

# Unraveling the chloroplast genome of *Actiniopteris radiata* from Saudi Arabia: Insights into genetic diversity in Pteridaceae

Fatmah Ahmed SAFHI<sup>1</sup>, Salha Mesfer ALSHAMRANI<sup>2\*</sup>

<sup>1</sup>Princess Nourah bint Abdulrahman University, College of Science, Department of Biology, Riyadh 11671, Saudi Arabia; [faalsafhi@pnu.edu.sa](mailto:faalsafhi@pnu.edu.sa)

<sup>2</sup>University of Jeddah, College of Science, Department of Biological Sciences, Jeddah, 21959, Saudi Arabia; [smalsbmrane@uj.edu.sa](mailto:smalsbmrane@uj.edu.sa) (\*corresponding author)

## Abstract

This study reports the chloroplast genome sequence of *Actiniopteris radiata* from Jazan, Saudi Arabia, marking it as the inaugural fern species of the *Actiniopteris* genus to be sequenced and analyzed. The chloroplast genome spans 148,219 bp with a GC content of 40.8% and contains 131 genes: 87 protein-coding genes (PCGs), 36 transfer RNAs (tRNAs), and 8 ribosomal RNAs (rRNAs). Comparative analyses with nine related species revealed a genome size range from 148,219 bp (*A. radiata*) to 154,106 bp (*Pteris vittata*), with GC content spanning 36.7% (*Ceratopteris cornuta*) to 44.4% (*Adiantum shastense*). The analysis identified 538 simple sequence repeats (SSRs) concentrated in the large single-copy (LSC) region. Codon usage analysis showed significant preferences, including an RSCU value of 0.36 for GCT in alanine codons. SNP analysis revealed 25,137 SNPs across the genome, yielding a dS/dN ratios indicative of purifying selection. Notable genes, such as *psbD* and *psaA*, exhibited high dS/dN ratios (46 and 8, respectively), suggesting adaptive evolution in photosynthetic pathways. Phylogenetic analysis confirmed the distinct evolutionary position of *A. radiata* within the Pteridoideae subfamily, highlighting its unique lineage in the absence of close relatives in existing databases. This research establishes a foundational resource for understanding fern evolution in the Pteridaceae family.

**Keywords:** adaptive evolution; codon usage bias; comparative genomics; cpSSR markers; phylogenetics; plastome; pteridophytes

## Introduction

Ferns, classified under the class Polypodiopsida, represent an ancient lineage of vascular plants that have persisted on Earth for over 360 million years. Their evolutionary history is marked by their unique reproductive strategy, which relies on spores rather than seeds, setting them apart from other vascular plants. This spore-based reproduction allows ferns to occupy a wide range of ecological niches, from lush tropical rainforests to arid deserts, demonstrating their remarkable adaptability to diverse environmental conditions (Fujiwara *et al.*, 2021). The evolutionary significance of ferns is underscored by their position as intermediates between

Received: 01 Feb 2025. Received in revised form: 16 Mar 2025. Accepted: 13 Jun 2025. Published online: 22 Jun 2025.

From Volume 49, Issue 1, 2021, Notulae Botanicae Horti Agrobotanici Cluj-Napoca journal uses article numbers in place of the traditional method of continuous pagination through the volume. The journal will continue to appear quarterly, as before, with four annual numbers.

bryophytes and seed plants, providing critical insights into the evolutionary transitions that have shaped the diversity of vascular plants (Thodberg *et al.*, 2020).

The chloroplast genome, or plastome, is a crucial element in understanding plant evolution due to its relatively conserved structure and gene content across different species. Fern plastomes typically exhibit a canonical quadripartite structure, consisting of a large single-copy (LSC) region, a small single-copy (SSC) region, and two inverted repeats (IRs) (Grewe *et al.*, 2013). However, ferns are distinctive among land plants for their higher rates of plastome rearrangements, which include inversions, deletions, and gene losses (Ojosnegros *et al.*, 2023). These genomic alterations provide valuable insights into the evolutionary pressures and adaptations that have influenced fern diversification and ecological specialization (Wang 2024).

Recent advancements in high-throughput sequencing technologies have enabled the complete sequencing of plastomes from various fern species, revealing intriguing genomic features such as additional introns, pseudogenes, and expanded intergenic regions (Hong *et al.*, 2022). Comparative analyses of these plastomes have illuminated phylogenetic relationships within ferns and between ferns and seed plants. For instance, studies on genera such as *Adiantum*, *Pteris*, and *Ceratopteris* have uncovered lineage-specific adaptations, including variations in gene content and structural rearrangements that may underlie their ecological specialization (Gago *et al.*, 2013). These findings highlight the importance of plastome studies in elucidating the evolutionary history and ecological roles of ferns.

Beyond their evolutionary significance, ferns play vital ecological roles in maintaining biodiversity and stabilizing ecosystems. They contribute to nutrient cycling, provide habitats for various organisms, and play a crucial role in water retention within tropical forest ecosystems (Larama *et al.*, 2017). Certain fern species, such as *Pteris vittata*, have been identified as hyperaccumulators of heavy metals, making them subjects of increasing interest for phytoremediation studies (Leroux *et al.*, 2013). Despite their ecological importance, there remains a significant gap in plastome data for many fern species, emphasizing the need for ongoing genomic exploration to fully understand their evolutionary and ecological dynamics (Doi *et al.*, 2015).

The Jazan Region in southwestern Saudi Arabia is recognized for its rich and diverse flora, attributed to its varied topography and climate. Approximately 858 wild species across 441 genera and 98 families have been documented in this region, representing about 37% of Saudi Arabia's total plant species (Jin *et al.*, 2020). Among these, 15 species are pteridophytes, indicating a notable presence of ferns in the area. The west-facing slopes of the mountains in Jazan receive moisture-laden winds from the Red Sea, creating microhabitats that support several endemic and rare species, including ferns such as *Adiantum capillus-veneris* and *Pteris vittata* (Qin 2024). This unique environmental context underscores the ecological adaptability of ferns and highlights the importance of studying their genomic features in relation to their habitats.

*Actiniopteris radiata*, commonly known as the ray fern, is among the pteridophytes recorded in the Jazan region. This species is typically distributed in tropical and subtropical regions but has also been reported in unusual and arid locations, such as Saudi Arabia's Jazan Region (Flora of Saudi Arabia, 2024). Its presence in such diverse landscapes underscores the ecological adaptability of ferns and highlights the unique environmental conditions of Jazan that support such species (Vejvodová 2024). Such atypical occurrences may suggest unique ecological adaptations, potentially reflected in the plastome structure and gene content. Despite the documentation of fern species in Jazan, comprehensive studies on their molecular identity remain limited, presenting an opportunity to explore their genomic features and evolutionary relationships (Bystriakova *et al.*, 2010).

The plastome of *A. radiata*, a member of the Pteridaceae family, remains uncharacterized to date. Comprehensive plastome analyses can provide valuable insights into its evolutionary trajectory, ecological strategies, and phylogenetic position within ferns. This study aims to report the first complete chloroplast genome sequence of *A. radiata*, marking the first plastome characterization within the genus *Actiniopteris*. By conducting a comparative analysis with nine other fern species from different genera, we aim to uncover key genomic features, including structural variations, gene content, and evolutionary trends. This work provides a

foundational resource for future studies on fern genomics, evolution, and ecological adaptation, contributing to a deeper understanding of the evolutionary processes that shape fern diversity and their ecological roles in various environments (Kinosian and Wolf 2022). The unique characteristics of fern plastomes, coupled with their ecological importance, underscore the necessity for continued research in this area. As we explore the genomic features of ferns like *A. radiata*, we not only enhance our understanding of their evolutionary history but also contribute to broader ecological and conservation efforts aimed at preserving the rich biodiversity represented by these ancient plants (Han *et al.*, 2020).

## Materials and Methods

### *Sample collection and DNA extraction*

Full grown plant of *Actiniopteris radiata* was collected from the Jazan Region (Saudi Arabia) in May 2024. Total genomic DNA was extracted from young leaves of a single specimen using the WizPrep™ gDNA Mini Kit (Cell/Tissue, WIZBIO, Seoul, South Korea). DNA quality was assessed through 1% TBE agarose gel electrophoresis, and its concentration was quantified using a Quantus™ Fluorometer (Promega, USA). The extracted DNA was stored at –20 °C for further analysis. Voucher specimens were deposited in the herbarium collection at Department of Biology, College of Science, Princess Nourah bint Abdulrahman University, coded as SIV-101.

