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Comparative chloroplast genome analysis and phylogenomics of Nigerian *Aframomum* (Zingiberaceae): insights into chloroplast genome evolution and species divergence

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Abstract

Background *Aframomum* is a genus of ecologically and economically important plants in the Zingiberaceae family, yet its genomic evolution remains poorly understood. Previous chloroplast genome studies in the family have focused mainly on Asian genera such as *Alpinia* and *Amomum*, leaving the African lineage unexplored.

Methods This study presents a comparative analysis of the complete chloroplast genomes of five *Aframomum* species, including *A. alboviolaceum*, *A. angustifolium*, *A. daniellii*, *A. melegueta*, and *A. sceptrum*. High-throughput Illumina sequencing was used to assemble the chloroplast genomes, which were annotated and analyzed for structural features, sequence divergence, codon usage, simple sequence repeats (SSRs), long repeats, and selection pressure on protein-coding genes. Phylogenetic relationships and divergence times were inferred using Bayesian methods implemented in MrBayes and BEAST2, respectively.

Results All five chloroplast genomes exhibited a conserved quadripartite structure with genome sizes ranging from 161,101 bp to 166,130 bp, but displayed lineage-specific variation at inverted repeat/single-copy boundaries, particularly the expanded *ycf1* and *rps19* gene junctions in *A. alboviolaceum*. Comparative analyses revealed high sequence conservation, with notable divergence hotspots in non-coding regions, including *ycf1-rps15*, *atpH-atpI*, and *trnT-trnL*. Codon usage patterns indicated strong AT-bias, and selection analyses revealed that most genes are under purifying selection, whereas *ycf1*, *rpoC2*, and *rpl16* show signals of positive selection, suggesting adaptive evolution in photosynthetic and translational functions. SSR analysis revealed predominantly A/T-rich mononucleotide repeats, with slight interspecific variation. Phylogenomic analysis strongly supported the monophyly of *Aframomum* and its close relationship to *Amomum*. Divergence time estimation indicates that *Aframomum* began diversifying around 26 Mya, with major radiation during the Miocene, contemporaneous with the African climatic shift that likely drove speciation.

Conclusions This study provides valuable insights into the chloroplast genome evolution and phylogeny of *Aframomum*, highlighting regions of high variability that are potential targets for molecular marker development.

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The results also reinforce the utility of chloroplast genome data in resolving evolutionary relationships and estimating divergence times within Zingiberaceae.

Keywords Aframomum, Chloroplast genome, Divergence time, Phylogeny, Selective pressure, Zingiberaceae

Introduction

Aframomum K. Schum. (Zingiberaceae: Alpinieae) as presently circumscribed, it is a genus of aromatic plants native to tropical Africa, comprising about 62 species, and it is one of the most ecologically and morphologically diverse taxa in the Zingiberaceae family [1, 2]. It is the largest genus of Zingiberaceae in Africa, serving as a key ecological component of the continent's forests. The genus was formerly included in the genus *Amomum*, and later was described to accommodate African species of the genus [3]. *Aframomum* is usually characterized by the absence of hypodermal sclerenchyma on the leaves, branched rhizomes, thick-walled fruit, and leafy stems in clumps from the rhizome [4]. Members of the genus mainly occur at the forest margins, old fields, and along roads in a moist environment. The species varied by the shape and size of leaves, stilt roots, and the architecture of rhizomes. *Aframomum* is well known for its economic and medicinal values, being used in folk medicine, food, and perfumery due to its bioactive compounds and aromatic properties [5]. Species of *Aframomum* are used in traditional medicine across the African continent in the treatment of diseases such as fever, stomach ache, diarrhea, inflammatory conditions and sexual stimulation [6]. *Aframomum* species also play a vital role in the African spice and food industry. *Aframomum melegueta* is a major export spice from West Africa, particularly Ghana, Nigeria, and Cameroon, contributing significantly to local economies and traditional trade networks. In Ghana alone, the annual export value of *A. melegueta* has been estimated at over 3 million USD, driven by demand in Europe and the Middle East for its pungent seeds used as a high-value substitute for black pepper and cardamom [7–9]. Other species, such as *A. daniellii* and *A. angustifolium*, are widely sold in local and regional markets as condiments and traditional herbal products [10, 11]. Beyond their culinary role, *Aframomum* species serve as important sources of essential oils, antioxidants, and pharmacologically active compounds used in perfumery, food preservation, and phytomedicine industries across Africa [5, 12].

The genus is known for bioactivities such as antiulcer, antiplasmodial, antinociceptive, antimicrobial, hepatoprotective and anticancer activities [13]. Members of *Aframomum* contain several phytochemicals such as diterpenoids, flavonoids, arylalkanoids and sesquiterpenoids [14]. Despite the importance of the genus, the chloroplast genomic resources of *Aframomum* were not reported, and the phylogenetic relationships and species divergence within the genus were not evaluated. Most research on the genus has mainly been on its chemical composition and nutritional values.

Nigeria, a biodiversity hotspot within the Guineo-Congolian region, hosts multiple *Aframomum* species such as *A. angustifolium*, *A. melegueta*, and *A. daniellii*, which were likely shaped by Pleistocene climatic fluctuations and habitat fragmentation [15]. The species of the genus in Nigeria are of cultural and medicinal value [16]. Despite their ecological and economic importance, the chloroplast genome characteristics and phylogenetic positions of the species remain unresolved. Understanding chloroplast genome variation among these species is essential for clarifying evolutionary relationships and informing taxonomic classification and conservation strategies. Moreover, estimating divergence times can provide context for the genus's radiation in relation to paleoclimatic and ecological shifts in tropical Africa.

Comparative chloroplast genome analysis can elucidate whether these species arose from recent radiations or ancient divergence, while also detecting signatures of selection in genes related to stress tolerance (e.g., *ndh* complex) and secondary metabolism (e.g., *accD*). Such insights are crucial for conservation and sustainable utilization, particularly as wild populations face threats from deforestation and overharvesting. In addition, Comparative chloroplast genome analysis offers a powerful approach to unraveling phylogenetic relationships, divergence patterns, and adaptive mechanisms in this genus, particularly in the context of West Africa's dynamic biogeographic history.

Plastid genomes are highly conserved, yet their structural variations, nucleotide substitutions, and selective pressures provide critical insights into plant evolution [17]. The chloroplast genome contained genes that regulate the transformation of light energy to chemical energy in plants [18, 19], providing nutrition to the plant. The circular genome exhibits a quadripartite structure consisting of a large single copy, a small single copy, and a pair of inverted repeats, with an overall size ranging from 125 kb to 180 kb, and encodes 115–135 genes [20, 21]. The chloroplast genome is an important tool in plant systematics and evolutionary studies due to its conserved structure, moderate mutation rates and uniparental inheritance [22]. Despite the conserved nature of the genome, there are variations in its size, structure, gene contents and substitution rate which are as a result of evolutionary events [23, 24]. The genome offers high-resolution data for phylogenetic analysis, species delimitation, molecular

marker development, and insights into genome evolution. In Zingiberaceae, chloroplast genome studies have resolved taxonomic uncertainties and identified key adaptive loci in related genera [25–28]. However, *Aframomum* has received no or limited genomic attention, leaving gaps in our understanding of its chloroplast genome, diversification and ecological adaptation. The extent of chloroplast genome variability and evolutionary divergence within this genus remains underexplored, particularly in West Africa, where many endemic species occur. This study aims to fill these knowledge gaps by conducting a comparative analysis of the complete chloroplast genomes of five *Aframomum* species, including four from Nigeria. Specifically, we (i) examine chloroplast genome structure, gene content, codon usage, and repeat elements; (ii) identify sequence divergence hotspots and evaluate evolutionary selection pressure on protein-coding genes; (iii) reconstruct phylogenetic relationships using whole chloroplast genome data; and (iv) estimate divergence times to understand the timing and pattern of speciation events in *Aframomum*. The findings provide new insights into chloroplast genome evolution and the phylogenomic framework of *Aframomum* and contribute to broader evolutionary studies in Zingiberaceae.

