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INTEGRATIVE CHLOROPLAST GENOMIC, EVOLUTIONARY AND STRUCTURAL ANALYSIS OF PLASTID-ENCODED RNA POLYMERASE GENES IN ELITE INDONESIAN SUGARCANE (*SACCHARUM* SPP. HYBRID) 'KIDANG KENCANA' AND 'BULULAWANG'

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ABSTRACT

Sugarcane (*Saccharum* spp. hybrid) is a major tropical C4 crop with high sucrose productivity, sustained by efficient photosynthesis occurring within the chloroplasts. Plastid-encoded RNA polymerase (PEP), encoded by the *rpo* gene family (*rpoA*, *rpoB*, *rpoC1* and *rpoC2*), plays a key role in chloroplast gene expression. Despite the agronomic importance of elite Indonesian sugarcane such as 'Kidang Kencana' and 'Bululawang', specific genomic information on the chloroplast *rpo* gene family is still lacking. In this study, we performed an integrative analysis of the *rpo* gene family to evaluate the genomic characteristics, structural conservation and evolutionary relationships. Long-read sequencing yielded approximately 8.0 Gb of data, enabling the assembly of complete circular chloroplast genomes with an average sequencing depth of 178.6×. The assembled chloroplast genomes were deposited in GenBank under accession numbers PZ344155 ('Kidang Kencana') and PZ364438 ('Bululawang'), providing the reference sequences for comparative analyses of the *rpo* gene family. The four *rpo* genes were identified as single-copy, intronless genes located in the LSC region, ranging in length from 1,020 bp (*rpoA*) to 4,605 bp (*rpoC2*). Protein motif analysis identified 30 conserved motifs (p-values $\leq 2.12\times10^{-158}$), while codon usage patterns were highly similar between cultivars (ΔRSCU = −0.57 to 0.36), indicating conserved translational preferences. Predicted protein structures exhibited high prediction confidence (pLDDT >90) with low structural divergence. Phylogenetic analysis produced a well-supported topology (ML = 100%, BI = 1.00), clearly resolving the evolutionary relationships among the analyzed *Saccharum* taxa. These findings expand genomic resources for Indonesian sugarcane and provide a foundation for future evolutionary studies and chloroplast marker development.

***Keywords*:** Conserved motifs, Molecular evolution, Plastid-encoded RNA polymerase, Protein structure, Sugarcane.

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1. INTRODUCTION

Sugarcane (*Saccharum officinarum* and modern *Saccharum* hybrids) is widely recognized as a C4 plant, known for its high capacity to accumulate sucrose and serves as a major feedstock for the production of sugar (80%), bioethanol (40%), bioenergy, and various derivative products (Desalegn et al., 2023; Wu et al., 2024; Perera et al., 2025; Zhou et al., 2025). This high biomass accumulation and sucrose yield are primarily driven by efficient photosynthesis, a process that converts light energy to sustain plant growth and sugar synthesis (Khan et al., 2023; Croce et al., 2024; Zhang et al., 2025). Chloroplasts, as the central organelle of this process, not only regulate photosynthesis but also participate in a range of essential metabolic pathways (Sun et al., 2023; Zhang et al., 2025). The sugarcane chloroplast genome is approximately 141 kb in size and exhibits a typical quadripartite structure, consisting of a large single copy (LSC) region, a small single copy (SSC) region, and two inverted repeat (IRa/b) regions, harboring around 110-120 genes (Lohmaneeratana et al., 2024; Li et al., 2025). These genes can be broadly grouped into three categories based on the molecular functions: (1) components involved in plastid gene expression

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machinery, including RNA polymerase, ribosomal proteins, tRNAs and rRNAs; (2) subunits of photosynthetic complexes, such as Rubisco, photosystem II, the cytochrome b6f complex, photosystem I, NADPH dehydrogenase, and ATP synthase; and (3) additional proteins supporting these processes, including *ClpP1, ycf1, ycf2* and *ycf3* (Dobrogojski et al., 2020).

The relatively low rates of nucleotide substitution and recombination in chloroplast genomes make them particularly useful for studying various biological aspects in plants, including gene families and their functions, as well as genome structure and evolution (Wonok et al., 2023). Recent advances in chloroplast genomics have facilitated comparative chloroplast genome analyses across numerous species within the Poaceae family, including bamboo (*Phyllostachys aureosulcata*) (Chen et al., 2025), wheat (*Triticum aestivum*) (Kim et al., 2026), Japanese silver grass (*Miscanthus sinensis*) (Kim et al., 2025), little millet (*Panicum sumatrense*) (Sathyamurthy et al., 2025), and Kentucky bluegrass (*Poa pratensis*) (Wang et al., 2026). These studies have expanded current knowledge of chloroplast genome organization, sequence conservation, and evolutionary relationships among economically important grasses. Within the genus *Saccharum*, the increasing availability of complete chloroplast genomes from Asian cultivars has provided valuable genomic resources for comparative analyses of molecular evolution, sequence conservation, and plastid gene diversity (Xu et al., 2019; Li et al., 2025). Despite these advances, most sugarcane plastid genomic studies have focused primarily on whole-plastome characterization, leaving comprehensive analyses of plastid gene families involved in chloroplast gene expression largely unexplored, particularly in elite Indonesian cultivars (Judith et al., 2026; Sawitri et al., 2026).