### *Library construction, chloroplast genome assembly, and annotation*

DNA libraries were constructed using the TruSeq Library Preparation Kit (Illumina, San Diego, California, USA), with 350 bp fragmented short inserts. Sequencing was performed on the Illumina HiSeq 4000 platform with 150 bp paired-end reads (Novogene, China). High-quality reads were utilized for single-contig assembly following the pipeline described by (Magdy *et al.*, 2019, 2022; Magdy and Ouyang 2020). The assembly was performed using Geneious Prime software (Kearse *et al.*, 2012), which also facilitated coding sequence confirmation and correction. Annotation of the assembled chloroplast genome was completed using GeSeq (Tillich *et al.*, 2017). The tRNA scan-SE 2.0 search server was employed to confirm anticodon sequences and the characteristic cloverleaf secondary structures of all tRNA genes (Lowe and Chan 2016).

### *Tandem repeats and codon usage bias analysis*

Long repeat sequences were predicted using REPuter with a minimum repeat size of 30 and a Hamming distance of 50 (Kurtz 2001). Simple sequence repeats (SSRs) were identified using the Microsatellite Identification Software (MISA) (Beier *et al.*, 2017). Relative synonymous codon usage (RSCU) was analyzed for *Actiniopteris radiata* protein-coding sequences using MEGA X (Kumar *et al.*, 2018).

### *Chloroplast genome comparative analysis and phylogenetics*

The boundaries and junctions of IR/SSC and IR/LSC regions were compared across *A. radiata* and nine related fern species retrieved from organelle database (NCBI) using IRscope (<https://iscope.shinyapps.io/irapp/>, accessed December 2024). The complete chloroplast genomes and individual regions (LSC, SSC, and IR) were aligned using the Mauve genome aligner (Darling *et al.*, 2004). Phylogenetic analysis was conducted using FastTree V2 software with default parameters (Price *et al.*, 2010).

### *Genetic polymorphism and evolutionary pressures*

The coding sequences of the chloroplast genome were aligned using MAFFT v7.3 (Katoh and Standley 2014). Single nucleotide polymorphisms (SNPs) among the sequenced samples and the nine related fern species and their effects on protein translation were determined using Geneious Prime. Synonymous (dS) and

nonsynonymous (dN) substitution rates (dS/dN ratios) were calculated using the CODEML program in the PAML package (Yang 2007). The interpretation of selective pressures was set according to previous studies (Nielsen 2005).

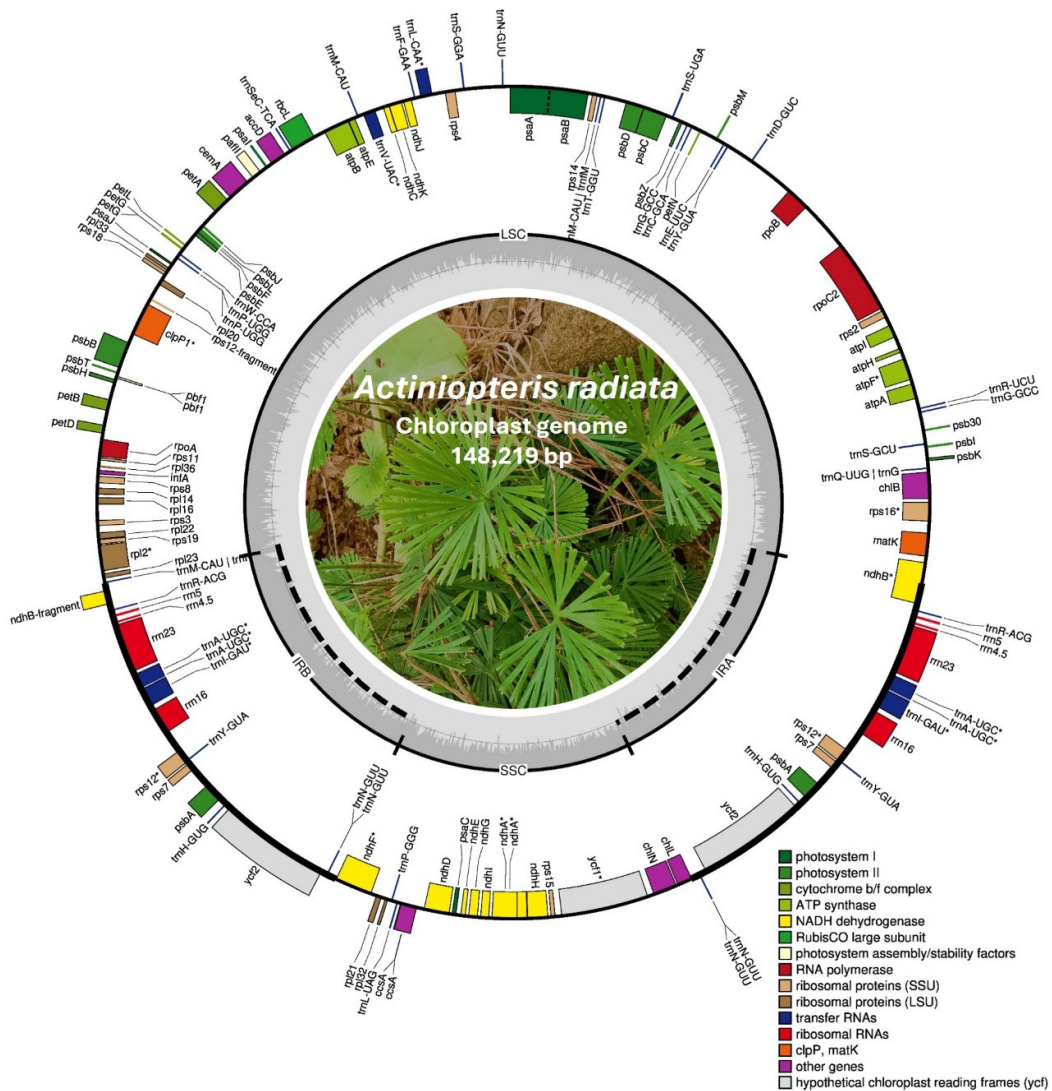
## Results

### *Characteristics of the Actiniopteris radiata chloroplast genome*

The newly sequenced chloroplast genome of *Actiniopteris radiata* (referred to as SIV) from Jazan Region, Saudi Arabia, is 148,219 bp in length with a GC content of 40.8%. Its genome structure follows the typical quadripartite organization, including a Large Single Copy (LSC) region of 83,354 bp, a Small Single Copy (SSC) region of 20,817 bp, and two Inverted Repeat (IR) regions of 22,024 bp each. The SIV genome contains 131 genes, which include 87 Protein-Coding Genes (PCGs), 36 transfer RNA (tRNA) genes, and 8 ribosomal RNA (rRNA) genes. These features represent foundational genomic stability while also allowing for unique adaptations reflective of the fern's ecological niche (Table 1; Figure 1).

**Table 1.** Comparative chloroplast genome characteristics of *Actiniopteris radiata* and nine related fern species from family Pteridaceae

Sub family	Species/ NCBI accession	GC%	Length bp	LSC bp	SSC bp	IR bp	Genes	PCGs	tRNA	rRNA
Pteridoideae	<i>Actiniopteris radiata</i>	40.8	148219	83354	20817	22024	131	87	36	8
	<i>Pteris multifida</i> MZ848380	42.2	153916	82027	21129	25380	134	91	35	8
	<i>Pteris vittata</i> MH173082	41.7	154106	82602	20954	25275	131	89	34	8
Parkerioideae	<i>Ceratopteris cornuta</i> MH173068	36.7	149424	83623	21227	22287	130	89	33	8
	<i>Ceratopteris pteridoides</i> MZ636516	36.7	149426	83631	21217	22289	134	90	36	8
Cryptogrammoideae	<i>Cryptogramma acrostichoides</i> MH173081	42.3	150162	87841	21168	20581	131	89	34	8
	<i>Llavea cordifolia</i> MH173088	41.9	149387	81944	21027	23208	132	89	35	8
Vittarioideae	<i>Adiantum capillus-veneris</i> AY178864	42	150568	82282	21390	23448	131	88	35	8
	<i>Adiantum sbastense</i> MG432483	44.4	150414	82113	21539	23381	132	89	35	8
	<i>Haplopteris ensiformis</i> OM867544	39.4	148805	80986	20773	23523	129	90	31	8



