavonoid profiles with additional site-related variation, while interspecific comparisons showed substantial heterogeneity. Similarity patterns were not strictly associated with geographic proximity, suggesting that flavonoid composition primarily reflects intrinsic biochemical traits rather than purely environmental effects. This study provides an effective qualitative framework for interspecific discrimination and chemotaxonomic assessment in *Selaginella*. Although compound identity and abundance were not resolved, the approach offers a practical foundation for future targeted phytochemical, bioactivity-oriented, and integrative chemotaxonomic investigations.

Keywords: Chemotaxonomy, flavonoids, HPLC fingerprinting, retention time, *Selaginella*

INTRODUCTION

Selaginella is one of the most diverse genera of lycophytes, comprising more than 700 species distributed predominantly in tropical and subtropical regions (Jermy 1990; Banks 2009; Weststrand and Korall 2016a). As an early-diverging lineage of vascular plants, *Selaginella* exhibits distinctive physiological and biochemical traits that have attracted increasing scientific interest, particularly in relation to secondary metabolites (Banks et al. 2011). Among these, flavonoids represent one of the most prominent and chemically diverse groups reported from the genus (Cao et al. 2010; Setyawan 2011).

Flavonoids in *Selaginella* are dominated by biflavonoids and related phenolic compounds, which have been associated with a wide range of bioactivities, including antioxidant, anti-inflammatory, cytotoxic, and neuroprotective effects (Chikmawati et al. 2012; Zheng et al. 2012; Křížkovská et al. 2020). In addition to their pharmacological relevance, flavonoids play important ecological roles in UV protection, stress tolerance, and defense against herbivores and pathogens, particularly in species inhabiting exposed or heterogeneous environments (Close and McArthur 2002; Mierziak et al. 2014). This dual functional relevance places flavonoids at the intersection of chemical ecology, adaptive biology, and applied phytochemistry in *Selaginella*.

Despite growing interest, most flavonoid studies on *Selaginella* have focused on individual species or a limited number of taxa, primarily emphasizing compound isolation and structural elucidation (Shim et al. 2018; Muema et al. 2022). While these studies provide valuable information on compound diversity, they offer limited insight into broader interspecific patterns of flavonoid composition. Consequently, current knowledge remains fragmented, underscoring the need for comparative approaches capable of capturing chemical variation across multiple *Selaginella* species within a unified analytical framework.

Phytochemical investigations of *Selaginella* have traditionally relied on targeted strategies, including

spectroscopic identification and LC-MS-based profiling aimed at known flavonoids (Cao et al. 2010; Almeida et al. 2013; Shim et al. 2018). Although indispensable for structural and bioactivity studies, such approaches are less effective for comparative analysis across numerous species, as they often prioritize dominant or previously reported compounds. Moreover, the lack of standardized comparative matrices hampers holistic assessment of chemical similarity, species discrimination, and chemotaxonomic interpretation within the genus, particularly in species-rich tropical regions where chemical diversity may parallel ecological differentiation (Wolfender et al. 2003; Kim et al. 2010; Allard et al. 2016).

From a methodological perspective, the application of chromatographic fingerprinting remains surprisingly scarce in *Selaginella* research. Comparative RT-based flavonoid fingerprinting across multiple *Selaginella* species is still poorly explored, and few studies have constructed retention time matrices or applied similarity indices and clustering analyses to evaluate interspecific chemical relationships (Liang et al. 2004; Tistaert et al. 2011; Goodarzi et al. 2013). As a result, chemotaxonomic signals and interspecific chemical affinities within *Selaginella* remain insufficiently resolved.

High-Performance Liquid Chromatography (HPLC) fingerprinting offers a robust and reproducible approach for comparative analysis of complex phytochemical mixtures by emphasizing overall chromatographic patterns rather than individual compound identities (Gong et al. 2003; Xie et al. 2006). Retention time-based fingerprinting is particularly suitable for multi-species comparisons, as it facilitates pattern recognition, similarity assessment, and clustering analysis across large datasets (Xie et al. 2006; D'Orazio et al. 2016). In the context of *Selaginella*, this approach provides a practical strategy to overcome the limitations of compound-centered studies and to support comparative, chemotaxonomic, and exploratory screening objectives.

The present study aims to comparatively evaluate flavonoid profiles across multiple *Selaginella* species using Retention Time (RT)-based HPLC fingerprinting. Specifically, this study seeks to (i) construct a retention time matrix of flavonoid peaks, (ii) identify shared and species-specific chromatographic features, and (iii) assess interspecific chemical similarity and clustering patterns using similarity indices and multivariate analysis. We hypothesized that RT-based HPLC flavonoid fingerprints would exhibit (i) stable species-level core patterns across samples of the same *Selaginella* species collected from different localities, and (ii) significant interspecific differentiation in flavonoid composition that is not strictly associated with geographic proximity, reflecting intrinsic biochemical traits rather than environmental factors alone. The novelty of this study lies in its integrative multi-species design, representing one of the first applications of RT-based HPLC fingerprinting combined with similarity and cluster analyses to explore flavonoid diversity within *Selaginella*. By adopting a comparative framework, this work contributes to improved chemotaxonomic understanding and provides a methodological foundation for future studies linking chemical diversity with bioactivity screening and evolutionary interpretation.

MATERIALS AND METHODS

Plant materials and species identification

Plant materials used in this study consisted of multiple *Selaginella* species representing a range of habitats and elevational zones. Species selection was designed to capture interspecific variation in flavonoid profiles across contrasting ecological settings, including lowland, montane, and high-altitude environments. Details of the analyzed species, sampling localities, habitat characteristics, and elevation ranges are summarized in Table 1.

Field sampling was conducted across different geographic locations, with site selection based on the natural distribution of each *Selaginella* species (Alves and Rosa 2007). For each species, healthy and mature aerial parts were collected to ensure sufficient biomass for phytochemical analysis, following recommended botanical collection practices (Hudson et al. 2021). Habitat descriptions were recorded in situ, including dominant vegetation type, light conditions, and substrate characteristics, as these factors may influence secondary metabolite production (Gershenzon 1984). Elevation was determined using a handheld GPS device or altimeter and is reported in meters above sea level (masl) to provide ecological context for each species (Hijmans et al. 2005).

Species identification was conducted using a combination of diagnostic morphological characters, including leaf arrangement, the shape and dimorphism of lateral and median leaves, stem architecture, and overall growth form. Identification *Selaginella* followed the earliest and authoritative taxonomic from the Malay Archipelago, particularly by Alston (1934, 1935a, b, 1937, 1940). Specimens were further compared with authenticated collections housed at Herbarium Bogoriense (BO), Indonesia, with special emphasis on specimens determined by A.G.H. Alston. Additional confirmation was obtained by consulting more recent taxonomic references covering *Selaginella* in the Malay Archipelago and adjacent regions, including Wong (1982, 2010), Tsai and Shieh (1994), Li and Tan (2005), Chang et al. (2012), Setyawan (2012), et al. (2013), and Zhang et al. (2013).

Voucher specimens for all analyzed species were prepared and deposited at the Herbarium of Universitas Sebelas Maret, Indonesia (UNS; herbarium code: SO) to ensure reproducibility and taxonomic verifiability of the study. Each voucher specimen was assigned a unique collection number and labeled with detailed locality information, collector name, and collection date. Duplicate specimens (selected herbarium copies) will be forwarded to Herbarium Bogoriense (BO) for reference and long-term curation. Voucher accession numbers corresponding to each species are provided in Table 1. The deposition of voucher specimens ensures that future studies can re-examine the taxonomic identity of the analyzed material and facilitates integration of phytochemical data with taxonomic and ecological research.