Materials and methods

Collection of plant material, DNA extraction and sequencing

Four species of *Aframomum* (*Aframomum melegueta*, *Aframomum sceptrum*, *Aframomum daniellii*, and *Aframomum angustifolium*) were collected in the forest areas of Nigeria (Cross River National Park). The collections were identified at the Herbarium of Umaru Musa Yaradua University, Katsina, Nigeria, and a voucher specimen was prepared for the species and deposited in the herbarium (*A.angustifolium* UMYUH 2414, A. *sceptrum* UMYUH 2415, *A. melegueta* UMYUH 2416, *A. daniellii* UMYUH 2417). The name of the herbarium curator is Dr Mustapha Haruna (Mustapha.haruna@umyu.edu.ng). During field collection, fresh young leaves were sampled from each species and were put in a container containing ice and transported to Yunnan University. Total genomic DNA was extracted from the fresh leaves using the Tiangen DNA extraction kit following the manufacturer's protocol. The quality of the DNA was checked using 1% agarose gel electrophoresis and a NanoDrop meter, and the concentration of the DNA was measured using a DNA Assay kit in Qubit. For the library preparation, a total of 0.2 μg of the DNA was used as input material, and the sequencing library was generated using NEB Next® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer's recommendations, and index codes were added to each sample. The clustering of the index-coded samples was performed on a CBOT Cluster Generation System using Illumina PE Cluster Kit (Illumina, USA) according to the manufacturer's instructions. After cluster generation, the DNA libraries were sequenced on the Illumina platform, and 150 bp paired-end reads were generated.

Chloroplast genome assembly and gene annotation

The complete chloroplast genome of the four species was assembled using Novoplasty version 4.2 [29]. The chloroplast genome of *A. angustifolium* was first assembled using the complete chloroplast genome of *Amomum longipetiolatum* (PP542016.1) as seed and reference with a k-mer of 39. The assembled chloroplast genome of *A. angustifolium* was used as seed and reference in the assembly of the chloroplast genomes of the three species with a k-mer of 39. All the assemblies were first checked for correct orientation in the CHLOROBOX, and the position of the inverted repeat b (IRb) was corrected in Geneious Prime version 2024.0.7 [30] using the chloroplast genome of *Amomum* as a reference. The final FASTA file generated from Geneious was used for the annotation of the genes in the chloroplast genome in GeSeq of the CHLOROBOX [31]. Sequin version 15.50 [32] was further used to correct the errors of the annotation generated by GeSeq by manually adjusting the positions of start and stop codons. Finally, tbl2asn was used to generate files for submission to NCBI for issuance of GenBank accession numbers. Organellar Genome Draw (ORGDRAW) [33] was used to generate the gene map of the chloroplast genomes.

Sequence polymorphic site analyses

For sequence divergence analysis of the five *Aframomum* chloroplast genomes, the mVISTA online tool [34] (Berkeley, CA, USA) was used to compare the chloroplast genome sequences of the species using the annotation of *A. angustifolium* as reference in the Shuffle LAGAN mode [35]. For the identification of the polymorphic hotspot site, the chloroplast genomes of the five *Aframomum* species were aligned using MAFFT version 7 [36] and analyzed using DnaSP version 6.0 [37] using 600 bp and 200 bp for the window length and step size, respectively [38].

Evaluation of genes under purifying and positive selection

To identify the genes that undergo either purifying or positive selection, the 79 shared protein-coding genes were extracted using Python. The protein-coding genes of the earliest diverged species (*A. alboviloceum)* were used as a reference and analyzed with those of *A. angustifolium*, *A. daniellii*, and *A. melegueta*. Each of the sets of protein-coding genes was aligned in Geneious, and the ka/ks values were calculated using DnaSP version 6.0 [37].

Analyses of simple sequence repeats and long repeats

For the identification of simple sequence repeats (1–6 bp) an online tool MISA [39] was used for the analysis using the following parameters: eight for mononucleotides, five for dinucleotides, four for trinucleotides, and three for tetra, penta, and hexanucleotides SSR motifs. Long repeat sequences (forward, reverse, complement and palindromic) were analyzed using REPuter (https://bibiserv.cebitec.uni-bielefeld.de/reputer) [40] with default parameters.

Codon usage

Relative synonymous codon usage values (RSCU), base composition, and codon usage were analyzed using MEGA version 7.0.26 [41].

Phylogenetic analysis and divergence time estimation

To infer the phylogenetic relationship between *Aframomum* and related genera within the tribe Alpineae, 21 complete chloroplast genomes of the tribe were used for the construction of the phylogenetic tree. Two species of the genus *Zingiber*, *Zingiber specabile* (NC_020363.1) and *Zingiber officinale* (NC_044775.1), were used as outgroups. The chloroplast genome sequences of all the species were aligned using MAFFT version 7 [36], and the phylogenetic tree was constructed using the Bayesian inference approach in MrBayes version 3.2 [42] using the GTR + I = I substitution model and Markov Chain Monte Carlo (MCMC) chains of 50 million generations with sampling every 5,000 generations. For the divergence time estimation, BEAST version 2.7.6 was used for the Bayesian molecular dating analysis. The chloroplast genome sequences of five *Aframomum* species and related genera within Alpineae, and two species of *Zingiber* were aligned using MAFFT version 7. The analysis was performed on a relaxed molecular clock and a substitution model of GTR + T with 4 gamma categories, and for the base frequency, the empirical option was used. For the priors, the Yule model was selected, and the time calibration was applied using a lognormal option. This prior was modeled using a lognormal distribution, allowing the 95% highest posterior density (HPD) interval to capture uncertainty across the estimates. The crown node of Zingiberaceae was calibrated using a secondary calibration (mean = 1.2, standard deviation = 0.8, offset = 55 Mya) based on molecular-dating studies that consistently place the family's crown age between 55 and 65 Mya [43, 44]. Additional fossil calibrations were not available for the genus *Aframomum* or the tribe Alpineae; hence, the single secondary calibration was adopted to maintain consistency with prior family-level divergence studies. MCMC chains were run for 100 million generations, sampling every 10,000 generations. Tracer v1.7.2 was used to assess parameter convergence and effective sample sizes (ESS > 200) for all estimated parameters, confirming adequate mixing and convergence of the chains.

Results

General characteristics of *Aframomum* chloroplast genomes

The length of the chloroplast genome of the five studied Aframomum species ranged from 161, 101 bp in *A. melegueta* to 166, 130 bp in *A. alboviolaceum* (Table 1). The chloroplast genomes of the four Nigerian species are similar in length, with a variation of about 60 to 170 bp, while *A. alboviolaceum* is lengthier with about 5, 000 bp. All the chloroplast genomes are circular with a quadripartite structure, consisting of a large single-copy, a small single-copy, and a pair of inverted repeat regions. The length of the LSC ranged from 86, 277 (*A. sceptrum*) to 88, 644 (*A. angustifolium)*. For the SSC region, the chloroplast genome of *A. alboviolaceum* is the longest with 22, 118 bp, and the four Nigerian species have almost the same length (17, 602 bp – 17, 615 bp). The length of the inverted repeats ranged from 27, 489 bp in *A. angustifolium* to 27, 672 in *A. daniellii* and *A. sceptrum*. The overall GC content is similar among species but unevenly distributed across the genome, with the four Nigerian species having 36.1% and the *A. alboviolaceum* having 35.8%.

Table 1 The general characteristics of the five *Aframomum* Chloroplast genomes

Characteristics	A. angustifolium	A. daniellii	A. melegueta	A. sceptrum	A. alboviolaceum
Total length (bp)	161, 232	161, 295	161, 101	161, 224	166, 130
Length of LSC (bp)	88, 644	88, 336	88, 291	88, 277	88, 634
Length of SSC (bp)	17, 610	17, 615	17, 602	17, 603	22, 118
Length of IRa (bp)	27, 489	27, 672	27, 604	27, 672	27, 689
Length of IRb (bp)	27, 489	27, 672	27, 604	27, 672	27, 689
Number of genes	134	134	134	134	134
Protein-coding genes	89	89	89	89	89
tRNA genes	35	35	35	35	35
rRNA genes	8	8	8	8	8
GC content (%)	36.1	36.1	36.1	36.1	35.8

The gene number in the chloroplast genome of *Aframomum* is highly conserved across all the species, containing 134 genes (89 are protein-coding genes, 35 tRNA genes and 8 rRNA genes). Among the 134 genes, 96 genes are located in the single-copy regions, while the remaining were duplicated in the inverted repeat regions, and 19 of the genes contained an intron (*trnK-UUU*, *rps16*, *trnG-UCC*, *atpF*, *rpoC1*, *trnL-UAA*, *trnV-UAC*, *rps12*, *petB*, *petD*, *rpl16*, *rpl2*,*ndhB*, *trnI-GAU*, *trnA-GUC*, *rrn23*, *ndhA)* have a single intron, while (*ycf3* and *clpP*) have two introns (Fig. 1).