**Figure 1.** The complete chloroplast genome map of *Actiniopteris radiata* (SIV) with a total length of 148,219 bp. The inner circle represents the GC content (40.8%) and is defined by the chloroplast regions: large single-copy (LSC), small single-copy (SSC), and inverted repeat regions (IRA and IRB). The outer circle represents the complete chloroplast sequence, with genes annotated outside the circle representing forward-strand genes and those inside the circle representing reverse-strand genes. All genes are color-coded according to their functional groups. The GC content of 40.2% and is shown proportionally within the genome structure

#### Comparative characteristics of other fern chloroplast genomes

The comparative analysis of *Actiniopteris radiata* with nine related species highlights both similarities and distinctive features. Genome sizes among the studied species ranged from 148,219 bp (*A. radiata*) to 154,106 bp (*Pteris vittata*). The GC content displayed moderate variability, spanning from 36.7% (*Ceratopteris cornuta* and *Ceratopteris pteridoides*) to 44.4% (*Adiantum shastense*), reflecting species-specific adaptations in genome composition. The LSC region showed significant consistency across species, with the smallest observed in *Haplopteris ensiformis* (80,986 bp) and the largest in *Cryptogramma acrostichoides* (87,841 bp). Similarly, the

SSC region exhibited a narrow range of sizes, from 20,773 bp (*Haplopteris ensiformis*) to 21,539 bp (*Adiantum shastense*). The IR regions were also highly conserved, varying between 20,581 bp (*Cryptogramma acrostichoides*) and 25,380 bp (*Pteris multifida*). In terms of gene content, most species possessed between 129 and 134 genes, encompassing 87–91 PCGs, 31–36 tRNA genes, and 8 rRNA genes. *Ceratopteris pteridoides* and *Pteris multifida* had the highest number of total genes (134), while *Haplopteris ensiformis* had the fewest (129). This level of gene conservation underscores the evolutionary constraints on chloroplast genomes in ferns (Table 1).

#### SSR and Tandem Repeat Sequence Analysis

The chloroplast genome of *Actiniopteris radiata* (SIV) revealed a total of 538 simple sequence repeats (SSRs), distributed across the large single-copy (LSC), small single-copy (SSC), and inverted repeat (IR) regions (Table 2). These repeats were categorized based on repeat unit sizes, ranging from mononucleotide to octanucleotide repeats. Mononucleotide repeats were the most prevalent, accounting for 258 (48%) of the total SSRs, followed by pentanucleotide repeats (72, 13%) and hexanucleotide repeats (69, 13%). Trinucleotide and tetranucleotide repeats contributed equally with 48 (9%) and 47 (9%) occurrences, respectively. The least frequent repeats were septanucleotide repeats (6, 1%) and octanucleotide repeats (12, 2%). The distribution of SSRs across the genomic regions showed a pronounced accumulation in the LSC region, which hosted 324 repeats (60.2% of the total), emphasizing its role as a hotspot for repetitive elements. The IR region contained 140 SSRs (26%), while the SSC region had the fewest, with 74 repeats (13.8%). This pattern aligns with findings in other fern plastomes, where the LSC region consistently demonstrates higher densities of SSRs.

**Table 2.** Distribution and frequency of simple sequence repeats (SSRs) across the chloroplast genome regions (LSC, SSC, and IR) in *Actiniopteris radiata*

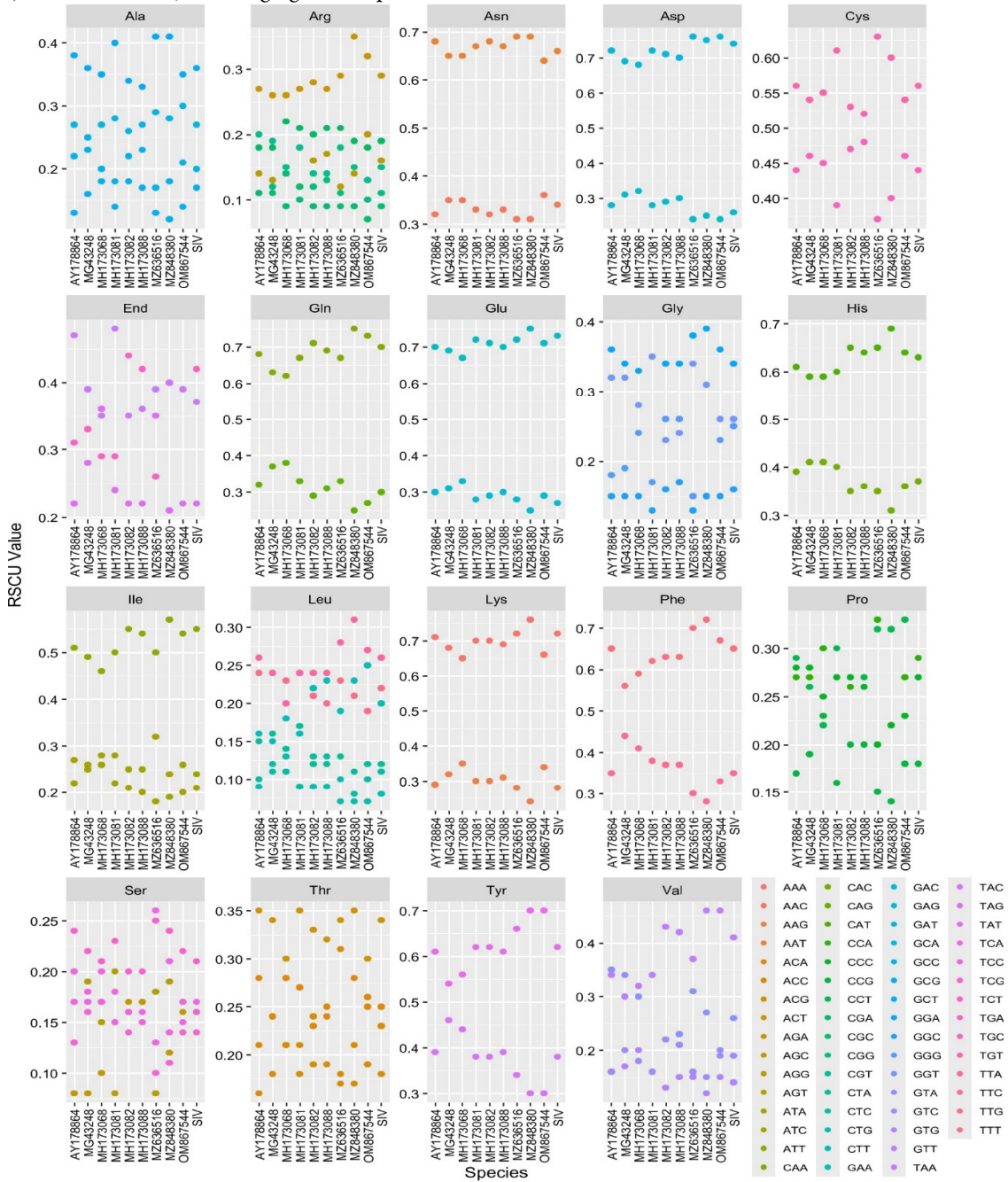
Repeats Unit	LSC	SSC	IR	Grand Total	Percentage
Mono-nucleotide	166	40	52	258	48%
Di-nucleotide	16	4	6	26	5%
Tri-nucleotide	24	4	20	48	9%
Tetra-nucleotide	31	10	6	47	9%
Penta-nucleotide	35	9	28	72	13%
Hexa-nucleotide	41	6	22	69	13%
Septa-nucleotide	4	0	2	6	1%
Octa-nucleotide	7	1	4	12	2%
Grand Total	324	74	140	538	100%
Percentage	60.2%	13.8%	26%	100%	-

In addition to SSRs, the analysis identified 14 tandem repeats in the *A. radiata* plastome, with period sizes ranging from 15 bp to 42 bp (Table 3). These repeats showed high levels of conservation, with consensus matches ranging from 90% to 100% and entropy values between 1.37 and 2.0, indicating varying sequence complexity. The repeats exhibited notable motif diversity, with the longest and highest-scoring repeats found in the IRa region at positions 95301–95429 and its inverted repeat IRb at 136145–136273, each spanning 42 bp and achieving a score of 199. The LSC region hosted the majority of these tandem repeats, consistent with its role as a genomic hotspot for repetitive sequences.