Table 1. List of *Selaginella* species, collection sites, and ecological characteristics

Species name	Collection locality	Province (Indonesia)	Habitat type	Elevation (masl)	Voucher specimen
Selaginella opaca	Karangtengah: Sikidang Crater	Central Java	*Albizia lophantha* forest above a volcanic crater	2,124	ADS 22
S. remotifolia	Karangtengah: Sikidang Crater	Central Java	Dirt inspection road of the geothermal steam pipeline	2,071	ADS 23
S. intermedia-01	Citalahab-Malasari: Nirmala Tea Plantation	West Java	Forest trail and tea plantation margin	1,118	ADS 117
S. subalpina-01	Banyukuning: Gedongsongo Temple	Central Java	Shaded area under the bamboo canopy	1,309	ADS 45
S. involvens-01	Kledung Village: Temporary tobacco field	Central Java	Drainage edge of the tobacco field	1,493	ADS 38
S. wildenowii	Citalahab-Malasari: Nirmala Tea Plantation	West Java	Forest trail and tea plantation margin	1,118	ADS 116
S. mayeri	Bogor Botanical Gardens	West Java	Wild growth among botanical garden collections	262	ADS 111
S. ornata	Citalahab-Malasari: Nirmala Tea Plantation	West Java	Forest trail and tea plantation margin	1,118	ADS 118
S. plana	Gondowido: Lake Ngebel	East Java	Lake margin	786	ADS 52
S. frondosa	Bogor Botanical Gardens	West Java	Wild growth among botanical garden collections	262	ADS 112
S. subalpina-02	Gunung Bunder 2: Mount Halimun Salak National Park	West Java	Cliff in Damar Forest	869	ADS 136
S. intermedia-02	Gunung Bunder 2: Mount Halimun Salak National Park	West Java	Cliff in Damar Forest	869	ADS 135
S. aristata	Berjo: Ngargoyoso Grand Forest Park	Central Java	Roadside cliff	1,220	ADS 8
S. ciliaris	Gentan: Batuseribu Park	Central Java	Small river cliff	182	ADS 46
S. repanda	Kulurejo: Riparian agroforestry	Central Java	Hills near a small stream	185	ADS 134
S. involvens-02	Baturiti: Batu Kahu Nature Reserve	Bali	Montane forest floor	1,403	ADS 155
S. ketra-ayam	Mount Menumbing	Bangka Belitung Islands	Hill forest	400	ADS 146
S. velutina	Cycloops Mountain Nature Reserve	Papua	Lower montane forest	281	ADS 141
Selaginella sp. (Mt. Meja)	Mount Meja Protected Forest	West Papua	Lowland protected forest	102	ADS 145
S. caudata	Mount Meja Protected Forest	West Papua	Lowland protected forest	102	ADS 144

Note: Numeric suffixes (e.g., -01, -02) indicate distinct samples of the same species collected from different localities or collection events, following the original sample labeling. Elevation is expressed in meters above sea level (masl)

Sample preparation and flavonoid extraction

Plant materials of each *Selaginella* sample were processed following a standardized protocol to ensure comparability of flavonoid profiles across species. Fresh aerial parts, including stems and leaves, were cleaned to remove adhering soil and debris, then air-dried under shade at ambient temperature to minimize degradation of phenolic compounds. Dried samples were subsequently ground into a fine powder using a laboratory mill and stored in airtight containers at room temperature until extraction.

Flavonoid extraction was carried out using a solvent-based maceration method commonly employed in phytochemical studies (Stalikas 2007; Azmir et al. 2013). Approximately 0.5 g of powdered plant material was extracted with 10 mL of analytical-grade methanol, selected for its effectiveness in solubilizing flavonoid and other phenolic compounds (Dai and Mumper 2010). The mixture was sonicated for 30 min at room temperature to enhance extraction efficiency, followed by static maceration for 24 h in the dark (Shehzadi et al. 2025). After centrifugation at 4,000 rpm for 10 min, the supernatant was collected for subsequent analysis (Stalikas 2007).

To ensure sample consistency, all extracts were filtered through 0.45 µm PTFE syringe filters prior to HPLC analysis to remove particulates and protect the chromatographic system (Stalikas 2007; Snyder et al. 2010). Filtrates were transferred into amber vials and stored at 4°C to minimize degradation of phenolic compounds before analysis (Dai and Mumper 2010). No further purification or fractionation was applied, as the aim was to obtain holistic flavonoid fingerprints rather than isolated compounds, thereby preserving the overall chromatographic pattern and enabling direct comparison of retention time-based profiles among samples (Liang et al. 2004; Xie et al. 2006).

Extraction conditions, solvent type, sample-to-solvent ratio, and filtration procedures were kept identical for all samples to minimize methodological bias. Such standardization is essential for reliable interpretation of interspecific differences in flavonoid fingerprints derived from HPLC analysis.

HPLC instrumentation and analytical conditions

The HPLC analysis of flavonoid extracts was performed using a reversed-phase system equipped with a quaternary pump, an autosampler, a column oven, and a photodiode array (PDA) detector. Chromatographic separation was achieved on a C18 reversed-phase column (250 × 4.6 mm, 5 µm particle size), which is commonly employed for the analysis of flavonoids and related phenolic compounds due to its robustness and broad applicability (Snyder et al. 2010).

The gradient system and mobile phase composition followed commonly applied RP-HPLC conditions for flavonoid fingerprinting (Liang et al. 2004; Stalikas 2007). The mobile phase consisted of solvent A (water containing 0.1% formic acid) and solvent B (acetonitrile). A linear gradient elution was applied to ensure adequate separation of flavonoid constituents with varying polarities. The gradient program was as follows: 10% B at 0 min, increased to 30% B over 15 min, then to 50% B at 30 min, and finally to 70% B at 45 min, followed by a 5 min re-equilibration to initial conditions. The flow rate was maintained at 1.0 mL min^{-1}, the column temperature was set at 30°C, and the injection volume was 10 µL.

Detection was carried out using a PDA detector, with chromatograms recorded at 270 nm, a wavelength commonly applied for monitoring flavonoid aglycones and biflavonoids due to their strong UV absorbance in this region (Liang et al. 2004; Stalikas 2007). For fingerprint analysis, chromatograms obtained at 270 nm were primarily used, providing adequate sensitivity for comparative assessment of major flavonoid constituents across samples. Representative chromatograms presented in this study were selected to reflect the range of profile complexity observed across the entire dataset.

To support RT stability and analytical consistency across runs, a reference standard of amentoflavone was analyzed under identical chromatographic conditions (Figure 1). The standard produced a single, well-resolved peak with a consistent retention time (≈7.27 min), serving as an external RT reference to monitor system performance and alignment reliability. The use of this reference was intended solely to support RT reproducibility and calibration, not for compound identification within sample chromatograms.

Method reproducibility was further evaluated through replicate injections of selected samples under identical chromatographic conditions. Retention time stability was assessed by calculating the Relative Standard Deviation (RSD) of major peak retention times, which consistently remained below 2%, indicating that the analytical conditions were sufficiently stable to support reliable comparison of RT-based flavonoid fingerprints across all Selaginella samples (Xie et al. 2006).

Peak detection and RT alignment

Peak detection was conducted on HPLC chromatograms obtained under standardized analytical conditions using the instrument's integrated chromatographic software. A consistent signal-to-noise threshold was applied uniformly across all samples, and only peaks with well-defined shapes and intensities clearly exceeding baseline noise were retained to minimize the inclusion of artefacts.

The RT alignment was performed to enable accurate comparison of chromatographic features among samples. Minor RT shifts arising from instrumental variability or matrix effects were accommodated by applying a general tolerance window of ±0.2 min for peak matching across chromatograms. Peaks falling within this tolerance range were treated as representing the same chromatographic feature, a criterion commonly adopted in chromatographic fingerprinting studies to balance alignment robustness and sensitivity (Liang et al. 2004; Xie et al. 2006).

For RT-based fingerprint construction, a narrow RT bin (7.25-7.40 min) was defined as an operational alignment feature (RT3) corresponding to the elution region of the amentoflavone reference peak. This restricted window was intentionally selected to capture only consistently aligned peaks and to exclude borderline signals occurring slightly earlier or later than the reference RT. The use of a narrow RT bin is critical for preserving feature homology across samples and for preventing artificial inflation of similarity values in subsequent similarity, clustering, and ordination analyses. Accordingly, RT3 represents a conservative alignment feature rather than an exhaustive indicator of amentoflavone occurrence.

Aligned peaks were further inspected visually to confirm consistency in peak shape and relative elution order across samples. This semi-automated approach reduced the risk of misalignment associated with fully automated algorithms, particularly when handling complex plant extracts.