Contraction and expansion of the single-copy and the inverted repeats regions

One of the major events in the chloroplast genome evolution is the contraction and expansion of the boundary of the single-copy and the inverted repeat regions, leading to a shift in the position of the genes and also variation of the size of the genome. The comparative analysis of inverted repeat (IR) boundaries among five *Aframomum* species reveals a generally conserved chloroplast genome structure with subtle variations. The length of the IR regions remains relatively stable (ranging from 27,489 to 27,689 bp), but genes located at the junctions, particularly *rps19*, *ycf1*, and *ndhF*, exhibit minor positional shifts (Fig. 2), particularly between the Nigerian species and the *A. alboviolaceum*. For instance, in four Nigerian species, the fragment of the *ycf1* pseudogene was found in the inverted repeat b region and extends into the SSC region with 46 to 108 bp, while in *A. alboviolaceum* it extends into the SSC region with 3, 680 bp, and the *ndhF* gene was contained in the SSC region and overlaps with the *ycf1* gene in the JSB. The *ycf1* genes of *A. alboviolaceum* is longer than those of the Nigerian species, with about 3, 711 bp. The *rps19* gene was found in the IRb region of *A. alboviolaceum*, *A. sceptrum* and *A. melegueta* with about 100 bp away from the JLB border and the gene extends into the LSC region with 64 bp in *A. daniellii* and 43 bp in *A. angustifolium*, indicating slight expansions or contractions of the IRs. Similarly, the other duplicated *rps19* gene extends into SSC in the JLA of *A. daniellii* and *A. angustifolium*. At the JSA boundary, the *ycf1* gene of all five species extends into IRa from the SSC, and most of the sequences are in the SSC region. These boundary shifts, although minor, represent evolutionary microstructural changes that can inform phylogenetic relationships and chloroplast genome evolution within the *Aframomum*.

Sequence polymorphic site analyses

The mVISTA alignment of complete chloroplast genomes of five *Aframomum* species revealed a high degree of overall sequence conservation, particularly in coding regions. However, several non-coding intergenic regions exhibited substantial variability. Among the most divergent regions were *atpH–atpI*, *trnT–trnL*, *ycf4–cemA*, *psaC–ndhE*, and *ycf1–rps15*, and the hypervariable gene *ycf1*. These regions showed noticeable drops in sequence identity, indicating elevated substitution rates and indels. As expected, the inverted repeat (IR) regions displayed high conservation, especially among ribosomal RNA and ribosomal protein genes *rrn16*, *rrn23*, *rps7*, and *rpl2*, reflecting their evolutionary stability. The variability observed in the single-copy regions—especially in the LSC and SSC intergenic spacers—highlights their potential utility in molecular marker development for *Aframomum* species. These divergent hotspots may serve as informative regions for DNA barcoding, species delimitation, and phylogenetic studies within the genus and the broader Zingiberaceae family. The conserved structure across chloroplast genomes, coupled with localized divergence in specific regions, supports the notion of evolutionary conservation interspersed with lineage-specific changes in non-coding chloroplast DNA (Fig. 3).

The nucleotide diversity plot across the aligned chloroplast genomes of five *Aframomum* species reveals clear variation in sequence conservation across different genomic regions with nucleotide diversity values ranging from 0 to 0.058 (Fig. 4). The single-copy regions, particularly the small single-copy, exhibited greater nucleotide diversity compared to the highly conserved inverted repeat (IR) regions, consistent with observations in other Zingiberaceae. Comparing the coding and non-coding regions, the highly variable regions are located within the intergenic spacer regions, notably *atpH–atpI*, *trnT–trnL*, and *ycf4–cemA* in the LSC, and *psaC–ndhE* and *ycf1–rps15* in the SSC region. Among all loci, the *ycf1* gene displayed the highest nucleotide diversity, marking it as the most variable coding region across the five *Aframomum* chloroplast genomes. The IR regions, particularly IRb and IRa, showed minimal variation, reflecting their structural and evolutionary stability. These findings highlight a set of divergent hotspots primarily located in the SSC and LSC intergenic regions that could serve as valuable molecular markers for species identification, population genetics, and phylogenetic studies within *Aframomum*. The variability observed in the *ycf1* gene also supports its growing utility as a universal barcode region in land plants.

Codon usage analyses

The analysis of codon usage among the chloroplast genomes of five *Aframomum* species revealed that the total number of codons present in the chloroplast genomes ranged from 53, 740 in *A. sceptrum* to 55, 376 in *A. alboviolaceum*. Codons coding for the amino acid leucine were the most prevalent codons in all the genomes, and those of tryptophan were the least abundant. This is

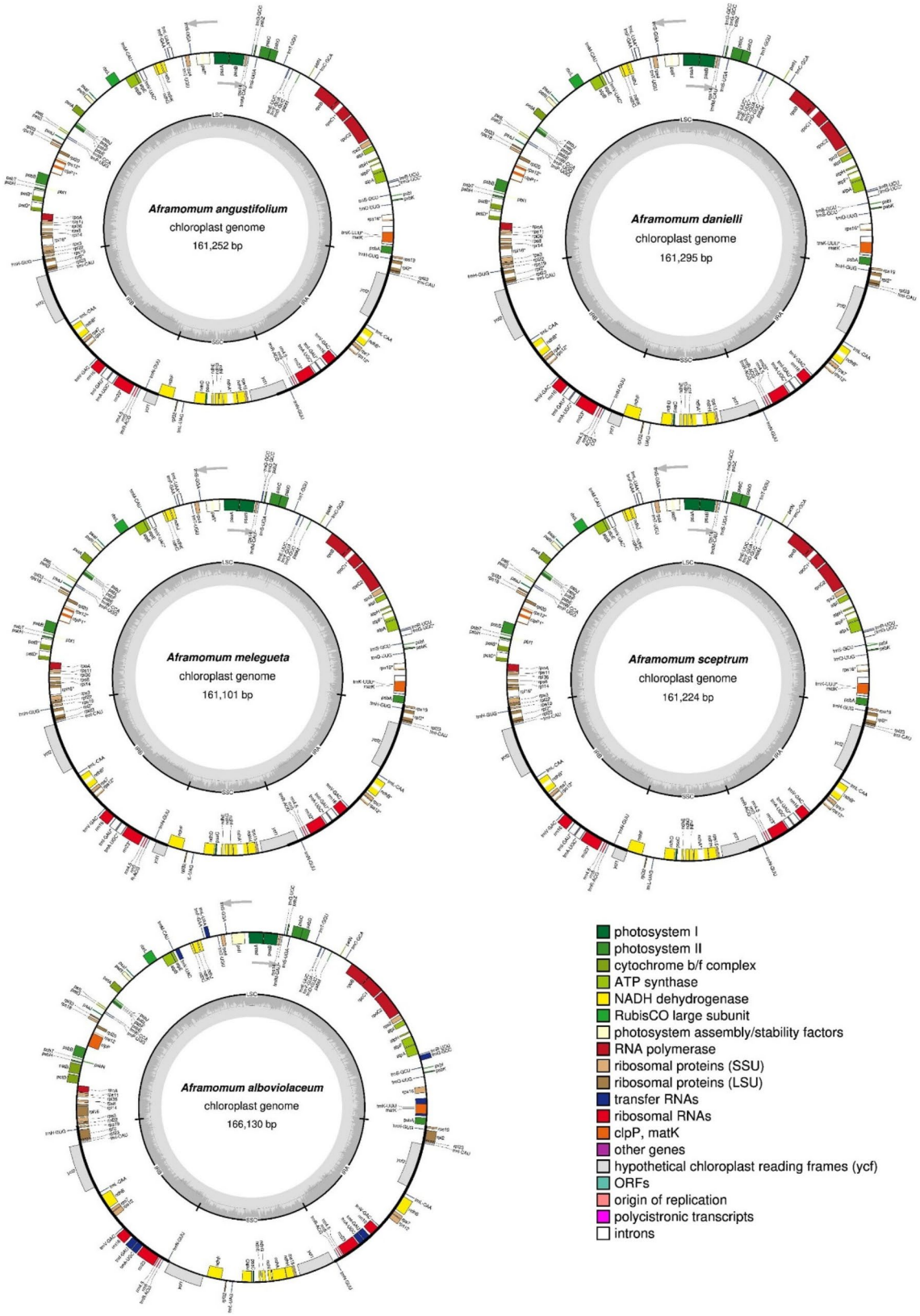

Fig. 1 The chloroplast genome maps of five *Aframomum* species, showing the numbers and categories of genes present in the genomes. Inside the circle are genes transcribed in the clockwise direction, and genes outside the circle are transcribed in an anti-clockwise direction