#### Codon usage bias analysis

Methionine (Met) and tryptophan (Trp), encoded by single codons (ATG and TGG, respectively), had uniform RSCU values of 1 across all species, reflecting no variation in their usage, thus were excluded from Figure (2). Alanine (Ala) codons demonstrated a varied distribution of preference across species. The codon GCG displayed the lowest RSCU value in *Actiniopteris radiata* (SIV, 0.17), similar to *A. capillus-veneris*

(AY178864, 0.13) and *P. multifida* (MZ848380, 0.12), indicating minimal preference. In contrast, GCT had the highest RSCU value (0.36 in SIV), comparable to *C. pteridoides* (MZ636516, 0.41) and *P. multifida* (MZ848380, 0.41), showing significant preference.



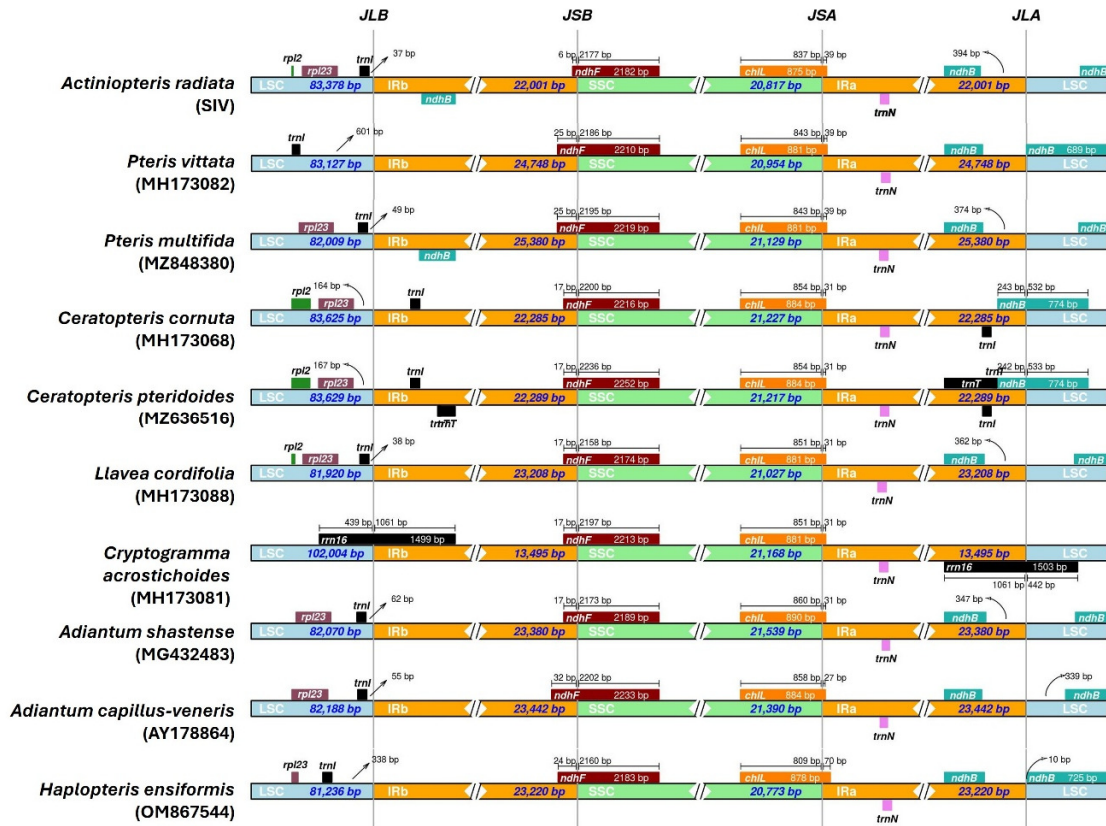
**Figure 2.** RSCU (Relative Synonymous Codon Usage) values for various codons across species of ferns grouped by their corresponding amino acids. Each mini-plot represents a specific amino acid, excluding Met and Trp. Species are denoted by their scientific names alongside GenBank accession identifiers: *Actiniopteris radiata* (SIV), *Pteris multifida* (MZ848380), *Pteris vittata* (MH173082), *Ceratopteris cornuta* (MH173068), *Ceratopteris pteridoides* (MZ636516), *Cryptogramma acrostichoides* (MH173081), *Llavea cordifolia* (MH173088), *Adiantum capillus-veneris* (AY178864), *Adiantum shastense* (MG432483), and *Haplopteris ensiformis* (OM867544)

Moderate usage was noted for GCA (0.27 in SIV), with *H. ensiformis* (OM867544, 0.30) showing the highest value. GCC, with an RSCU of 0.20 in SIV, was consistently lower across most species. Cysteine (Cys) codons exhibited a stronger bias toward TGT, with an RSCU of 0.56 in SIV, matching values in *A. capillus-veneris* (AY178864, 0.56) and *P. multifida* (MZ848380, 0.60). Conversely, TGC showed reduced preference, with an RSCU of 0.44 in SIV, consistent with *A. capillus-veneris* (AY178864, 0.44) and *A. shastense* (MG43248, 0.46). Aspartic acid (Asp) codons showed a clear preference for GAT, which had an RSCU value of 0.74 in SIV, the highest among species like *H. ensiformis* (OM867544, 0.76) and *C. pteridoides* (MZ636516, 0.76). GAC was underrepresented across all species, with SIV showing an RSCU of 0.26, similar to *H. ensiformis* (OM867544, 0.24) and *C. pteridoides* (MZ636516, 0.24). Glutamic acid (Glu) displayed a strong preference for GAA, which had an RSCU value of 0.73 in SIV, similar to most species. The alternative codon GAG showed a significantly lower RSCU in SIV (0.27), aligning with values observed in *P. multifida* (MZ848380, 0.25).

For phenylalanine (Phe), the codon TTT was highly preferred, with an RSCU of 0.65 in SIV, matching *A. capillus-veneris* (AY178864, 0.65). On the other hand, TTC showed reduced preference, with SIV displaying an RSCU of 0.35. Glycine (Gly) codons also displayed varied bias. GGA was the most favored codon for glycine in SIV (0.34), similar to *A. capillus-veneris* (AY178864, 0.36). GGG exhibited minimal usage, with SIV showing an RSCU of 0.25, slightly higher than *C. pteridoides* (MZ636516, 0.15) and *P. multifida* (MZ848380, 0.15). Leucine (Leu) codons showed moderate preference for TTG (0.22) and TTA (0.26) in SIV, whereas CTC and CTG were underrepresented, with RSCU values of 0.11 and 0.08, respectively. This pattern was consistent across species, with *A. shastense* (MG43248) and *A. capillus-veneris* (AY178864) showing similar trends. Lysine (Lys) codons exhibited a strong bias for AAA in SIV (0.72), with much lower representation for AAG (0.28). Similar trends were observed across species, with *P. multifida* (MZ848380, 0.76) showing the highest preference for AAA. Serine (Ser) codons showed significant preference for TCT in SIV (0.21), while AGC and AGT displayed lower values (both 0.16). This pattern was observed across species, with *C. cornuta* (MH173068) and *L. cordifolia* (MH173088) showing similar trends. For stop codons, TAA showed the highest RSCU in SIV (0.37), comparable to *P. multifida* (MZ848380, 0.40). TAG and TGA were less preferred, with SIV displaying values of 0.22 and 0.42, respectively.