The resulting aligned RT dataset formed the basis for constructing a binary presence–absence fingerprint matrix used in subsequent similarity and clustering analyses. By focusing on RT-defined features rather than absolute peak areas, this approach emphasizes qualitative pattern recognition and interspecific comparability, which are central to the objectives of this study.

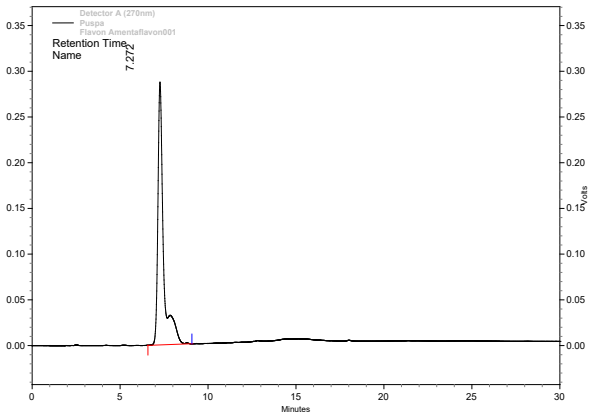

Figure 1. HPLC chromatogram of amentoflavone reference standard (270 nm) showing a single peak at RT 7.27 min under the chromatographic conditions applied in this study

Construction of RT-based fingerprint matrix

An RT-based fingerprint matrix was constructed using aligned chromatographic data to facilitate comparative analysis of flavonoid profiles across *Selaginella* species. Retention Times (RTs) of detected peaks were compiled for all samples, and a tolerance threshold of ±0.2 min was applied to define equivalent chromatographic features among different samples. Peaks eluting within this RT window were considered to represent the same flavonoid-related signal, following established practices in chromatographic fingerprinting studies (Liang et al. 2004; Xie et al. 2006).

Based on this criterion, a binary presence-absence matrix was generated, in which each row corresponded to an individual *Selaginella* sample and each column represented a distinct RT-defined peak. The presence of a peak within the defined RT window was coded as "1," while its absence was coded as "0." Peak intensity or area was not included in the matrix to avoid bias arising from concentration differences or matrix effects, thereby emphasizing qualitative pattern recognition rather than quantitative abundance.

The resulting RT matrix summarizes the distribution of flavonoid-related chromatographic features across all analyzed species and samples. This matrix is presented in Table 2, which serves as the primary dataset for subsequent similarity analysis and cluster construction. By standardizing RT thresholds and using a binary scoring approach, the fingerprint matrix provides a robust and reproducible framework for evaluating interspecific similarities and differences in flavonoid profiles among *Selaginella* species.

Similarity analysis and multivariate statistics

Similarity analysis was performed using the RT-based binary fingerprint matrix derived from aligned chromatographic peaks. Pairwise similarity among *Selaginella* samples was quantified using both Sørensen and Jaccard similarity coefficients, which are widely applied to presence-absence data in comparative chemical and ecological studies (Jaccard 1901; Sørensen 1948). These indices emphasize shared features between samples while minimizing the influence of joint absences, making them suitable for RT-based chromatographic fingerprints.

The Sørensen coefficient was calculated as $2a/(2a+b+c)$, whereas the Jaccard coefficient was computed as $a/(a+b+c)$, where a represents the number of shared RT-defined peaks between two samples, and b and c represent peaks unique to each sample. Similarity matrices generated from these coefficients formed the basis for subsequent multivariate analyses.

Hierarchical cluster analysis was conducted to visualize patterns of chemical relatedness among *Selaginella* species. Clustering was performed using the Unweighted Pair Group Method with Arithmetic (UPGMA) mean based on the similarity matrices, a method commonly used for chemotaxonomic and fingerprinting studies due to its interpretability and robustness (Everitt et al. 2011; Legendre and Legendre 2012). The resulting dendrogram illustrates the grouping of samples according to overall flavonoid fingerprint similarity and was used to identify major chemotypic clusters among the analyzed species.

To complement hierarchical clustering and to provide an ordination-based visualization of overall similarity patterns, Non-Metric Multidimensional Scaling (NMDS) was additionally applied to the RT-based binary fingerprint matrix. NMDS was performed using a Sørensen dissimilarity matrix, which is appropriate for presence-absence data and emphasizes shared chromatographic features while minimizing the influence of joint absences. The analysis was conducted in a two-dimensional solution, and ordination quality was evaluated using the stress value as a measure of goodness-of-fit. NMDS was employed as an exploratory visualization tool rather than a formal hypothesis-testing framework, with the aim of assessing whether major similarity patterns observed in clustering analysis were consistently reflected in an ordination space.

RESULTS AND DISCUSSION

Overview of HPLC chromatographic profiles

The HPLC chromatographic profiles of flavonoid extracts from the analyzed *Selaginella* species exhibited pronounced qualitative differences in peak distribution, profile complexity, and overall chromatographic architecture. Representative chromatograms illustrating these interspecific variations are shown in Figure 2. All samples produced multiple resolved peaks under identical extraction and analytical conditions, confirming that flavonoids constitute a prominent component of the secondary metabolite profiles across the genus.

Clear variation was observed in the number and distribution of peaks along the retention time axis. Several species displayed complex chromatographic profiles characterized by numerous peaks distributed across mid to late retention times, indicating a diverse assemblage of flavonoid-related constituents. In contrast, other species exhibited simpler profiles dominated by a limited number of peaks, reflecting a more restricted flavonoid composition. These differences suggest substantial heterogeneity in flavonoid profiles among *Selaginella* species, even when methodological variables are fully standardized.

Samples representing the same nominal species but collected from different localities, as indicated by numerical suffixes, generally shared a similar overall chromatographic framework. Major retention time regions were often conserved between conspecific samples, while additional peaks or minor shifts in peak occurrence contributed to subtle intra-specific variation. This pattern indicates that species-level flavonoid fingerprints are broadly stable, with local environmental conditions potentially influencing the presence of secondary or low-frequency peaks without altering the core chromatographic structure.