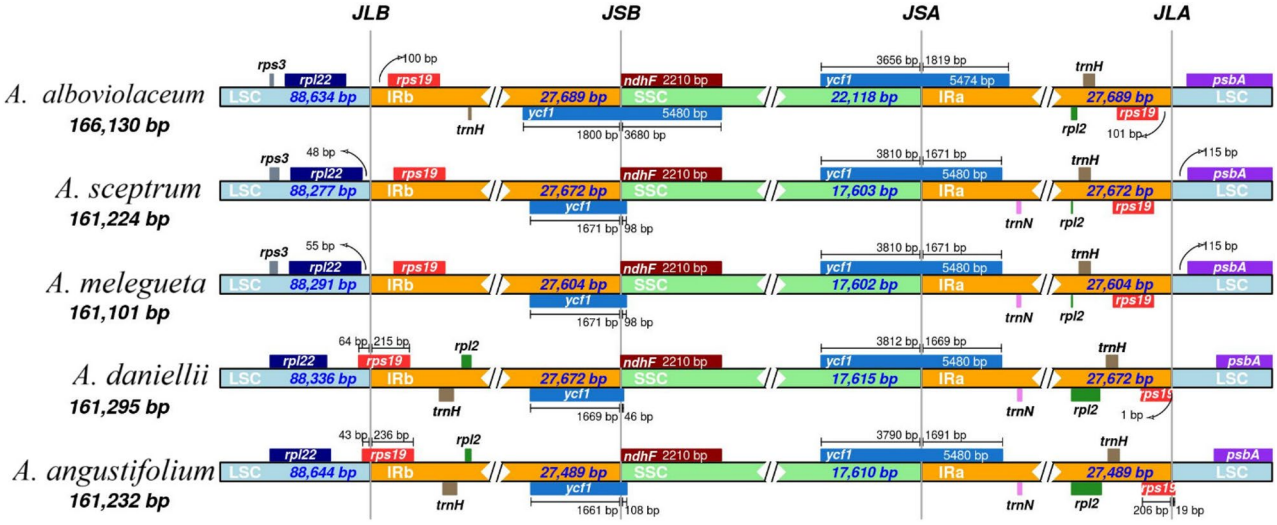

Fig. 2 Comparison of junction boundaries of SSC, LSC, IRa and IRb in the chloroplast genome of five *Aframomum* species. JLB= LSC/IRb intersection; JSB= SSC/IRb intersection; JLA= LSC/IRa intersection; JSA= SSC/IRa intersection. SSC: small single copy, LSC: large single copy, IRb: inverted repeat B, IRa: inverted repeat A

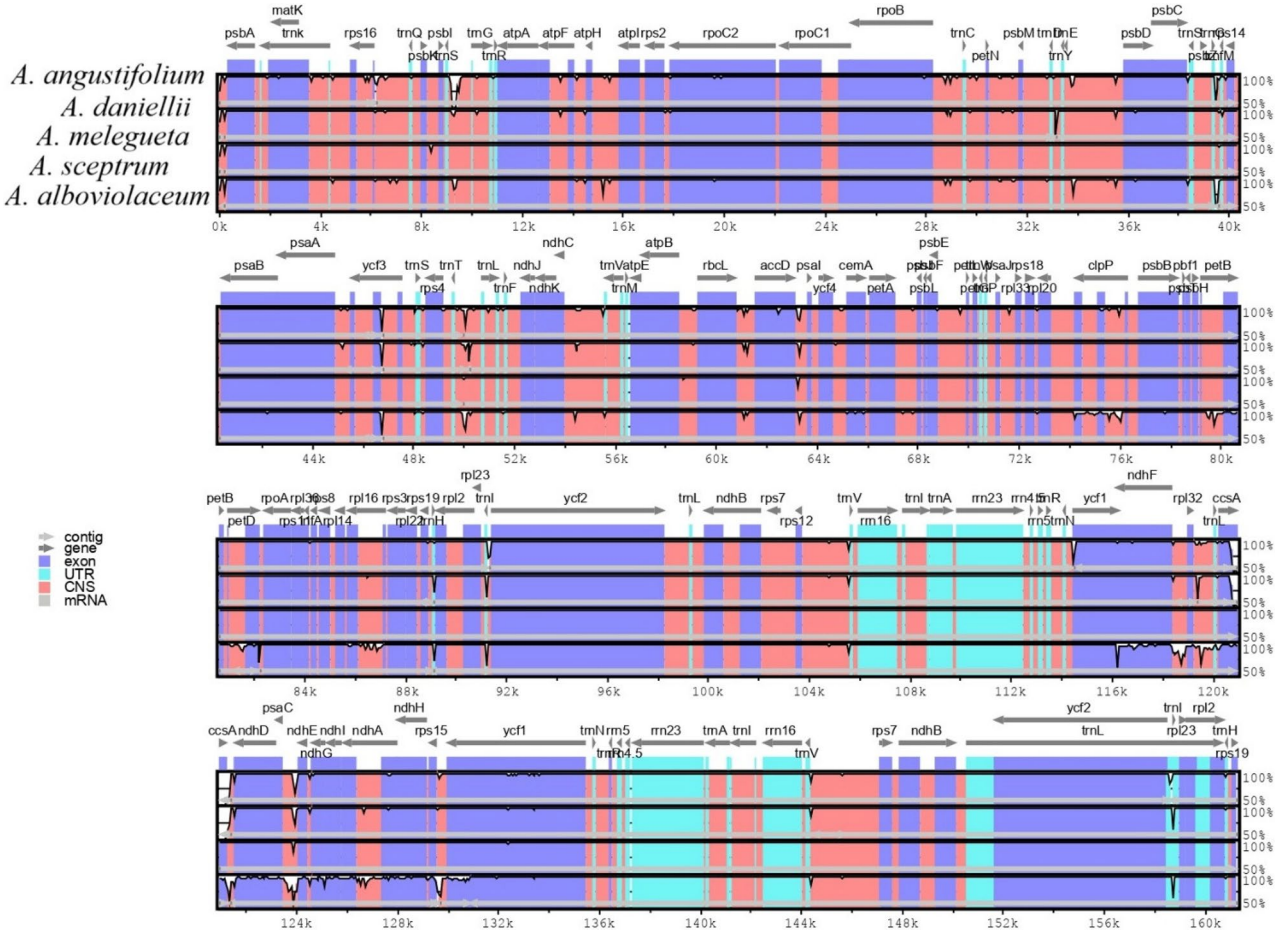

Fig. 3 Sequence alignment of five *Aframomum* species chloroplast genomes generated with mVISTA with annotation *A. angustifolium* as reference. The top arrow shows transcription direction, blue colour indicates protein coding, pink colour shows conserved non-coding sequence CNS and light green indicates tRNAs and rRNAs. The x-axis represents the coordinates in the cp. genome, while y- axis represents the percentage identity within 50–100%

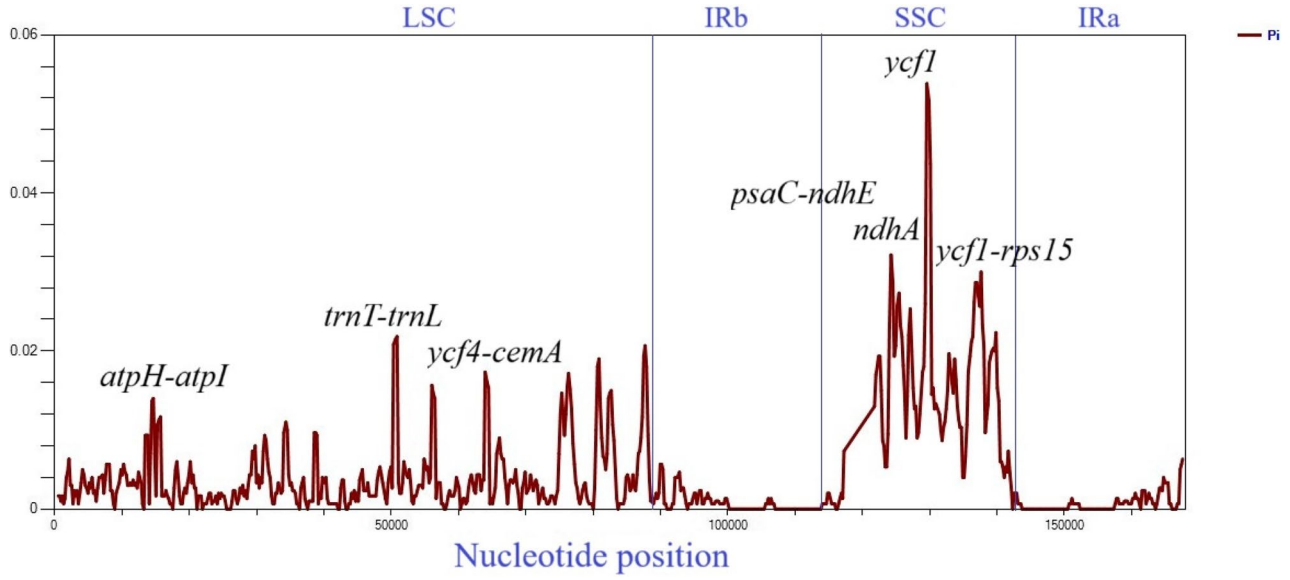

Fig. 4 The nucleotide diversity of the five *Aframomum* chloroplast genomes generated with a step length of 200 bp and a window length of 600 bp. SSC: small single copy, LSC: large single copy, IRb: inverted repeat B, IRa: inverted repeat A