#### *IR expansion and contraction*

The inverted repeat (IR) regions of chloroplast (cp) genomes are known for their conserved structure, yet their boundaries often exhibit dynamic expansions and contractions that contribute to genome size variation during evolution. The critical junctions delineating transitions between the regions -JLB (IRb-LSC), JSB (IRb-SSC), JSA (SSC-IRa), and JLA (IRa-LSC)- were analyzed across the chloroplast genomes. The positioning of key genes such as *ndhF*, *rpl2*, and *chlL* at these junctions varied across species, although the general boundaries remained conserved. In *A. radiata*, the JLB junction placed *rpl2* 37 bp into the IRb region, while the JLA junction extended *ndhB* into the LSC by 394 bp. This pattern contrasts with *P. multifida*, where *rpl2* was shifted 49 bp into IRb, reflecting a subtle adjustment in IR boundary positioning. The JSB boundary in *C. pteridoides* showed a notable elongation, with the *ndhF* gene extending 52 bp further into the IRb compared to *P. vittata*. The *L. cordifolia* exhibited a contraction at the JSA boundary, reducing the overlap of *ndhB* in the SSC to 38 bp. The most striking divergence was observed in *C. acrostichoides*, which displayed an exceptionally short IR region, as evident by a contracted JLB boundary and reduced *ndhF* extension into the IRb region. Meanwhile, *A. shastense* and *A. capillus-veneris* showed more conservative IR boundary shifts, with JSA and JLA junctions comparable to *H. ensiformis* (Figure 3).



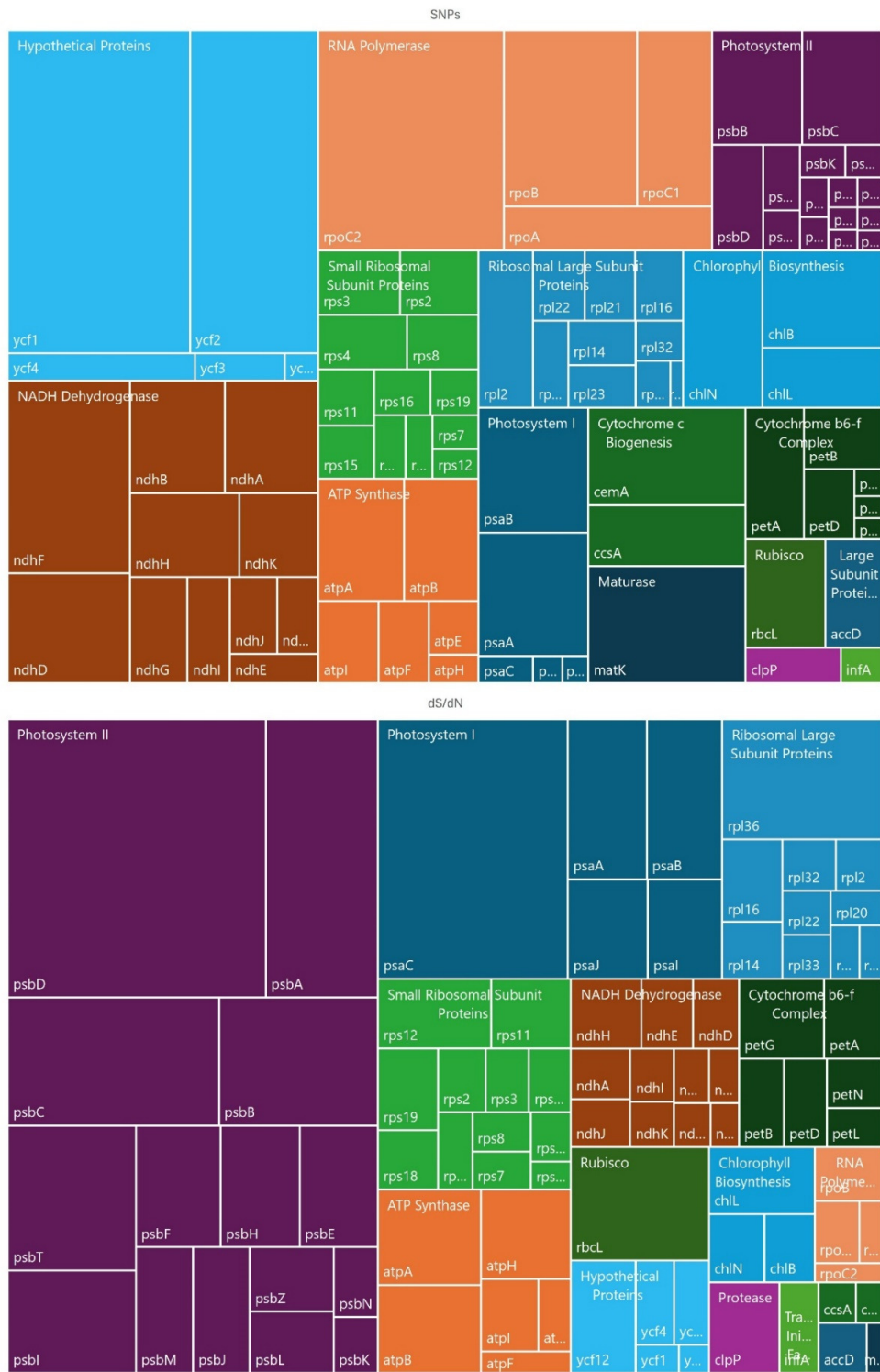
**Figure 3.** Comparative analysis of IR boundary expansion and contraction in chloroplast genomes of ten fern species. The diagram illustrates the positions of the junctions (JLB, JSB, JSA, JLA) and associated genes (*rpl2*, *ndhB*, *ndhF*, *chlL*, *trnI*, and *trnN*) across the species *Actiniopteris radiata* (SIV), *Pteris vittata* (MH173082), *Pteris multifida* (MZ848380), *Ceratopteris cornuta* (MH173068), *Ceratopteris pteridoides* (MZ636516), *Llavea cordifolia* (MH173088), *Cryptogramma acrostichoides* (MH173081), *Adiantum shastense* (MG432483), *Adiantum capillus-veneris* (AY178864), and *Haplopteris ensiformis* (OM867544)

#### *Genetic polymorphism and substitution rates (dS/dN)*

Within the fern family Pteridaceae, the SNP analysis of *Actiniopteris radiata* chloroplast genome (Figure 4) revealed a total of 25,137 SNPs distributed across various genes, including 15,370 synonymous (dS) and 9,767 nonsynonymous (dN) mutations, yielding an overall dS/dN ratio of approximately 2. This result suggests a general prevalence of purifying selection across the genome, with notable variations among different functional gene groups.

The ATP synthase genes exhibited a wide range of SNP counts, with *atpA* showing the highest total SNP count (463), most of which were synonymous, leading to a dS/dN ratio of approximately 6. This indicates strong positive selection. Conversely, *atpF* had a lower SNP count (181) and a near-neutral dS/dN ratio of 1.3. The lowest SNP count within this group was observed in *atpH* (62), with a dS/dN ratio of 5, suggesting significant selection pressure in this gene.

The NADH dehydrogenase complex displayed a high degree of variation in SNP counts and selection pressures. *ndhF* had the highest SNP count (1039) in this group, with a moderate dS/dN ratio of 1.2, indicating weak positive selection. In contrast, *ndhG* had a lower SNP count (268) and a dS/dN ratio of 0.8, suggestive of purifying selection. Interestingly, *ndhH* had a comparatively low SNP count (405) but a high dS/dN ratio of 3, indicating strong selection pressure.



**Figure 4.** Genetic polymorphism (SNPs) and genes under selection (dN/dS) ratio of chloroplast protein-coding genes of nine fern species of the family Pteridaceae compared to *Actiniopteris radiata* cp genome

The RNA polymerase genes varied significantly in SNP counts and selection patterns. *rpoC2* had the highest SNP count (1793) but a low dS/dN ratio of 0.8, indicating purifying selection. In contrast, *rpoB* exhibited a dS/dN ratio of 2.4, suggesting moderate positive selection.