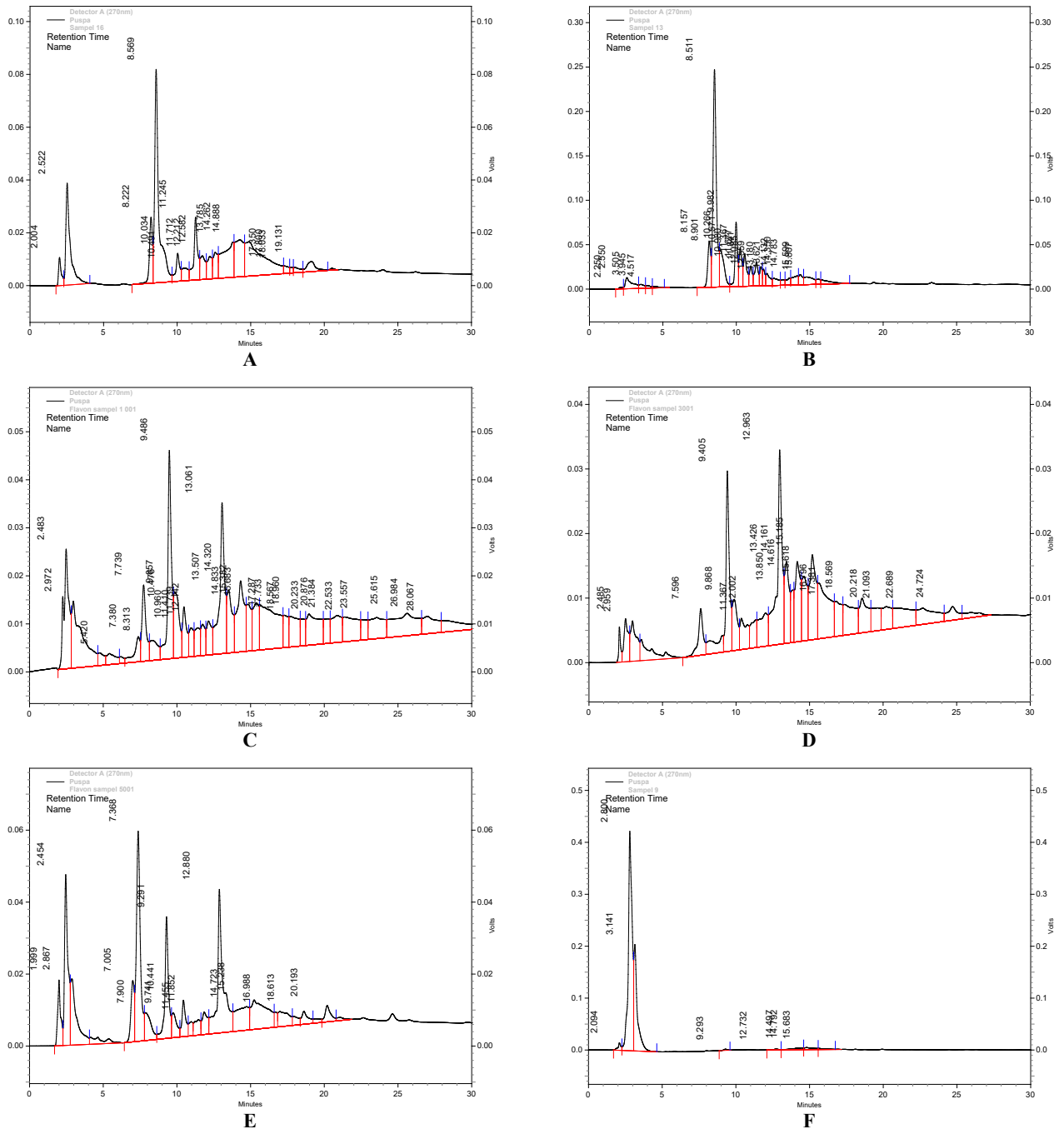

Figure 2. Representative HPLC chromatograms of flavonoid extracts from selected *Selaginella* species recorded at 270 nm. A. *S. ciliaris*, B. *S. aristata*, C. *S. opaca*, D. *S. intermedia*-01, E. *S. involvens*-01, F. *S. plana*

Across the dataset, several retention time regions were recurrently occupied by prominent peaks in multiple species, suggesting the presence of shared flavonoid-related components within the genus. At the same time, distinct peaks confined to one or a few species contributed to unique chromatographic signatures. Together, these patterns demonstrate that HPLC-based profiling effectively captures both shared and species-specific features of flavonoid composition in *Selaginella* and provides a qualitative foundation for subsequent RT-based fingerprint matrix construction and similarity analysis.

RT-based flavonoid fingerprint matrix

Alignment of HPLC chromatographic peaks across all *Selaginella* samples resulted in a discrete set of 11 RT-defined flavonoid features, representing chromatographic signals that are either shared among multiple species or restricted to particular taxa. Using a tolerance window of ±0.2 min, peaks eluting within the same RT interval were treated as equivalent features and compiled into a binary RT-based fingerprint matrix (Table 2). This matrix summarizes the qualitative presence-absence distribution of flavonoid-related peaks across all analyzed samples and

constitutes the primary dataset for subsequent similarity and clustering analyses.

As shown in Table 2, the RT-based fingerprint matrix reveals clear heterogeneity in flavonoid composition among *Selaginella* species. Several of the 11 RT features are detected in multiple species, indicating the occurrence of shared flavonoid components that may reflect conserved biosynthetic traits within the genus. In contrast, other RT features are present only in one or a few species, highlighting species-specific or low-frequency flavonoid constituents that contribute to distinct chromatographic signatures.

Conspecific samples collected from different localities, such as *Selaginella intermedia*-01 versus -02 and *S. involvens*-01 versus -02, exhibit partially overlapping RT profiles within the 11-feature matrix (Table 2). These samples share a set of core RT peaks while differing in the presence of auxiliary peaks, indicating intraspecific variability superimposed on broader interspecific differentiation. This pattern suggests that species-level flavonoid fingerprints are generally stable, with additional variation likely influenced by local environmental or microhabitat factors.

The RT-based fingerprint matrix comprising 11 aligned RT bins provides a standardized qualitative framework for evaluating similarities and differences in flavonoid profiles across *Selaginella* species. By explicitly resolving shared and unique RT features (Table 2), this matrix forms the analytical basis for similarity coefficient calculation and hierarchical clustering presented in subsequent sections.

Similarity matrix among *Selaginella* species

Pairwise similarity among all Selaginella samples was quantified using Sørensen and Jaccard coefficients derived from the RT-based binary fingerprint matrix. Both indices yielded comparable patterns of qualitative chemical relatedness; therefore, the Sørensen similarity matrix is presented as the primary result to represent overall similarity relationships among samples, while Jaccard values are used as complementary support in the interpretation (Table 3).

Similarity values spanned a broad range across sample pairs, indicating substantial heterogeneity in flavonoid fingerprint composition within the dataset. Conspecific samples collected from different localities, such as *S. intermedia*-01 versus -02 and *S. involvens*-01 versus -02, consistently exhibited moderate to high similarity values, reflecting the presence of shared core flavonoid features accompanied by additional variation at the individual level.

In contrast, comparisons among ecologically and geographically distinct samples generally resulted in lower similarity values, suggesting pronounced differentiation in overall flavonoid composition. Nevertheless, several sample pairs displayed relatively high similarity coefficients despite geographic separation, indicating the presence of chemical affinities that are not strictly structured by spatial proximity. Such patterns suggest that RT-based flavonoid fingerprints may capture chemotaxonomic signals that extend beyond local environmental variation.

Table 2. RT-based flavonoid fingerprint matrix across *Selaginella* species

Species	RT1 (2.05-2.25)	RT2 (2.45-2.60)	RT3 (7.25-7.40)	RT4 (8.45-8.55)	RT5 (9.90-10.05)	RT6 (11.30-11.45)	RT7 (12.25-12.35)	RT8 (13.45-13.55)	RT9 (14.30-14.40)	RT10 (14.75-14.85)	RT11 (15.60-15.75)
Selaginella opaca	1	1	1	0	1	1	0	1	1	1	1
S. remotifolia	0	1	0	0	1	0	1	1	0	1	1
S. intermedia-01	0	1	0	0	1	0	1	1	1	0	0
S. subalpina-01	1	1	1	0	1	0	1	0	1	1	0
S. involvens-01	1	1	0	0	1	0	0	0	0	1	0
S. wildenowii	1	1	0	0	1	0	0	0	1	1	0
S. mayeri	1	1	0	0	1	0	0	0	1	0	0
S. ornata	1	1	0	0	0	0	0	0	1	0	0
S. plana	1	0	0	0	0	0	0	0	0	0	0
S. frondosa	1	1	0	0	1	0	1	0	1	1	0
S. subalpina-02	1	1	0	1	0	1	1	0	1	1	0
S. intermedia-02	1	1	0	1	0	1	0	0	1	1	0
S. aristata	1	1	0	1	1	1	0	0	1	1	1
S. ciliaris	1	1	1	1	1	1	1	0	1	1	1
S. repanda	1	0	0	0	0	0	0	0	1	0	0
S. involvens-02	1	1	0	1	1	1	0	0	1	1	0
S. ketra-ayam	1	1	0	1	1	0	0	0	1	1	0
S. velutina	0	1	0	1	1	1	1	0	1	1	1
Selaginella sp. (Mt. Meja)	1	1	0	0	0	0	0	0	1	1	0
S. caudata	1	1	0	0	0	0	0	0	1	1	0

Note: Binary scores (1/0) indicate the presence or absence of a chromatographic peak within each RT bin for a given species, based on aligned HPLC chromatograms using a tolerance of ±0.2 min. RT3 corresponds to the retention time of the amentoflavone reference peak (~7.27 min) and was used as an alignment anchor. The matrix represents qualitative RT-based features and does not imply compound identity beyond the reference anchor

Table 3. Sørensen similarity matrix of RT-based flavonoid fingerprints among 20 *Selaginella* samples