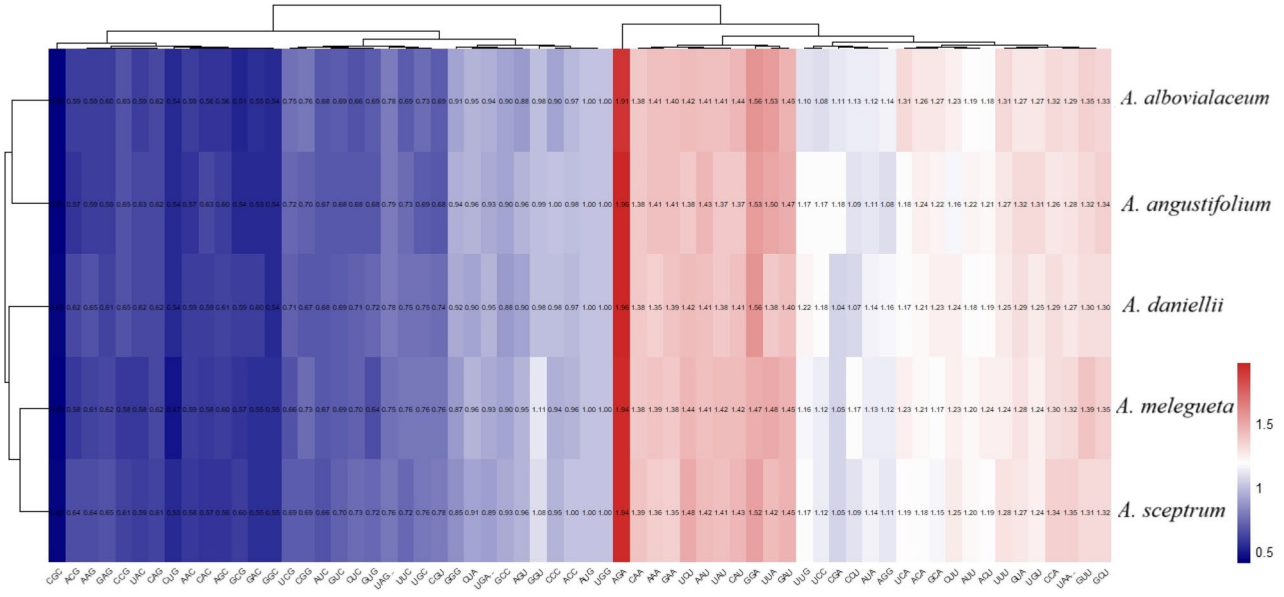

Fig. 5 Heatmap showing the relative synonymous codon usage values in the chloroplast genome of five *Aframomum* species, revealing the codon usage bias

attributed to the fact that Leucine is coded by 6 different codons, while tryptophan is encoded by a single codon. The result further revealed a conserved and slightly variable pattern of relative synonymous codon usage (RSCU) values in all five genomes, indicating evolutionary conservation. The RSCU values of 30 codons in *A. albovialaceum*, *A. angustifolium*, and *A. daniellii* were greater than 1, and the genomes of *A. melegueta* and *A. sceptrum* have 31 codons with RSCU values greater than 1 (Fig. 5). The codons coding for methionine and tryptophan have an RSCU value of 1 in all the species, while one of the codons coding for the amino acid proline (CCC) in *A.angustifolium* has an RSCU value of 1. Similarly, the ACC codon, coding for threonine in *A. sceptrum*, also has an RSCU value of 1. The following codons: UUA (Leu), AGA (Arg) and AUU (Ile) were the most frequently used across all the species, with RSCU values significantly greater than 1, indicating a strong codon usage bias favoring A- or U-ending codons. On the other hand, CUG (Leu), AUC (Ile), GGC (Gly), CGC (Arg), CGA (Arg), and AGG (Arg) were the codons with the lowest RSCU values less than 1 (Fig. 5), signifying underrepresentation. The

bias towards the A/U codon is a result of the high abundance of A/T content in the genome, and this phenomenon is common in angiosperm chloroplast genomes. Minor interspecific variances in codon usage seen in leucine (UUG vs. UUA) and isoleucine (AUA vs. AUU) may reflect subtle lineage-specific evolutionary pressures or mutational constraints. The clustering pattern in the heatmap indicates the close phylogenetic relationship among the *Aframomum* species.

Evaluation of genes under purifying and positive selection

To assess the selective pressure acting on chloroplast protein-coding genes among *Aframomum* species, we calculated pairwise Ka/Ks (nonsynonymous/synonymous substitution rate) ratios using *Aframomum alboviolaceum* as the reference against *A. angustifolium*, *A. daniellii*, and *A. melegueta*. The majority of the genes analyzed displayed Ka/Ks values significantly below 1.0, indicating strong purifying selection and functional conservation across these species. However, a few genes showed elevated Ka/Ks values, suggesting the genus may be under positive selection. Notably, genes such as *clpP*, *rpoC2*, *ndhA*, *ycf1*, and *rpl16* displayed relatively high Ka/Ks ratios in all the comparisons, with *rpl16* in the *A. alboviolaceum* vs. *A. daniellii* comparison exceeding 1.0, indicative of adaptive evolution. Similarly, *rpoC2* in *A. alboviolaceum* vs. *A. melegueta* undergoes positive selection. In contrast, essential photosynthetic genes such as *psaA*, *psbA*, and *rbcL* maintained low Ka/Ks ratios across all comparisons, consistent with strong functional constraints. Additionally, moderate Ka/Ks values (~0.3–0.6) were detected for *matK*, *rpoA*, *rpoB*, and several *ndh* genes, implying potential shifts in functional constraint or environmental adaptation (Fig. 6).

Analyses of simple sequence repeats and long repeats

The analyses of the simple sequence repeats (SSRs) among the five *Aframomum* species reveal a highly conserved SSR composition in the chloroplast genome of the species. Mononucleotide repeats are the most dominant, ranging from 173 in *A. daniellii* to 185 in *A. alboviolaceum*, and the majority are typically A and T motifs (Fig. 7A). Dinucleotide and tetranucleotide repeats occur at moderate frequencies and display consistent patterns across all species, having 32 and 19, respectively. *A. melegueta* and *A. daniellii* have the same frequency of pentanucleotide of 2, while *A. alboviolaceum*, *A. angustifolium*, and *A. sceptrum* have the same number of pentanucleotides of 3. Hexanucleotide is not present in the chloroplast genome of *A. daniellii* and *A. alboviolaceum*, and it has a frequency of 2 in the chloroplast genome of *A. melegueta*, and it occurred once in the genomes of *A. angustifolium* and *A. sceptrum*. The conserved SSR distribution indicates structural similarity and limited divergence in these chloroplast genomes. The SSRs heatmap (Fig. 8) illustrates that most species share a conserved pattern of SSR motif occurrence, with a slight variation in the counts of less frequent motifs such as AAATT/ATTTT, AATG/ATTC, and AAAG/CTTT. These minor differences contribute to the hierarchical clustering observed, which groups species with similar SSR profiles. The overall conservation of SSR motif types, especially among mono- and dinucleotide repeats, suggests a high degree of chloroplast genome structural stability within the genus. The species-specific motifs and the SSR loci may serve as informative molecular markers for phylogenetic, taxonomic, and population genetics studies within the *Aframomum* genus and the broader Zingiberaceae family. The analysis of long repeats in the chloroplast genome of the five *Aframomum* species revealed that only *A. melegueta* possessed all four types of repeats (Palindromic, Forward, Reverse and Complement). *A. sceptrum* and *A. angustifolium* have three types of repeats (Palindromic, Forward and Reverse) while *A. daniellii* and *A. angustifolium* have only two types of repeats (Palindromic and Forward). *A melegueta* and *A.*