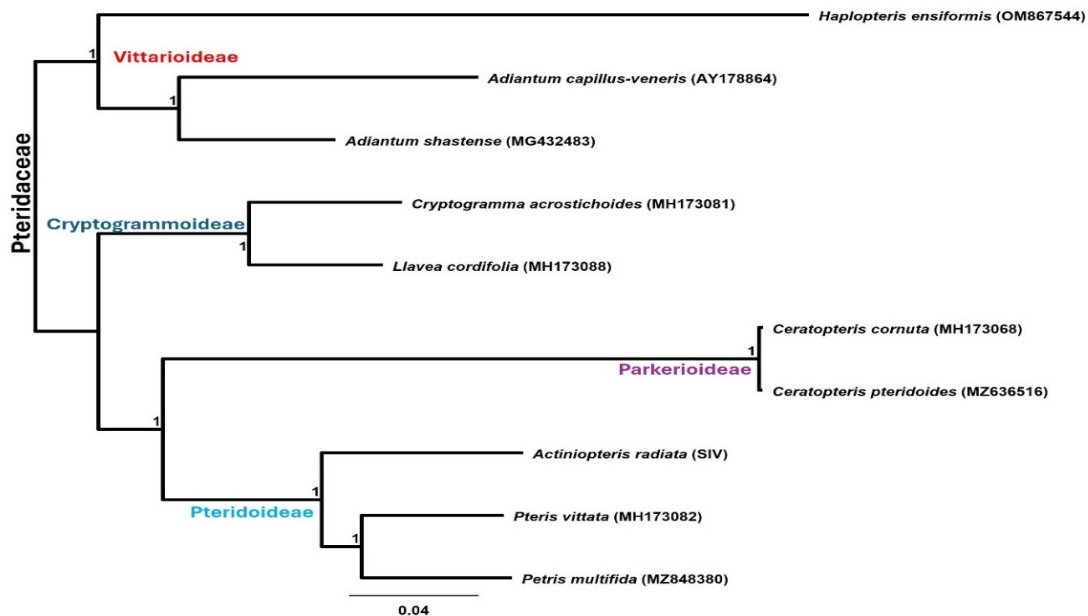
The large and small ribosomal subunit genes also displayed interesting selection dynamics. Among the large subunit genes, *rpl2* had the highest SNP count (379) with a dS/dN ratio of 1.6, while *rpl36* had the lowest SNP count (27) and a dS/dN ratio of 12, indicating strong positive selection. For the small subunit genes, *rps12* had a low SNP count (61) and a dS/dN ratio of 5, indicating significant selection, while *rps4* exhibited a moderate SNP count (212) and a near-neutral dS/dN ratio of 1.7.

Among the hypothetical proteins, *ycf1* stood out with the highest SNP count across the entire genome (2589) but a low dS/dN ratio of 0.8, indicative of purifying selection. In contrast, *ycf3* had fewer SNPs (107) but a higher dS/dN ratio of 1.9, showing moderate positive selection. Photosystem I and II genes stood out with extreme dS/dN ratios.

Within Photosystem I, *psaA* and *psaB* exhibited SNP counts of 596 and 607, respectively, and high dS/dN ratios of 8 and 7, reflecting significant positive selection. Photosystem II genes, particularly *psbD* and *psbC*, demonstrated the most pronounced selection pressures, with *psbD* having a remarkably high dS/dN ratio of 47 and *psbC* at 18. These results emphasize their functional importance in photosynthesis adaptation.

#### Phyloplastomic relationships of Pteridaceae species under study

The phylogenetic tree constructed from plastome data depicts well-supported relationships among species of the Pteridaceae family, with all clades supported by a bootstrap value of 1. The Vittarioideae subfamily forms a cohesive clade comprising *H. ensiformis* (OM867544), *A. capillus-veneris* (AY178864) and *A. shastense* (MG432483), reflecting their close evolutionary relationship as members of the same genus. This grouping was used to root the presented tree (Figure 5).



**Figure 5.** Phylogenetic relationships among species of the Pteridaceae family based on plastome data. Clades correspond to the following subfamilies: Vittarioideae (*Adiantum* species), Cryptogrammoideae (*Cryptogramma* and *Llavea*), Parkerioideae (*Ceratopteris* species), and Pteridoideae (*Pteris* species and *Actiniopteris radiata*). The tree is rooted with *H. ensiformis* (OM867544) as an outgroup. Bootstrap values of 1 are indicated at all nodes. *A. radiata* (SIV) occupies a distinct position within the Pteridoideae subfamily, reflecting its lack of closely related genera in the database

The Cryptogrammoideae subfamily also forms a distinct clade that includes *C. acrostichooides* (MH173081) and *L. cordifolia* (MH173088). This clade demonstrates the genetic similarity between the two genera within Cryptogrammoideae, supported by high bootstrap values. Within the Parkerioideae subfamily, *C. cornuta* (MH173068) and *C. pteridooides* (MZ636516) group together tightly, consistent with their placement in the same genus. The Pteridoideae subfamily shows greater diversity. The *P. vittata* (MH173082) and *P. multifida* (MZ848380) form a robust clade within the subfamily, indicating their shared genus and evolutionary history. Interestingly, *A. radiata* (SIV) branches separately from this clade, as it does not have a closely related genus in the current database. This isolated position highlights the distinct lineage of Actiniopteris within the Pteridoideae and reflects the need for expanded genomic data for related genera.

## Discussion

This study represents a comprehensive investigation into the chloroplast genome of *A. radiata* and its comparative genomics within the Pteridaceae family. The results provide insights into genome structure, polymorphism, and evolutionary trends, laying the groundwork for understanding the functional and ecological significance of chloroplast genome variation in ferns. Below, we discuss the findings in the context of current literature and propose potential applications and future research directions.

The chloroplast genome of *A. radiata* exhibits a typical quadripartite structure with large single copy (LSC), small single copy (SSC), and inverted repeat (IR) regions. The genome size of 148,219 bp and a GC content of 40.8% align with the ranges reported in other ferns, indicating its conserved nature. Similar findings in ferns have been documented, emphasizing the evolutionary stability of their chloroplast genomes (Fan *et al.*, 2021). However, the distinct gene content and organization in *A. radiata*, such as its specific IR boundary arrangements, highlight the potential influence of adaptive pressures in its arid ecological niche. This is consistent with studies suggesting that variations in chloroplast genome structure may be linked to ecological adaptations (Gu 2023). For instance, Gu's research indicates that adaptive evolution in chloroplast genomes is often driven by environmental factors, leading to significant genomic rearrangements (Gu 2024).

The comparative analysis revealed moderate variation in genome size, GC content, and gene content among the studied species. These differences are likely driven by IR expansions and contractions and varying levels of repetitive elements, as seen in other plastid genome studies (Hong *et al.*, 2020). The high number of simple sequence repeats (SSRs) in *A. radiata* (538) and their predominance in the LSC region underscore the importance of this region in accommodating repetitive sequences. SSRs play a critical role in plastome evolution and species differentiation, as they are hotspots for mutation and recombination (Zhu *et al.*, 2021). The identified SSRs in *A. radiata* may serve as valuable molecular markers for population genetics and phylogeographic studies in ferns, similar to their utility in angiosperms (Fan *et al.*, 2022). This aligns with findings by Zhu *et al.*, 2021, who emphasized the significance of SSRs in understanding phylogenetic relationships within ferns (Fan *et al.*, 2021; Zhu *et al.*, 2021).

Codon usage patterns reflect a balance between mutation, selection, and genetic drift. In *A. radiata*, the relative synonymous codon usage (RSCU) analysis revealed significant biases for certain amino acids, such as alanine (Ala), cysteine (Cys), and leucine (Leu). Methionine (Met) and tryptophan (Trp), encoded by single codons, were uniformly utilized, which aligns with the findings in other plant species (Luo 2023). These biases are indicative of selective pressures to optimize translational efficiency and accuracy in the chloroplast genome. The observed variability among species in codon preferences for synonymous codons such as GCT (Ala) and TGT (Cys) suggests evolutionary divergence in translational optimization. Further research could explore how

such codon biases impact protein function and adaptation in diverse environments, as highlighted by Liu *et al.*, in their comparative analyses of chloroplast genomes (Zhu *et al.*, 2021).

IR boundary dynamics are a key driver of chloroplast genome evolution. The variations in IR boundaries across the studied species, particularly the extended *ndhB* gene in *A. radiata* and the contracted IR regions in *Ceratopteris pteridoides*, exemplify how these shifts can contribute to genome size variability. Such boundary dynamics have been correlated with lineage-specific adaptations and evolutionary rates (Hong *et al.*, 2020). The isolated position of *A. radiata* within the Pteridoideae subfamily, marked by unique IR arrangements of the species and possibly the genus, underscores its evolutionary distinctiveness and warrants further genomic exploration within its genus. This observation is supported by recent studies that have documented the dynamic nature of IR regions in ferns, suggesting that these regions are not as stable as previously thought (Fan *et al.*, 2021; Peng 2024).