One of the most functionally important plastid gene families involved in chloroplast gene expression is the *rpo* gene family, which encodes the core components of the plastid-encoded RNA polymerase (PEP). Chloroplast genes are transcribed by two distinct types of RNA polymerases: the nucleus-encoded RNA polymerase (NEP) and the plastid-encoded RNA polymerase (PEP) (Palomar et al., 2022; Wang et al., 2024). During early chloroplast development, NEP transcribes essential housekeeping genes, including members of the *rpo* gene family such as *rpoA*, *rpoB*, *rpoC1* and *rpoC2* (Yu et al., 2014; Ali et al., 2024). The mRNA transcripts of these *rpo* genes are subsequently translated into four core subunits of plastid-encoded RNA polymerase (PEP), namely α (38 kDa), β (120 kDa), β′ (85 kDa) and β″ (185 kDa), which assemble into a functional enzyme that serves as the primary transcription machinery responsible for transcribing more than 80% of photosynthesis-related chloroplast genes (Börner et al., 2015; Loiacono & Bock, 2024; Zhang et al., 2025).

PEP is a bacterial-type RNA polymerase that retains the catalytic core inherited from its cyanobacterial endosymbiotic ancestor, reflecting the evolutionary origin of chloroplasts and the conservation of the plastid transcription machinery across photosynthetic plant (Palomar et al., 2022). This enzyme stably interacts with several PEP-associated proteins (PAPs) encoded by nuclear genes, which contribute to the stability, regulation, and proper functioning of the transcription complex, reflecting the complex integration between the chloroplast and nuclear genomes (Vergara-Cruces et al., 2024; Wang et al., 2024). Unlike PAPs and other accessory transcription factors that primarily regulate promoter recognition, complex assembly, or transcriptional elongation, the plastid-encoded *rpo* genes constitute the catalytic core responsible for RNA synthesis itself, making them the structural and functional foundation of the chloroplast transcription machinery (Yu et al., 2014; Tadini et al., 2020; Zhang et al., 2025). The central role of PEP emphasizes the importance of characterizing the *rpo* gene family, as its presence and function regulate the transition from young chloroplasts to mature chloroplasts capable of efficient photosynthesis (Yu et al., 2014). Consistent with this essential role, studies in the model plant *Nicotiana tabacum* have shown that mutations in *rpo* genes disrupt chloroplast gene expression and result in albino or chlorotic phenotypes, highlighting the indispensable function of this gene family in chloroplast biogenesis and photosynthetic development (Tadini et al., 2020; Vergara-Cruces et al., 2024; Wang et al., 2024). The essential role of the *rpo* gene family is further supported by recent structural studies demonstrating that the catalytic core encoded by these genes is highly conserved across evolutionarily distant land plants, including dicots, monocots, and bryophytes. This remarkable conservation highlights the strong evolutionary constraint acting on the *rpo* gene family despite diversification of the surrounding regulatory machinery (Moon et al., 2026).

Indonesia harbors a range of elite sugarcane cultivars with advantageous agronomic traits, including the cultivars Kidang Kencana and Bululawang. These cultivars are widely cultivated across the major sugarcane-producing regions of Indonesia, including Java, Sumatra, Sulawesi, and Nusa Tenggara, owing to their superior agronomic performance and high sucrose accumulation (Riajaya, 2020; Widyasari et al., 2022). Reported sucrose yields reach 10.99±1.65% under irrigated conditions and 9.51±0.88% in dryland systems for 'Kidang Kencana', while 'Bululawang' shows approximately 7.51% under dryland cultivation (Widyasari et al., 2022). Despite these favorable agronomic traits, specific genomic information on the chloroplast *rpo* gene family is still lacking. Previous molecular studies in Indonesia have primarily investigated chloroplast genes, including *rbcL*, *matK*, and *trnK*, for DNA barcoding, cultivar identification, and genetic diversity assessment, providing valuable resources for

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germplasm discrimination (Aristya et al., 2020, 2024, 2025). However, these studies have not extended to comprehensive analyses of functionally important plastid gene families, limiting our understanding of their genomic characteristics, structural conservation and evolutionary relationships. Consequently, the plastid-encoded RNA polymerase (*rpo*) gene family, despite its essential role in chloroplast gene expression, remains largely unexplored in elite Indonesian sugarcane cultivars. Comprehensive characterization of this highly conserved gene family will expand genomic resources for Indonesian sugarcane and provide a foundation for evolutionary studies and future chloroplast marker development. Therefore, this study aims to perform an integrative analysis of the plastid-encoded RNA polymerase (*rpo*) gene family in the elite Indonesian sugarcane 'Kidang Kencana' and 'Bululawang'. Specifically, gene organization, protein motifs, codon usage patterns, selective pressure, predicted protein structures, and phylogenetic relationships were investigated to evaluate the genomic characteristics, structural conservation, and evolutionary relationships of the *rpo* gene family.