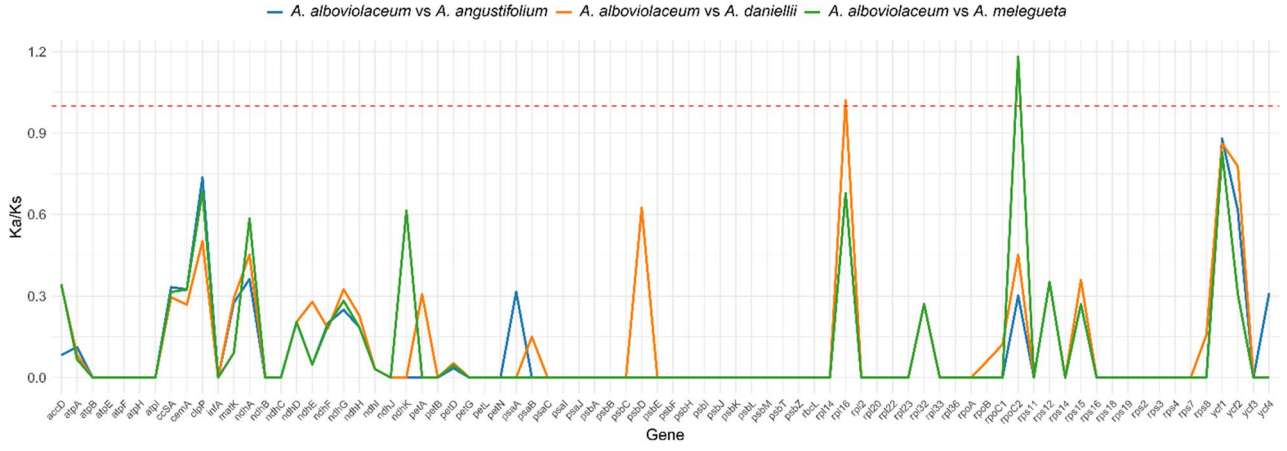

Fig. 6 The Ka/Ks ratio values of 79 protein-coding genes from the chloroplast genome of four *Aframomum* species

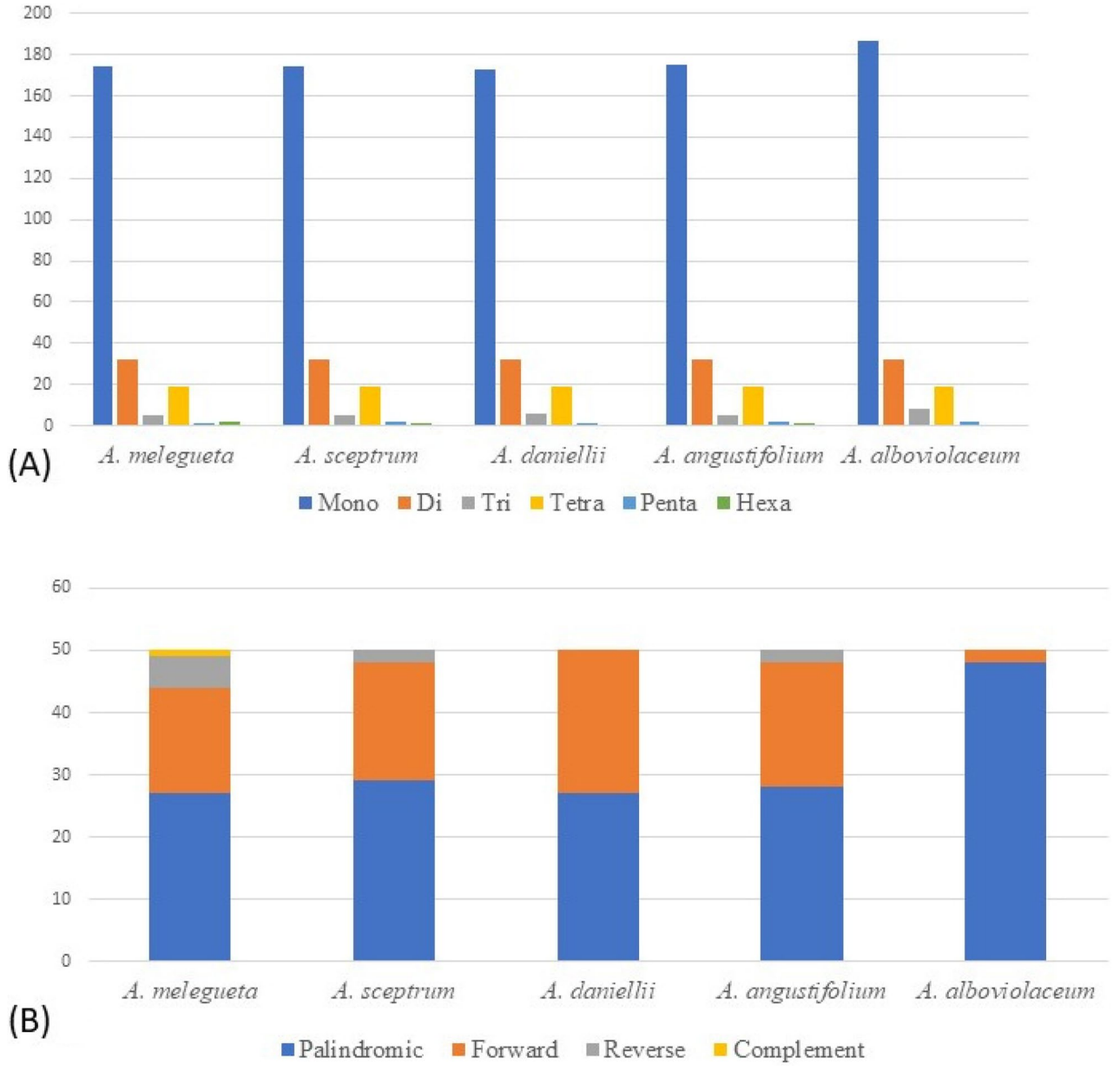

Fig. 7 (**A**) Frequency of different types of simple sequence repeats in the chloroplast genome of five *Aframomum* species; (**B**) Frequency of different long repeats in the chloroplast genome of five *Aframomum* species

angustifolium have the same frequency of Palindromic repeat, while *A. sceptrum* and *A. angustifolium* have the same number of Reverse repeats (Fig. 7B).

Phylogenetic analysis and divergence time Estimation

The phylogenetic tree constructed using chloroplast genome sequences delineates the evolutionary relationships within the Alpineae tribe. The tree is rooted with *Zingiber spectabile* and *Zingiber officinale*, forming the basal clade, confirming the Zingiber genus's distinct lineage from alpineae. A well-supported monophyletic clade (posterior probability = 1.0) encompasses the genus *Aframomum* (highlighted in yellow), indicating close evolutionary affinity among these species (Fig. 9). The phylogenetic tree supports the close relationship between *Aframomum* and *Amomum; this group is* sister to the genus *Alpinia* (Fig. 9). The genera *Amomum* and *Alpinia* appear in multiple clades, suggesting paraphyly or polyphyly, which may reflect taxonomic inconsistencies or recent diversification events. *Amomum longipetiolatum* and *A. velutinum* form a distinct clade (light pink), while other *Amomum* species, such as *A. krervanh* and *A. compactum*, cluster with *Wurfbainia* species (purple), indicating that potential taxonomic reclassification is

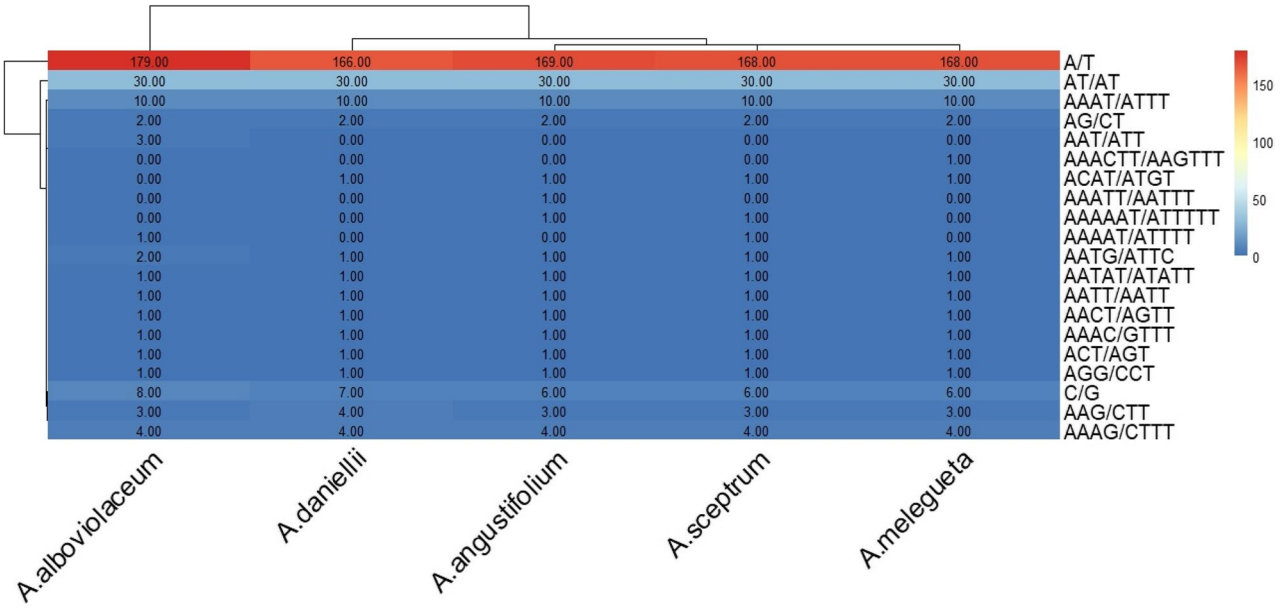

Fig. 8 Heatmap showing different forms of simple sequence repeat motifs in the chloroplast genome of five *Aframomum* species