Species	No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Selaginella opaca	1	1.000	0.667	0.571	0.750	0.615	0.714	0.615	0.500	0.200	0.667	0.625	0.667	0.824	0.842	0.364	0.750	0.667	0.706	0.615	0.615
S. remotifolia	2	0.667	1.000	0.727	0.615	0.600	0.545	0.400	0.222	0.000	0.667	0.462	0.333	0.571	0.625	0.000	0.462	0.500	0.714	0.400	0.400
S. intermedia-01	3	0.571	0.727	1.000	0.667	0.444	0.600	0.667	0.500	0.000	0.727	0.500	0.364	0.462	0.533	0.286	0.500	0.545	0.615	0.444	0.444
S. subalpina-01	4	0.750	0.615	0.667	1.000	0.727	0.833	0.727	0.600	0.250	0.923	0.714	0.615	0.667	0.824	0.444	0.714	0.769	0.667	0.727	0.727
S. involvens-01	5	0.615	0.600	0.444	0.727	1.000	0.889	0.750	0.571	0.400	0.800	0.545	0.600	0.667	0.571	0.333	0.727	0.800	0.500	0.750	0.750
S. wildenowii	6	0.714	0.545	0.600	0.833	0.889	1.000	0.889	0.750	0.333	0.909	0.667	0.727	0.769	0.667	0.571	0.833	0.909	0.615	0.889	0.889
S. mayeri	7	0.615	0.400	0.667	0.727	0.750	0.889	1.000	0.857	0.400	0.800	0.545	0.600	0.667	0.571	0.667	0.727	0.800	0.500	0.750	0.750
S. ornata	8	0.500	0.222	0.500	0.600	0.571	0.750	0.857	1.000	0.500	0.667	0.600	0.667	0.545	0.462	0.800	0.600	0.667	0.364	0.857	0.857
S. plana	9	0.200	0.000	0.000	0.250	0.400	0.333	0.400	0.500	1.000	0.286	0.250	0.286	0.222	0.182	0.667	0.250	0.286	0.000	0.400	0.400
S. frondosa	10	0.667	0.667	0.727	0.923	0.800	0.909	0.800	0.667	0.286	1.000	0.769	0.667	0.714	0.750	0.500	0.769	0.833	0.714	0.800	0.800
S. subalpina-02	11	0.625	0.462	0.500	0.714	0.545	0.667	0.545	0.600	0.250	0.769	1.000	0.923	0.800	0.824	0.444	0.857	0.769	0.800	0.727	0.727
S. intermedia-02	12	0.667	0.333	0.364	0.615	0.600	0.727	0.600	0.667	0.286	0.667	0.923	1.000	0.857	0.750	0.500	0.923	0.833	0.714	0.800	0.800
S. aristata	13	0.824	0.571	0.462	0.667	0.667	0.769	0.667	0.545	0.222	0.714	0.800	0.857	1.000	0.889	0.400	0.933	0.857	0.875	0.667	0.667
S. ciliaris	14	0.842	0.625	0.533	0.824	0.571	0.667	0.571	0.462	0.182	0.750	0.824	0.750	0.889	1.000	0.333	0.824	0.750	0.889	0.571	0.571
S. repanda	15	0.364	0.000	0.286	0.444	0.333	0.571	0.667	0.800	0.667	0.500	0.444	0.500	0.400	0.333	1.000	0.444	0.500	0.200	0.667	0.667
S. involvens-02	16	0.750	0.462	0.500	0.714	0.727	0.833	0.727	0.600	0.250	0.769	0.857	0.923	0.933	0.824	0.444	1.000	0.923	0.800	0.727	0.727
S. ketra-ayam	17	0.667	0.500	0.545	0.769	0.800	0.909	0.800	0.667	0.286	0.833	0.769	0.833	0.857	0.750	0.500	0.923	1.000	0.714	0.800	0.800
S. velutina	18	0.706	0.714	0.615	0.667	0.500	0.615	0.500	0.364	0.000	0.714	0.800	0.714	0.875	0.889	0.200	0.800	0.714	1.000	0.500	0.500
Selaginella sp. (Mt. Meja)	19	0.615	0.400	0.444	0.727	0.750	0.889	0.750	0.857	0.400	0.800	0.727	0.800	0.667	0.571	0.667	0.727	0.800	0.500	1.000	1.000
S. caudata	20	0.615	0.400	0.444	0.727	0.750	0.889	0.750	0.857	0.400	0.800	0.727	0.800	0.667	0.571	0.667	0.727	0.800	0.500	1.000	1.000

Note: Similarity values were calculated using the Sørensen coefficient based on the RT-based presence–absence fingerprint matrix (Table 2). Numeric suffixes indicate distinct samples of the same species collected from different localities

Conversely, samples characterized by simplified RT profiles or a limited number of shared chromatographic features showed consistently low similarity values when compared with most other taxa. The Sørensen similarity matrix provides a quantitative foundation for evaluating qualitative chemical relationships among Selaginella samples and serves as the basis for subsequent hierarchical clustering analysis.

Cluster analysis of flavonoid fingerprints

Hierarchical cluster analysis was performed to visualize patterns of chemical relatedness among Selaginella samples based on the Sørensen similarity matrix derived from the RT-based flavonoid fingerprint data (Table 3). Clustering was conducted using the Unweighted Pair Group Method with Arithmetic mean (UPGMA), which is widely applied in chemotaxonomic and fingerprinting studies due to its interpretability and suitability for similarity-based datasets. The resulting clustering pattern is illustrated in Figure 3.

The dendrogram reveals several distinct clusters that reflect differences and affinities in flavonoid fingerprint composition among samples and species. Samples belonging to the same nominal species but collected from different localities, such as *S. intermedia*-01 and -02, as well as *S. involvens*-01 and -02, were consistently grouped within the same or closely adjacent clusters. This pattern indicates that core species-level flavonoid signatures are generally preserved at the individual level despite site-specific variation, supporting the robustness of RT-based fingerprinting for comparative analysis.

At a broader level, clusters comprised multiple species sharing relatively high similarity values, suggesting the presence of broad chemotypic affinities that are not strictly aligned with geographic origin. Several samples collected from different regions were positioned within the same major cluster, indicating that flavonoid composition may capture intrinsic biochemical traits that are not exclusively structured by spatial proximity. Conversely, samples characterized by simplified RT profiles or a limited number of shared peaks tended to form isolated branches, reflecting their distinct flavonoid fingerprints.

The clustering pattern provides an integrated visualization of qualitative chemical relationships among Selaginella samples, which can be interpreted at the species level when conspecific samples exhibit consistent grouping. When interpreted together with the RT-based fingerprint matrix and similarity analysis, the dendrogram highlights the potential of flavonoid fingerprinting to contribute to chemotaxonomic assessment and comparative phytochemical studies within the genus.

NMDS ordination of RT-based flavonoid fingerprints

The NMDS ordination based on the Sørensen dissimilarity matrix provided a low-dimensional visualization of similarity relationships among Selaginella samples derived from the RT-based flavonoid fingerprint matrix (Figure 4). The ordination was constructed using the full Sørensen dissimilarity matrix (Table 3), and the two-dimensional solution yielded a low stress value (Stress-1 ≈ 0.087), indicating that the ordination adequately represents the multivariate similarity structure of the dataset.