The single nucleotide polymorphism (SNP) analysis across functional gene groups revealed varying dS/dN ratios, providing insights into selective pressures. Genes associated with photosynthesis, particularly *psbD* and *psbC*, showed remarkably high dS/dN ratios, indicating strong positive selection. This may reflect adaptive optimization of photosynthetic efficiency under the environmental conditions specific to the habitats of these ferns (Xiaoxu *et al.*, 2020). Similarly, the high dS/dN ratios observed in *psaA* and *psaB* suggest ongoing selective pressures on Photosystem I genes, potentially to enhance light harvesting in shaded or arid environments. In contrast, the NADH dehydrogenase complex displayed predominantly purifying selection, highlighting its functional conservation. The variation in selection pressures across functional groups mirrors findings in angiosperms, where photosynthetic genes often show rapid evolution compared to housekeeping genes (Wei *et al.*, 2020).

The phylogenetic analysis supports the monophyletic grouping of subfamilies within the Pteridaceae family, consistent with previous plastid-based studies (Wang *et al.*, 2023). The placement of *A. radiata* in a distinct branch within Pteridoideae reflects its unique evolutionary trajectory and the absence of closely related genera in current databases. This finding underscores the need for expanded genomic sampling within Pteridoideae to better resolve the phylogeny of this subfamily. The phylogenetic significance of the chloroplast genome structure in elucidating evolutionary relationships among ferns has been emphasized in various studies, indicating that comprehensive genomic data can enhance our understanding of fern phylogeny (Zhong *et al.*, 2020). Moreover, the unique phylogenetic position of *A. radiata* underscores its potential as a model for studying evolutionary divergence in ferns. Future research should prioritize expanding genomic sampling within the Pteridaceae family to fill phylogenetic gaps and validate the observed patterns of genome evolution. Functional studies on the positively selected photosynthetic genes could provide insights into their adaptive significance. Additionally, exploring the ecological roles of repetitive elements and their contribution to genome stability and plasticity in ferns would be valuable.

## Conclusion

This study presents the first published chloroplast genome sequence for *Actiniopteris radiata*, marking a significant milestone as the inaugural representative of its genus. The genome structure follows the conserved quadripartite arrangement seen in ferns, yet it reveals unique characteristics that shed light on the species' ecological and evolutionary adaptations. Comparative analyses highlighted both conserved and variable genomic traits within the Pteridaceae family. While the IR regions displayed evolutionary stability, subtle boundary shifts unique to *A. radiata* were observed, offering insights into plastome dynamics. The SSR and tandem repeat analyses further emphasized the LSC region as a hotspot for repetitive sequences, with species-specific variations that can serve as genetic markers. Codon usage bias and SNP analyses underscored the evolutionary pressures acting on the genome, with a genome-wide dS/dN ratio of approximately 2 indicating

strong purifying selection. However, adaptive evolution was evident in certain functional groups, such as photosystem genes, particularly *psbD* and *psaA*, which showed signs of positive selection. Phylogenetic analysis placed *A. radiata* within the Pteridoideae subfamily but highlighted its unique evolutionary lineage due to the absence of closely related genera in current databases. This underscores the need for expanded genomic studies in this clade. Overall, these findings provide valuable genomic resources and deepen our understanding of fern evolution, offering potential applications in plant systematics, ecology, and conservation efforts.

### **Data Availability**

The complete sequence of *Actiniopteris radiata* was deposited into figshare – DOI: <https://doi.org/10.6084/m9.figshare.28119764>

### **Authors' Contributions**

Conceptualization: FAS; Data curation: FAS; Formal analysis: FAS, SMA; Funding acquisition: FAS; Investigation: FAS, SMA; Methodology: FAS, SMA; Project administration: FAS; Resources: FAS, SMA; Software: SMA; Supervision: FAS; Validation: SMA; Visualization: SMA; Roles/Writing - original draft: FAS, SMA; and Writing - review & editing: FAS, SMA.

### **Funding**

This research was funded by Princess Nourah bint Abdulrahman University Researchers Supporting Project number (PNURSP2025R318), Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia

### **Ethical approval** (for researches involving animals or humans)

Not applicable.

### **Acknowledgements**

Authors are grateful for the supported provided by Princess Nourah bint Abdulrahman University Researchers Supporting Project number (PNURSP2025R318), Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia.

### **Conflict of Interests**

The authors declare that there are no conflicts of interest related to this article.

## References

- Beier S, Thiel T, Münch T, Scholz U, Mascher M (2017). MISA-web: a web server for microsatellite prediction, *Bioinformatics* 33(16):2583-2585. <https://doi.org/10.1093/bioinformatics/btx198>
- Bystriakova N, Schneider H, Coomes D (2010). Evolution of the climatic niche in scaly tree ferns (Cyatheaaceae, Polypodiopsida), *Botanical Journal of the Linnean Society* 165(1):1-19. <https://doi.org/10.1111/j.1095-8339.2010.01092.x>
- Darling ACE, Mau B, Blattner FR, Perna NT (2004). Mauve: multiple alignment of conserved genomic sequence with rearrangements. *Genome Research* 14:1394-1403. <https://doi.org/10.1101/gr.2289704>
- Doi M, Kitagawa Y, Shimazaki KI (2015). Stomatal blue light response is present in early vascular plants. *Plant Physiology* 169:1205-1213. <https://doi.org/10.1104/pp.15.00134>
- Fan R, Ma W, Liu S, Huang Q (2021). Integrated analysis of three newly sequenced fern chloroplast genomes: genome structure and comparative analysis. *Ecology and Evolution* 11:4550-4563. <https://doi.org/10.1002/ece3.7350>
- Fan X, Wang W, Wagutu GK, Li W, Li X, Chen Y (2022). fifteen complete chloroplast genomes of *Trapa* species (Trapaceae): Insight into genome structure, comparative analysis and phylogenetic relationships. *BMC Plant Biology* 22. <https://doi.org/10.1186/s12870-022-03608-7>
- Fujiwara T, Li H, Torres EIM, Morero RE, Vega AJ, Liang Z, Ebihara A, Leitch IJ, Schneider H (2021). Evolution of genome space occupation in ferns: linking genome diversity and species richness. *Annals of Botany* 131:59-70. <https://doi.org/10.1093/aob/mcab094>
- Gago J, Coopman RE, Cabrera HM, Hermida C, Molins A, Conesa MA, Galmes J, Ribas-Carbo M, Flexas J (2013). Photosynthesis limitations in three fern species. *Physiologia Plantarum* 149:599-611. <https://doi.org/10.1111/ppl.12073>
- Grewe F, Guo W, Gubbels EA, Hanses AK, Mower JP (2013). Complete plastid genomes from *Ophioglossum californicum*, *Psilotum nudum*, and *Equisetum hyemale* reveal an ancestral land plant genome structure and resolve the position of Equisetales among Monilophytes. *BMC Evolutionary Biology* 13. <https://doi.org/10.1186/1471-2148-13-8>
- Gu X (2023). Adaptive evolution and co-evolution of chloroplast genomes in Pteridaceae species occupying different habitats: overlapping residues are always highly mutated. *BMC Plant Biology* 23. <https://doi.org/10.1186/s12870-023-04523-1>
- Gu X (2024). The size diversity of the Pteridaceae family chloroplast genome is caused by overlong intergenic spacers. *BMC Genomics* 25. <https://doi.org/10.1186/s12864-024-10296-0>
- Han K, Shi C, Li L, Seim I, Lee SM-Y, Yang H, Fan G, Liu X (2020). Lineage-specific evolution of mangrove plastid genomes. *The Plant Genome* 13(2): e20019. <https://doi.org/10.1002/tpg2.20019>
- Hong Y, Wang Z, Li M, Su Y, Wang T (2022). First multi-organ full-length transcriptome of tree fern *Alsophila spinulosa* highlights the stress-resistant and light-adapted genes. *Frontiers in Genetics* 12:784546. <https://doi.org/10.3389/fgene.2021.784546>
- Hong Z, Wu Z, Zhao K, Yang Z, Zhang N, Guo J, Tembrock LR, Xu D (2020). Comparative analyses of five complete chloroplast genomes from the genus *Pterocarpus* (Fabaceae). *International Journal of Molecular Sciences* 21:3758. <https://doi.org/10.3390/ijms21113758>
- Jin Y, Li L, Wang S, Dong S, Chane Z, Patel N, Goffinet B, Liu H, Liu Y (2020). Draft genome of the aquatic moss *Fontinalis antipyretica* (Fontinalaceae, Bryophyta). *Frontiers in Genetics* 12:784546. <https://doi.org/10.1101/2020.04.29.069583>
- Katoh K, Standley DM (2014). MAFFT: iterative refinement and additional methods. in: Russell DJ (ed). *Multiple sequence alignment methods*. Humana Press, Totowa, NJ, pp 131-146.
- Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Durmmond A (2012). Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28:1647-1649. <https://doi.org/10.1093/bioinformatics/bts199>
- Kinoshian SP, Wolf PG (2022). The Biology of *C. Richardii* as a Tool to Understand Plant Evolution. *eLife* 11:e75019.. <https://doi.org/10.7554/elife.75019>

- Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018). MEGA X: Molecular Evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution* 35:1547-1549. <https://doi.org/10.1093/molbev/msy096>
- Kurtz S (2001). REPuter: the manifold applications of repeat analysis on a genomic scale. *Nucleic Acids Research* 29:4633-4642. <https://doi.org/10.1093/nar/29.22.4633>
- Larama G, Ostria-Gallardo E, Berríos G, Gutierrez A, Ensminger I, Bravo LA (2017). The transcriptional dynamics of two filmy ferns from Hymenophyllaceae with different niche preferences unravel key aspects of their desiccation tolerance and vertical distribution along host trees. Preprint BioRxiv 228866. <https://doi.org/10.1101/228866>
- Leroux O, Eeckhout S, Viane R, Popper ZA (2013). *Ceratopteris Richardii* (C-Fern): A model for investigating adaptive modification of vascular plant cell walls. *Frontiers in Plant Science* 4:367. <https://doi.org/10.3389/fpls.2013.00367>
- Lowe TM, Chan PP (2016). tRNAscan-SE On-line: Integrating search and context for analysis of transfer RNA genes. *Nucleic Acids Research* 44:W54-W57. <https://doi.org/10.1093/nar/gkw413>
- Luo Y (2023). Application of chloroplast genome analysis in plant taxonomy. *Theoretical and Natural Science* 8:290-294. <https://doi.org/10.54254/2753-8818/8/20240434>
- Magdy M, El-Sherbeny EA, Ramirez Sanchez A (2022). The complete chloroplast genome of the Egyptian henbane (*Hyoscyamus muticus* L., Solanaceae). *Mitochondrial DNA Part B* 7:1109-1111. <https://doi.org/10.1080/23802359.2022.2086493>
- Magdy M, Ou L, Yu H, Chen R, Zhou Y, Hassan H, Feng B, Taitano N, van der Knaap E, Zou X, Li F, Ouyang B (2019). Pan-plastome approach empowers the assessment of genetic variation in cultivated Capsicum species. *Horticulture Research* 6:108. <https://doi.org/10.1038/s41438-019-0191-x>
- Magdy M, Ouyang B (2020). The complete mitochondrial genome of the chiltepin pepper (*Capsicum annuum* var. *glabriusculum*), the wild progenitor of *Capsicum annuum* L. *Mitochondrial DNA Part B* 5:683-684. <https://doi.org/10/gbngh7>
- Nielsen R (2005). Molecular signatures of natural selection. *Annual Review of Genetics* 39:197-218. <https://doi.org/10.1146/annurev.genet.39.073003.112420>
- Ojosnegros S, Álvarez JMA, Grossmann J, Gagliardini V, Quintanilla LG, Grossnikalus U, Fernandez H (2023). Proteome and interactome linked to metabolism, genetic information processing, and abiotic stress in gametophytes of two woodferns. *International Journal of Molecular Sciences* 24:12429. <https://doi.org/10.3390/ijms241512429>
- Peng Y (2024). Characterization and analysis of multi-organ full-length transcriptomes in *Sphaeropteris brunoniana* and *Alsophila latebrosa* highlight secondary metabolism and chloroplast RNA editing pattern of tree ferns. *BMC Plant Biology* 24(1):73. <https://doi.org/10.1186/s12870-024-04746-w>
- Price MN, Dehal PS, Arkin AP (2010). FastTree 2 - approximately maximum-likelihood trees for large alignments. *PLoS ONE* 5:e9490. <https://doi.org/10.1371/journal.pone.0009490>
- Qin G (2024). Chromosome-scale genome of the fern *Cibotium barometz* unveils a genetic resource of medicinal value. *Horticulturae* 10:1191. <https://doi.org/10.3390/horticulturae10111191>
- Thodberg S, Sørensen M, Bellucci M, Crocoll C, Bendtsen AK, Nelson DR, Motawia MS, Moller BL, Neilson EHJ (2020). A flavin-dependent monooxygenase catalyzes the initial step in cyanogenic glycoside synthesis in ferns. *Communications Biology* 3(1):507. <https://doi.org/10.1038/s42003-020-01224-5>
- Tillich M, Lehwark P, Pellizzer T, Ulbricht-Jones ES, Fischer A, Bock R, Greiner S (2017). GeSeq - versatile and accurate annotation of organelle genomes. *Nucleic Acids Research* 45:W6-W11. <https://doi.org/10.1093/nar/gkx391>
- Vejvodová K (2024). High mountains of central Europe as a refuge of surprising cytotype diversity of *Huperzia selago* (Lycopodiaceae). *Alpine Botany*, 134(1):87-100. <https://doi.org/10.21203/rs.3.rs-3896707/v1>
- Wang M (2024). Whole genome duplication events likely contributed to the aquatic adaptive evolution of Parkerioideae. *Plants* 13:521. <https://doi.org/10.3390/plants13040521>
- Wang X, Xu K-W, Lee SY, Wu J, Li Q, Chen BJW (2023). Characterization of the chloroplast genome and phylogenetic analysis of *Ceratopteris pteridoides* (Pteridaceae). *Gene Reports* 30:101716. <https://doi.org/10.1016/j.genrep.2022.101716>
- Wei X, He M, Jin X (2020). The first complete chloroplast genome of a mangrove fern, *Acrostichum speciosum*. *Mitochondrial Dna Part B* 5:1413-1414. <https://doi.org/10.1080/23802359.2020.1735964>

- Xiaoxu H, Yinran H, Dong-yun L (2020). The complete chloroplast genome sequence of *Ulmus glabra* 'Pendula' (Ulmaceae). Mitochondrial Dna Part B 5(2):1765-1766. <https://doi.org/10.1080/23802359.2020.1749171>
- Yang Z (2007) PAML 4: phylogenetic analysis by maximum likelihood. Molecular Biology and Evolution 24:1586-1591. <https://doi.org/10.1093/molbev/msm088>
- Zhong Y, Yu R, Chen Y, Zhou R, Liu Y (2020). The complete chloroplast genomes of the mangrove fern *Acrostichum aureum*. Mitochondrial Dna Part B 5:2258-2259. <https://doi.org/10.1080/23802359.2020.1772140>
- Zhu M, Feng P, Ping J, Li J, Su Y, Wang T (2021). Phylogenetic significance of the characteristics of simple sequence repeats at the genus level based on the complete chloroplast genome sequences of Cyatheaaceae. Ecology and Evolution 11:14327-14340. <https://doi.org/10.1002/ece3.8151>
- Jazan Province - Flora of Saudi Arabia. Retrived in 2024 Dec 29 from <https://plantdiversityofsaudiarabia.info/jazan-province/>



The journal offers free, immediate, and unrestricted access to peer-reviewed research and scholarly work. Users are allowed to read, download, copy, distribute, print, search, or link to the full texts of the articles, or use them for any other lawful purpose, without asking prior permission from the publisher or the author.



**License** - Articles published in *Notulae Botanicae Horti Agrobotanici Cluj-Napoca* are Open-Access, distributed under the terms and conditions of the Creative Commons Attribution (CC BY 4.0) License.

© Articles by the authors; Licensee UASVM and SHST, Cluj-Napoca, Romania. The journal allows the author(s) to hold the copyright/to retain publishing rights without restriction.

**Notes:**

- **Material disclaimer:** The authors are fully responsible for their work and they hold sole responsibility for the articles published in the journal.
- **Maps and affiliations:** The publisher stay neutral with regard to jurisdictional claims in published maps and institutional affiliations.
- **Responsibilities:** The editors, editorial board and publisher do not assume any responsibility for the article's contents and for the authors' views expressed in their contributions. The statements and opinions published represent the views of the authors or persons to whom they are credited. Publication of research information does not constitute a recommendation or endorsement of products involved.