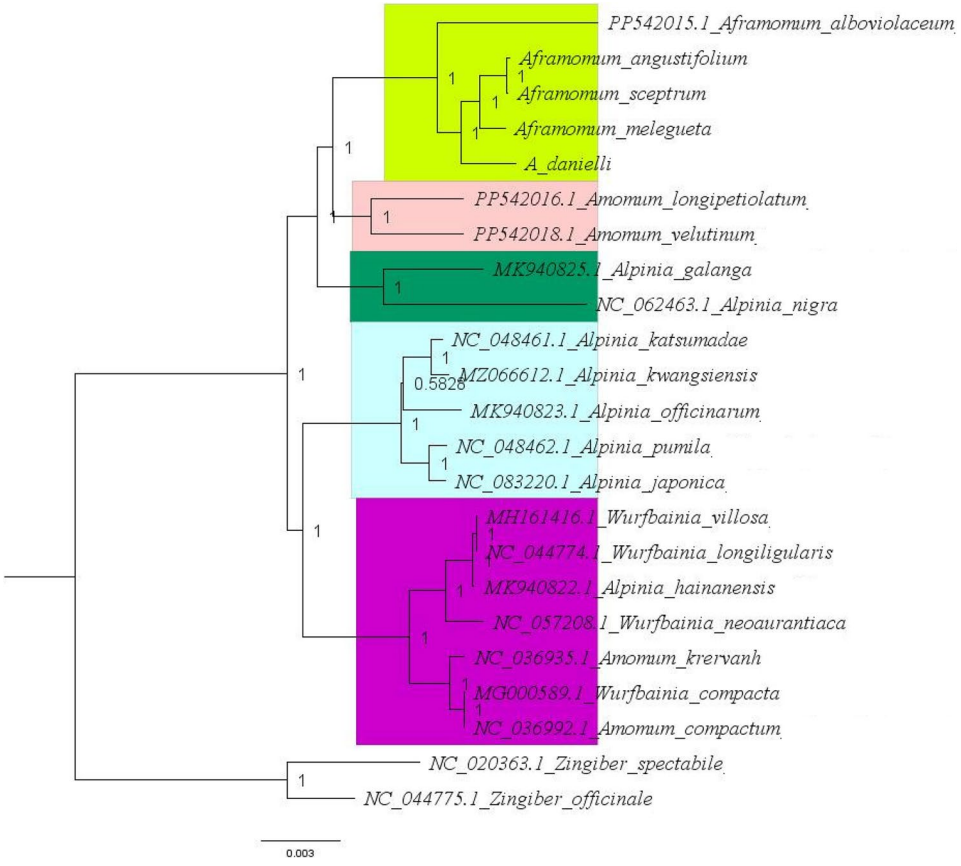

Fig. 9 Bayesian inference phylogenetic tree constructed using the complete chloroplast genome of Alpineae species with the genus *Zingiber* as outgroup

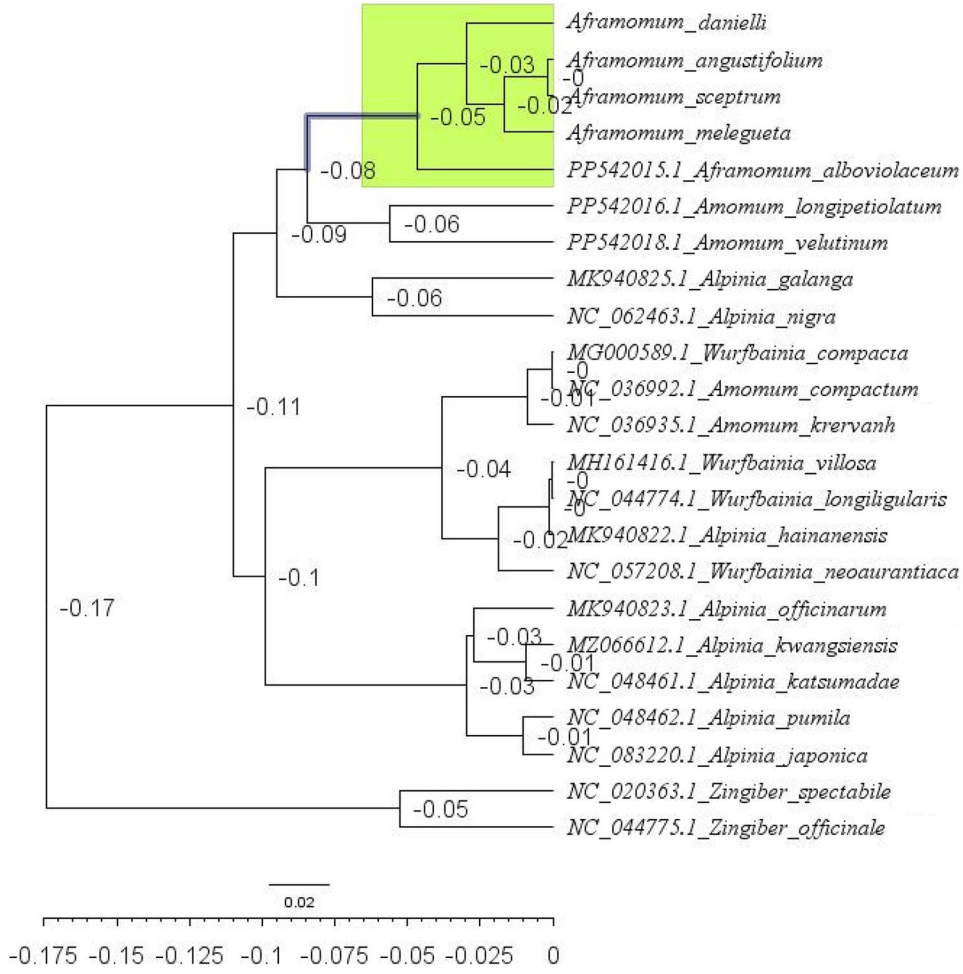

Fig. 10 Time-calibrated phylogeny of *Aframomum* and related taxa inferred using BEAST2.Node ages are shown in substitutions/site and scaled to millions of years ago (Mya) based on a lognormal prior calibration (M = 1.2, S = 0.8 and offset = 55 Mya) at the crown of Zingiberaceae

warranted. *Alpinia* species form several well-supported clades (dark green and light blue), with *A. galanga* and *A. nigra* clustering separately from *A. katsumadae*, *A. kwangsiensis*, *A. officinarum*, *A. pumila*, and *A. japonica*, again suggesting genus-level polyphyly. Notably, *Wurfbainia* species (*W. villosa*, *W. longiligularis*, *W. neoaurantiaca*, and *W. compacta*) are interspersed among *Amomum* species, reflecting close genetic relatedness and possibly recent divergence or historical misclassification. Overall, the tree underscores the complex evolutionary history of the Zingiberaceae family and highlights the necessity for further molecular and morphological studies to resolve genus-level relationships, particularly within *Amomum*, *Alpinia*, and *Wurfbainia*.

The time-calibrated phylogeny inferred using BEAST2 provides insights into the evolutionary history of the genus *Aframomum* within Zingiberaceae. Divergence times are estimated in millions of years ago (Mya), with calibration applied using a lognormal prior (M = 1.2, S = 0.8 and offset = 55 Mya) at the Zingiberaceae crown node. The root age of the tree is inferred to be approximately 55 Mya, corresponding to the node height of 0.17. Within the *Aframomum* clade (highlighted in green), the most recent common ancestor (MRCA) of the group comprising *A. daniellii*, *A. angustifolium*, *A. sceptrum*, and *A. melegueta* is estimated to have diverged around 16 Mya (node height = 0.05) (95% highest posterior density (HPD): 10.03–20.78 Mya) (Fig. 10). The earliest split within this group, between *A. melegueta* and *A. sceptrum* and *A. angustifolium*, occurred approximately 13 Mya (0.03) (95% HPD: 9.47–17.35 Mya), followed by more recent divergences of A. *sceptrum* and *A. angustifolium* that diverged about 6 to 9 Mya (0.02–0.03) (95% HPD: 3.47–12.35 Mya) (Fig. 10). The broader divergence of *Aframomum alboviolaceum* from this crown group is inferred at approximately 26 Mya (0.08) (95% HPD: 20.47–33.35 Ma), suggesting a deep genetic separation between *A. alboviolaceum* and the rest of the *Aframomum* species analyzed. This temporal pattern indicates a rapid radiation of several *Aframomum* species during the Miocene, a period known for significant climatic and ecological shifts in tropical Africa that may have driven

diversification. These divergence estimates, when placed in the context of geoclimatic events, support the hypothesis that Miocene environmental dynamics played a pivotal role in shaping the evolutionary trajectories within *Aframomum*. The findings also underscore the importance of time-calibrated phylogenies for reconstructing the biogeographical and ecological history of tropical plant lineages.