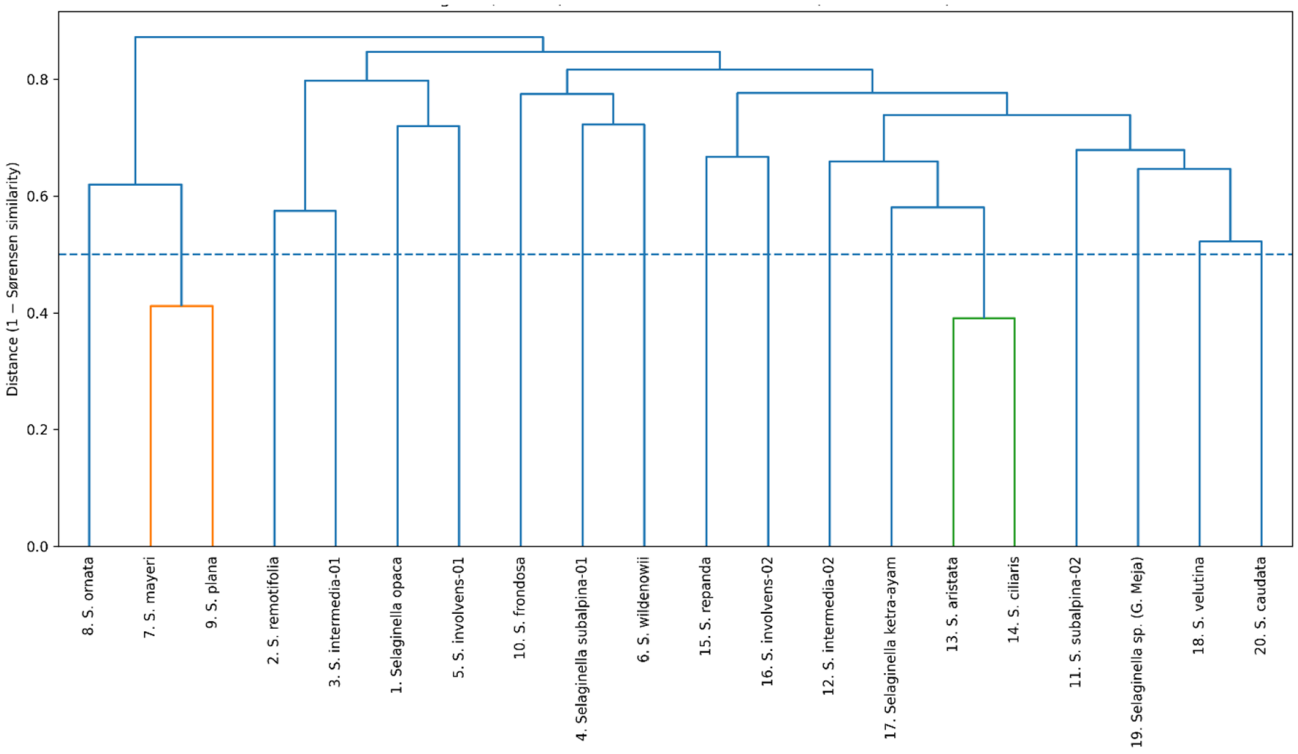

Figure 3. Dendrogram (UPGMA) showing hierarchical clustering of *Selaginella* species based on Sørensen similarity of RT-based flavonoid fingerprints (cut-off = 0.45)

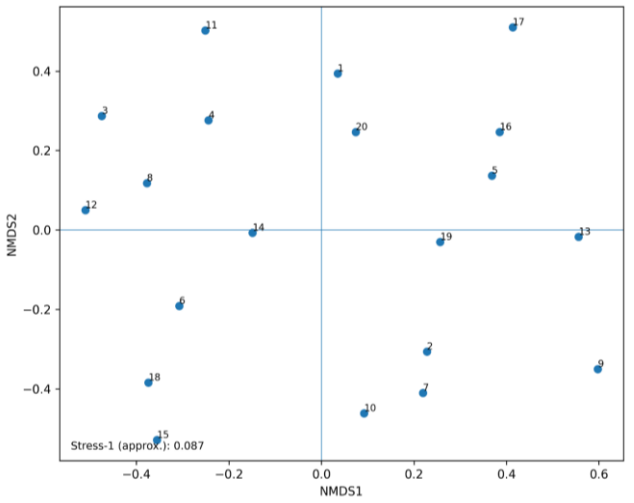

Figure 4. NMDS ordination of *Selaginella* samples based on Sørensen dissimilarity of RT-based flavonoid fingerprints (presence-absence matrix). Each point represents one sample (1-20), and inter-point distances reflect overall qualitative similarity in RT-defined features. The ordination is presented as an exploratory visualization complementary to hierarchical clustering. Stress-1 (approx.) = 0.087. Sample codes: 1. *S. opaca*, 2. *S. remotifolia*, 3. *S. intermedia*-01, 4. *S. subalpina*-01, 5. *S. involvens*-01, 6. *S. wildenowii*, 7. *S. mayeri*, 8. *S. ornata*, 9. *S. plana*, 10. *S. frondosa*, 11. *S. subalpina*-02, 12. *S. intermedia*-02, 13. *S. aristata*, 14. *S. ciliaris*, 15. *S. repanda*, 16. *S. involvens*-02, 17. *S. ketra-ayam*, 18. *S. velutina*, 19. *Selaginella* sp. (Mt. Meja), 20. *S. caudata*

Overall, samples that clustered closely in the UPGMA dendrogram also tended to occupy proximate positions in the NMDS ordination space. Conspecific samples collected from different localities showed partial overlap or close proximity, reflecting shared core flavonoid features with limited divergence. In contrast, samples characterized by simplified RT profiles tended to be positioned toward the periphery of the ordination, consistent with their lower similarity values observed in pairwise comparisons.

The NMDS ordination did not reveal a clear separation of samples based on geographic origin, supporting the inference that overall flavonoid fingerprint similarity is not primarily structured by spatial proximity. Instead, the ordination reflects broad qualitative chemical similarity among samples, in agreement with the clustering analysis, and is interpreted here as a complementary visualization rather than evidence of discrete chemotypic boundaries.

Summary of flavonoid fingerprint variation across *Selaginella* species

The integrated results of chromatographic profiling, RT-based fingerprint construction, similarity analysis, and hierarchical clustering collectively reveal structured patterns of qualitative flavonoid variation among the analyzed Selaginella samples. Visual comparison of chromatographic profiles demonstrated pronounced interspecific and inter-individual differences in peak distribution and overall profile complexity, indicating heterogeneous flavonoid compositions across the genus (Figure 2). These qualitative differences provided the empirical basis for subsequent RT alignment and comparative analysis.

The RT-based fingerprint matrix captures the distribution of aligned flavonoid-related peaks across all analyzed samples and highlights the coexistence of conserved and variable chemical features (Table 2). Several RT-defined peaks were consistently detected across multiple species, suggesting the presence of shared flavonoid components within Selaginella. In contrast, numerous peaks were restricted to a limited number of species or individual samples, contributing to distinct chromatographic signatures. This pattern reflects a dual structure of flavonoid diversity, comprising both genus-level conserved elements and species- or sample-specific differentiation.

Quantitative similarity analysis based on the Sørensen coefficient translated these qualitative patterns into measurable similarity relationships among samples (Table 3). Conspecific samples collected from different localities exhibited moderate to high similarity values, indicating that core flavonoid fingerprints are generally stable at the species level. At the same time, lower similarity values observed between ecologically or taxonomically distinct samples underscore pronounced divergence in overall flavonoid composition, reinforcing the discriminatory power of RT-based fingerprinting.

Hierarchical clustering analysis integrates these findings by providing a coherent visualization of chemical relatedness among *Selaginella* samples (Figure 3). The resulting dendrogram reveals clusters that group samples and species with similar flavonoid fingerprints, while also identifying chemically distinct taxa that form isolated branches. Importantly, these chemotypic groupings are not strictly aligned with geographic origin, suggesting that intrinsic biochemical characteristics play a substantial role in shaping flavonoid profiles.

The combined results demonstrate that RT-based HPLC fingerprinting provides a robust qualitative framework for assessing interspecific flavonoid diversity based on individual-level chemical data in Selaginella. Chromatographic profiles, fingerprint matrices, similarity indices, and clustering analysis consistently reveal patterns of shared and species-specific chemical variation, providing a basis for interpreting comparative chemotaxonomic patterns and exploratory ecological implications.

Discussion

Flavonoid fingerprinting as a tool for interspecific discrimination

The results of this study demonstrate that RT-based HPLC flavonoid fingerprinting provides an effective qualitative tool for discriminating among *Selaginella* species based on their secondary metabolite profiles. Distinct chromatographic patterns observed across species, as reflected in peak distribution, profile complexity, and similarity relationships, indicate that flavonoid fingerprints capture biologically meaningful variation at the interspecific level. This finding is consistent with previous phytochemical studies showing that flavonoid composition

often exhibits species-specific patterns linked to genetic background and evolutionary history (Markham 1988; Harborne and Williams 2000).

In vascular plants, flavonoids have long been recognized as valuable chemotaxonomic markers due to their structural diversity, relative stability, and taxonomic consistency (Harborne 1994; Harborne and Williams 2000). In pteridophytes, including *Selaginella*, flavonoids are among the most frequently reported secondary metabolites, yet their application for comparative and chemotaxonomic purposes has remained limited. Most existing studies on *Selaginella* flavonoids have focused on the isolation and structural elucidation of individual compounds or on targeted LC-MS screening of selected taxa (Cao et al. 2010; Zhao et al. 2013). While such approaches provide detailed chemical information, they are less suited for broad interspecific comparisons across multiple taxa.

The RT-based fingerprinting approach adopted in this study addresses this limitation by emphasizing holistic chromatographic patterns rather than individual compound identities. Similar fingerprinting strategies have been widely applied in medicinal plant authentication, species discrimination, and quality control, particularly when dealing with complex phytochemical mixtures (Gong et al. 2003; Liang et al. 2004; Xie et al. 2006). The present results align with these studies by showing that reproducible retention time patterns can be translated into binary matrices and similarity indices that effectively differentiate species based on overall chemical composition.

The moderate to high similarity values observed among conspecific samples collected from different localities further support the utility of flavonoid fingerprints for species-level discrimination. Such stability of core chromatographic features suggests that flavonoid profiles are largely governed by intrinsic biochemical and genetic factors, with environmental variation contributing secondary modifications. Comparable patterns have been reported in fingerprint-based studies of other plant groups, where interspecific variation exceeds intraspecific variability when standardized analytical protocols are applied (Zhao et al. 2006; Smillie and Khan 2010).

Importantly, the clustering patterns derived from similarity analysis reveal chemical affinities that are not strictly correlated with geographic proximity. This observation suggests that flavonoid fingerprinting may capture deeper chemotaxonomic signals reflecting shared evolutionary or biosynthetic traits among species, rather than merely environmental influences. Similar findings have been reported in chemotaxonomic studies of ferns and lycophytes, where flavonoid distribution patterns were found to complement morphological and molecular evidence (Wollenweber et al. 1998; Vetter 2018).

Taken together, these results highlight the value of RT-based HPLC flavonoid fingerprinting as a practical and informative approach for interspecific discrimination in *Selaginella*. By enabling comparative analysis across multiple species without reliance on full compound identification, this method provides a robust framework for exploring chemical diversity, supporting chemotaxonomic inference, and guiding the selection of taxa for further bioactivity-oriented phytochemical studies.

Chemotaxonomic implications of flavonoid variation in Selaginella

The observed patterns of flavonoid fingerprint variation among *Selaginella* species carry important implications for chemotaxonomic interpretation within the genus. The coexistence of shared and species-specific RT-defined peaks suggests that flavonoid composition reflects both conserved biochemical traits and lineage-specific differentiation. Such dual patterns are characteristic of secondary metabolite systems that are shaped by evolutionary constraints as well as diversification processes, making flavonoids particularly informative for chemotaxonomic assessment (Harborne 1994; Wink 2003).

In chemotaxonomy, the value of flavonoids lies not only in the presence of particular compounds but also in broader distributional patterns across taxa. In this study, several RT regions were consistently represented across multiple *Selaginella* species, indicating the presence of common flavonoid-related components at the genus level. These shared features may reflect conserved biosynthetic pathways that have been maintained throughout the evolutionary history of *Selaginella*, one of the earliest diverging lineages of vascular plants (Banks 2009; Weststrand and Korall 2016a, b). Comparable genus-level conservation of flavonoid patterns has been reported in ferns and other lycophytes, where flavonoid classes show phylogenetically structured distributions (Wollenweber et al. 1998; Vetter 2018).

At the same time, the occurrence of distinct RT peaks restricted to specific species or small species groups highlights the potential of flavonoid fingerprints to resolve finer-scale taxonomic relationships. Species-specific or narrowly distributed flavonoid features have long been recognized as valuable chemotaxonomic markers, particularly in groups where morphological differentiation is subtle or convergent (Wollenweber et al. 1998; Harborne and Williams 2000). In *Selaginella*, morphological plasticity and ecological adaptation often complicate species delimitation, suggesting that complementary chemical evidence may provide additional resolution when integrated with morphological and molecular data.

The clustering patterns observed in this study further support the chemotaxonomic relevance of flavonoid fingerprints. Species grouped within the same chemical clusters did not always share close geographic proximity, implying that chemical similarity is not merely a reflection of local environmental conditions. Instead, these groupings may correspond to shared evolutionary or biosynthetic traits, consistent with the view that secondary metabolite profiles can retain phylogenetic signals across broad spatial scales (Wink and Waterman 1999; Wink 2008). Similar chemotaxonomic clustering based on flavonoid profiles has been reported in fern genera and other plant groups, where chemical affinities complement phylogenetic reconstructions based on DNA sequence data (Zhao et al. 2006; Smillie and Khan 2010).

Nevertheless, it is important to recognize that chemotaxonomic interpretation based on fingerprinting approaches remains inherently qualitative. RT-based fingerprints capture overall compositional patterns but do not directly resolve compound identity or biosynthetic pathways. As such, the chemotaxonomic signals inferred here should be viewed as complementary rather than definitive. Integration with molecular phylogenetic frameworks and targeted structural identification of key flavonoid markers would further strengthen taxonomic inference in future studies (Wink and Waterman 1999; Xie et al. 2006; Burleigh et al. 2009).

The flavonoid fingerprint variation documented in this study supports the utility of secondary metabolite profiling as a chemotaxonomic tool in *Selaginella*. By revealing patterns of chemical similarity and differentiation that align imperfectly with geography, RT-based HPLC fingerprinting provides independent evidence of biochemical structuring within the genus. These findings reinforce the value of combining chemical, morphological, and molecular data to achieve a more comprehensive understanding of species relationships and evolutionary diversification in *Selaginella*.

Ecological and adaptive significance of flavonoid diversity in Selaginella

The observed diversity of flavonoid fingerprints among *Selaginella* species also carries important ecological and adaptive implications. Flavonoids are multifunctional secondary metabolites that play key roles in plant-environment interactions, including protection against ultraviolet radiation, oxidative stress, pathogens, and herbivores, as well as mediation of physiological responses to abiotic stress (Agati et al. 2012; Brunetti et al. 2013). In early-diverging vascular plants such as *Selaginella*, these functions may be particularly critical for survival across heterogeneous habitats.

The qualitative variation in flavonoid fingerprints documented in this study, reflected in differences in peak number, distribution, and species-specific features, suggests that adaptive responses to local environmental conditions may shape flavonoid composition. *Selaginella* species occupy a wide range of ecological settings, from shaded montane forests to open, seasonally dry, or disturbed habitats. Such environmental heterogeneity is likely to impose different selective pressures on secondary metabolite production, resulting in distinct flavonoid profiles optimized for specific ecological contexts (Winkel-Shirley 2002; Treutter 2005).

Conspecific samples collected from different localities exhibited broadly similar core flavonoid fingerprints, yet with additional variation in minor or low-frequency peaks. This pattern suggests that while fundamental flavonoid biosynthetic pathways are genetically conserved at the species level, fine-scale modulation of flavonoid expression may occur in response to microhabitat conditions such as light intensity, moisture availability, and temperature regimes. Similar patterns of environmentally modulated flavonoid variation have been reported in ferns and angiosperms, where stress-related flavonoids increase under high irradiance or nutrient limitation without altering the core metabolite profile (Close and McArthur 2002; Agati and Tattini 2010).

The lack of strict correspondence between chemical similarity and geographic proximity further indicates that spatial factors do not solely determine flavonoid diversity in *Selaginella*. Instead, adaptive convergence may occur among species occupying comparable ecological niches in different regions, leading to chemically similar flavonoid fingerprints despite geographic separation. Such convergence has been documented in other plant groups, where similar environmental pressures result in parallel secondary metabolite profiles across unrelated or distantly distributed taxa (Wink 2003; Lattanzio et al. 2006).