Discussion

This work represents the first comprehensive chloroplast genome-scale comparison within the African genus *Aframomum*. While earlier Zingiberaceae chloroplast genome studies concentrated on Asian taxa such as *Alpinia*, *Amomum*, and *Kaempferia* [27, 45, 46], none have examined African lineages. We identified a unique pattern of IR boundary expansion in *A. alboviolaceum*, in which the *ycf1* gene extends ~ 3.7 kb into the SSC region, and *rps19* overlaps the IR/LSC junction, features not reported in other family members. The detection of positively selected genes (*ycf1*, *rpoC2*, *rpl16*) further distinguishes *Aframomum*, implying lineage-specific adaptive evolution possibly linked to tropical African environments. Moreover, the divergence-time analysis reveals a Miocene radiation (~ 26–6 Mya) that provides new evidence for a distinct biogeographic history relative to Southeast Asian Zingiberaceae, which diversified earlier during the Oligocene. Collectively, these findings establish *Aframomum* as an evolutionarily and biogeographically unique lineage within the family, expanding current understanding of chloroplast genome structural dynamics and adaptive evolution in Zingiberaceae.

Structural conservation and variation in *Aframomum* chloroplast genomes

The chloroplast genomes of the five *Aframomum* species exhibit typical quadripartite structures and conserved gene content, consistent with previous reports in Zingiberaceae chloroplast genomes [47, 48]. While the Nigerian species (*A. melegueta*, *A. daniellii*, *A. sceptrum*, and *A. angustifolium*) showed minimal variation in genome size and structure, *A. alboviolaceum* displayed a noticeably longer genome, driven primarily by an expanded small single-copy (SSC) region. This observation highlights microstructural plasticity, which may result from IR boundary shifts, a common evolutionary feature in angiosperm chloroplast genomes [49]. Despite the conserved nature of the chloroplast genome of flowering plants, there is evidence of gene loss, structural rearrangement in some taxonomic groups. In *Aframomum*, the *ycf15* gene is absent, and this gene was not reported to be present in the chloroplast genome of Zingiberaceae [27, 45, 46]. The pair of *ycf1* genes in *A. alboviolaceum* is located in the SSC region, whereas in the four Nigerian species, one of the *ycf1* genes is located in the IRb region, and the other is extended into SSC from IRa. The expansion in the boundary of IRb and SSC of *A. alboviolaceum* makes it the species with the longest genome size not only in *Aframomum* but *Amomum*, inclusive due to the remarkable increase in the SSC region. In addition, the contraction and expansion in the IRb and SSC resulted in *rps19* extending into SSC from IRb in some species, while the gene is located in the SSC region in other species. The positioning of the *rps19* gene at the boundary of the single-copy and inverted repeats regions has been reported in the Zingiberaceae chloroplast genome [50]. *Aframomum* has two copies of the gene, as seen in other related genera [47, 48]. The genome of *Cautleya* contains only one copy of the gene in the LSC region [51]. Comparing the results of phylogenetic relationships and chloroplast genome size variation reveals that the expansion and contraction of the boundaries of the single-copy and inverted repeat regions played an important role in the evolution of *Aframomum*. The earliest-diverged species has the largest chloroplast genome, about 5000 bp, compared with the more recently diverged species. Interestingly, the basal species has a genome size (166, 130 bp) that is also longer than *Amomum* (~ 163, 000 bp) and *Alpinia* (~ 162, 000 bp), showing that there is an increase in genome size in the process of evolution of *Aframomum* from *Amomum* and subsequent reduction in size in the evolution of species within the *Aframomum*. The composition and types of long repeats and SSRs in the chloroplast genome of the studied *Aframomum* are consistent with their phylogenetic relationships; the four Nigerian species shared the same pattern and composition of the types of repeats compared with the *A. alboviloceum* which is the basal species in the genus. There is evidence of contraction and expansion among the chloroplast genomes of *Aframomum*, leading to the variation in sizes of the genomes as well as the positions of the genes in the boundary of the single-copy regions and the inverted repeat regions.

Divergence hotspots and marker potential

Sequence divergence analysis identified several hypervariable regions, including *ycf1-rps15*, *atpH-atpI*, and *trnT-trnL*, which reside mainly in intergenic regions of the LSC and SSC. These regions exhibited elevated nucleotide diversity and are consistent with known divergence hotspots in other monocot chloroplast genomes [52, 53]. Notably, *ycf1* emerged as the most variable gene, supporting its growing role as a universal barcode for land plants [54]. These variable loci offer potential for developing molecular markers for population genetics, phylogenetic inference, and species identification within *Aframomum*.

Codon usage and selective constraints

Codon usage analysis revealed a bias toward A/U-ending codons, particularly in leucine and isoleucine residues, reflecting AT-rich plastid genome composition, which is typical of angiosperms [55]. The RSCU values suggest conserved codon usage patterns among species, with minor lineage-specific deviations possibly shaped by mutational bias or translational selection. Ka/Ks analysis confirmed that most chloroplast genes are under purifying selection, especially essential genes involved in photosynthesis (*psaA*, *psbA*, *rbcL*), mirroring findings in related genera [56]. However, the elevated Ka/Ks ratios in genes such as *ycf1*, *rpoC2*, and *rpl16* suggest episodes of positive selection, potentially linked to adaptive divergence during speciation. The purifying selection of the majority of the genes can be attributed to the fact that the species shared the same habitat and the environmental conditions are similar; therefore, there is no threat facing the species that will drive positive selection to overcome the environmental challenges.

Phylogenetic relationships

The phylogenetic tree constructed using complete chloroplast genome sequences strongly supports the monophyly of *Aframomum*, with high posterior probability values. This monophyly is congruent with morphological and ecological traits shared among species [57], and with prior molecular phylogenies [58]. However, the paraphyletic placements of *Amomum* and *Alpinia* highlight unresolved taxonomy in Alpineae and support recent calls for reclassification based on genomic data [59]. The placement of *Aframomum* as sister to *Amomum*, and the interspersion of *Wurfbainia* within *Amomum* clades, reflects complex evolutionary histories and potentially rapid radiation events within the tribe. The earliest diverged species is *A. alboviloceum* followed by *A. daniellii*, and later *A. sceptrum* and *A. angustifolium* diverged. The recently diverged species have very little genetic diversity, and this corresponds to their morphological similarities.

Divergence time Estimation and evolutionary insights

The BEAST2-based time-calibrated phylogeny estimates the crown divergence of *Aframomum* at approximately 26 Mya, with diversification within the Nigerian species occurring between 6 and 16 Mya, largely during the Miocene epoch. This timeline aligns with major climatic fluctuations in tropical Africa that likely promoted ecological diversification and speciation in forest understorey herbs like *Aframomum* [60]. The observed interspecific variation in IR boundary configuration, particularly the expanded *ycf1* and *rps19* junctions in *A. alboviolaceum*, may reflect ancient divergence associated with early Miocene Forest fragmentation. During this period (approximately 23–16 Mya), the African rainforest belt experienced repeated contraction and expansion cycles driven by global cooling and aridification events [60, 61]. Such environmental oscillations likely created ecological barriers and promoted allopatric divergence among *Aframomum* lineages. Similarly, the strong purifying selection detected in photosynthetic genes and the positive selection in genes related to transcription and protein turnover may indicate adaptive responses to fluctuating light and moisture conditions within these dynamic forest habitats. The high conservation of SSRs and overall chloroplast genome structure among the Nigerian species suggests recent diversification and continued gene flow within the Guineo-Congolian forest corridor.

Abbreviations

RSCU	Relative synonymous codon usage values
LSC	Large single-copy
SSC	Small single-copy
IR	Inverted repeat
JLB	Junction between large single copy and inverted repeat B
JSB	Junction between small single copy and inverted repeat B
JLA	Junction between large single copy and inverted repeat A
JLB	Junction between large single copy and inverted repeat B
SSRs	Simple sequence repeats
Mya	Millions of years ago

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Authors' contributions

Conceptualization: SSY **;** Data curation: SSY **;** Formal analysis: SSY **;** Investigation: SSY; Methodology: SSY **;** Writing original draft: SSY **;** Writing review & editing: SSY.

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Data availability

The complete chloroplast genome sequences of *Aframomum* species reported in this study were deposited in GenBank with the following accession numbers: PV600844-PV600847.

Declarations

Ethics approval and consent to participate

Not applicable. The plant materials were collected from a public land and no special permission is required for the collection of plants from a public land.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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