The flavonoid fingerprint variation observed in this study supports the view that flavonoids contribute to the ecological flexibility and adaptive capacity of *Selaginella*. While RT-based fingerprinting does not directly resolve functional mechanisms, the detected patterns provide indirect evidence that flavonoid diversity is linked to environmental adaptation. These findings underscore the importance of considering ecological context when interpreting phytochemical variation and highlight the potential of comparative flavonoid profiling for exploring adaptive strategies in early vascular plant lineages.

Methodological strengths and limitations of RT-based HPLC fingerprinting

RT-based HPLC fingerprinting offers several methodological advantages for comparative phytochemical studies, particularly when dealing with multiple species and complex metabolite mixtures such as those found in *Selaginella*. One of the principal strengths of this approach lies in its ability to capture holistic chemical profiles without requiring full structural identification of individual compounds. By focusing on reproducible retention time patterns, fingerprinting enables efficient interspecific comparison and pattern recognition across large datasets (Liang et al. 2004; Xie et al. 2006).

The standardized analytical conditions applied in this study, including consistent extraction procedures, chromatographic parameters, and retention time alignment thresholds, contributed to the reproducibility of the resulting fingerprints. The use of a binary presence-absence matrix further reduced the influence of peak intensity variability, which can be affected by extraction efficiency, sample handling, or detector sensitivity. As a result, the RT-based fingerprints provided a robust qualitative framework for similarity analysis and clustering, suitable for chemotaxonomic and comparative purposes (Gong et al. 2003).

The methodological robustness of RT alignment was further strengthened by the inclusion of an amentoflavone reference standard, which served as an external retention time anchor for biflavonoid-rich regions of the chromatogram. Although amentoflavone was not introduced as an internal standard into each extract, its reproducible elution under identical chromatographic conditions confirms the stability and comparability of retention time patterns across analytical runs. This external

calibration approach enhances confidence in RT-based fingerprint interpretation while remaining consistent with the qualitative objectives of chromatographic fingerprinting.

Another methodological strength of RT-based fingerprinting is its scalability. Compared with targeted compound identification using LC-MS or NMR, fingerprinting requires less analytical time and interpretative effort, making it particularly useful for preliminary screening of chemical diversity across multiple taxa. This characteristic is especially relevant for understudied groups such as lycophytes, where comprehensive phytochemical coverage across species remains limited (Xie et al. 2006).

Despite these advantages, several limitations of the RT-based fingerprinting approach must be acknowledged. Retention time alone does not provide direct information on compound identity, structural class, or biosynthetic origin. Consequently, different compounds with similar chromatographic behavior may contribute to the same RT-defined peak, potentially obscuring finer chemical distinctions. In addition, minor shifts in retention time can occur due to column aging or subtle changes in mobile phase composition, necessitating careful calibration and alignment procedures (Gong et al. 2003).

Furthermore, the qualitative nature of binary scoring precludes quantitative assessment of relative flavonoid abundance. While this approach is appropriate for interspecific discrimination and pattern recognition, it cannot address questions related to metabolite concentration, dominance, or dose-dependent bioactivity. Therefore, RT-based fingerprinting should be viewed as a complementary tool rather than a replacement for targeted quantitative or structural analyses (Xie et al. 2006).

RT-based HPLC fingerprinting represents a methodologically sound and efficient strategy for comparative analysis of flavonoid diversity in *Selaginella*. When combined with rigorous analytical standardization, external RT referencing, and cautious interpretation, this approach provides valuable insights into interspecific chemical variation while laying the groundwork for subsequent targeted phytochemical and bioactivity-oriented investigations.

The inclusion of NMDS ordination further supports the robustness of the similarity patterns derived from RT-based fingerprinting. NMDS was applied conservatively as a complementary visualization tool, and yielded ordination patterns broadly consistent with hierarchical clustering results, with a low stress value indicating an adequate two-dimensional representation of the qualitative similarity structure. Importantly, the absence of sharp group boundaries or strong gradients in the ordination space reinforces the qualitative nature of the dataset and cautions against overinterpretation of fine-scale chemical distances. In this context, NMDS serves to confirm general similarity relationships rather than to infer discrete chemotypes or environmental drivers, which would require quantitative metabolite data or targeted compound identification.

Comparison with previous phytochemical studies on Selaginella

Previous phytochemical investigations on *Selaginella* have substantially expanded knowledge of flavonoid diversity within the genus, particularly through the isolation and structural elucidation of individual compounds. Numerous studies have reported biflavonoids, flavones, and related phenolic compounds from selected *Selaginella* species, often emphasizing their pharmacological potential, such as antioxidant, anti-inflammatory, and cytotoxic activities (Lin et al. 2000; Cao et al. 2010; Zhao et al. 2013; Shim et al. 2018). While these studies provide detailed chemical characterization, their scope has generally been limited to one or a few species and has rarely addressed comparative patterns across multiple taxa.

Targeted LC-MS-based studies have further contributed to flavonoid identification in *Selaginella* by enabling the detection of known compounds and their derivatives in complex extracts (Zhang et al. 2013; Yao et al. 2017; Křížkovská et al. 2021). However, such approaches typically rely on predefined metabolite classes and reference standards, which limit their applicability for holistic chemical comparison among diverse species (Dunn et al. 2013; Wolfender et al. 2013). Consequently, interspecific chemical relationships and broader patterns of flavonoid distribution within *Selaginella* remain insufficiently explored in the existing literature.

In contrast to these compound-centered approaches, the present study adopts a fingerprint-based strategy that emphasizes overall chromatographic patterns rather than individual metabolite identities. This methodological shift allows direct comparison of flavonoid profiles across a wider set of *Selaginella* species under standardized analytical conditions. By constructing an RT-based fingerprint matrix and applying similarity and clustering analyses, this study provides a comparative framework that complements earlier phytochemical reports and addresses a gap in genus-wide chemical assessment, consistent with established chromatographic fingerprinting approaches (Liang et al. 2004; Xie et al. 2006).

Comparative fingerprinting approaches have been successfully applied in other plant groups, particularly in medicinal plants and ferns, where they have proven effective for species discrimination, chemotaxonomic inference, and preliminary screening of chemical diversity (Liang et al. 2004; Zhao et al. 2006; Smillie and Khan 2010). The present results demonstrate that similar strategies are equally applicable to *Selaginella*, a lineage that has received relatively limited attention from a comparative phytochemical perspective.

The findings of this study do not contradict previous reports on flavonoid composition in *Selaginella*, but rather contextualize them within a broader comparative framework. Species previously reported to contain diverse flavonoid classes also tend to exhibit complex chromatographic fingerprints, while species with fewer documented compounds often show simpler profiles. This correspondence suggests that RT-based fingerprinting captures meaningful chemical signals consistent with

existing phytochemical knowledge, while extending it to a multi-species comparative scale (Harborne and Williams 2000; Xie et al. 2006).

This study complements earlier phytochemical investigations by shifting the analytical focus from isolated compounds to comparative flavonoid patterns across species. In doing so, it provides a foundation for integrating fingerprint-based screening with targeted structural and bioactivity studies, thereby advancing a more comprehensive understanding of flavonoid diversity and its taxonomic, ecological, and applied significance within *Selaginella*.

In conclusion, this study demonstrates that RT-based HPLC flavonoid fingerprinting provides a robust qualitative framework for comparing flavonoid profiles across *Selaginella* species. Analysis of 20 samples yielded a binary fingerprint matrix comprising 11 aligned RT-defined features generated using a retention time tolerance of ±0.2 min. Chromatographic profiles exhibited pronounced interspecific variation, while conspecific samples from different localities showed moderate to high similarity, indicating the presence of stable species-level core flavonoid signatures. Similarity and clustering analyses, supported by complementary NMDS ordination, further revealed broader chemical affinities that are not strictly associated with geographic origin, suggesting that flavonoid composition reflects intrinsic biochemical traits shaped by evolutionary history, with additional modulation by local environmental conditions. Although RT-based fingerprinting does not resolve compound identity, structural variation, or metabolite abundance, it effectively captures holistic chemical patterns and supports comparative and chemotaxonomic assessment. As a qualitative screening approach, it provides a practical foundation for future studies integrating LC-MS/MS dereplication, quantitative profiling, and molecular phylogenetic analyses to clarify the biosynthetic, functional, and evolutionary significance of flavonoid diversity in *Selaginella*.

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