2. MATERIALS AND METHODS

2.1. Plant Materials and DNA Extraction

Fresh young leaves of *Saccharum* hybrid 'Kidang Kencana' and 'Bululawang' were collected from PT Madubaru–PG Madukismo located in Bantul, Special Region of Yogyakarta, Indonesia (7°49′40″S, 110°20′14″E) (Fig. 1). The leaf samples were immediately placed into tubes containing liquid nitrogen and transported to the laboratory for DNA extraction. The samples were subsequently stored at −20 °C prior to DNA extraction. High-molecular weight genomic DNA was extracted from approximately 100 mg of leaf tissue using the Wizard® HMW DNA Extraction Kit (Promega Corporation, Madison, WI, USA) following the manufacturer's protocol. DNA quality and quantity were measured using a NanoDrop Lite Plus Spectrophotometer and a Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA integrity was further verified by electrophoresis on a 1% agarose gel using the Mupid-exU system (Advance Co., Ltd., Japan).

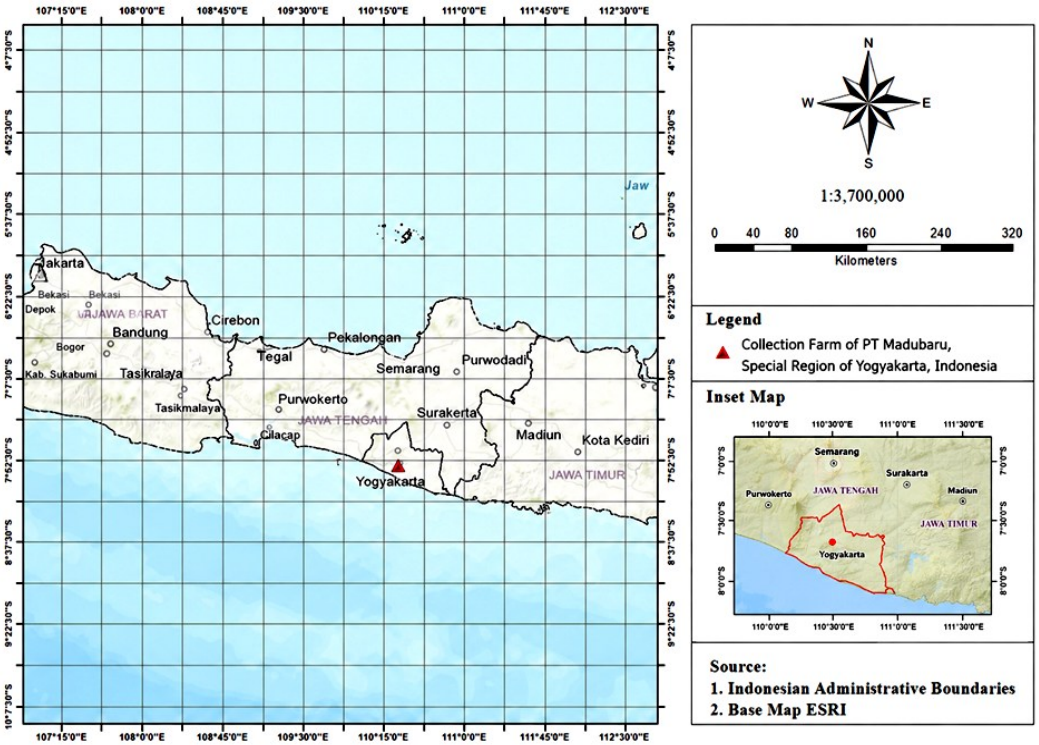

Fig. 1: Sampling locations of the Indonesian elite sugarcane 'Kidang Kencana' and 'Bululawang'. The red triangle indicates the collection site, and the inset map shows the administrative boundary of the Special Region of Yogyakarta.

2.2. Library Preparation and Long-Read Sequencing

DNA library preparation was performed using the Ligation Sequencing Kit V14 (SQK-LSK114; Oxford Nanopore Technologies, UK) according to the manufacturer's instructions. The library preparation included DNA repair and end-preparation, adapter ligation, flow-cell priming, and loading. Sequencing was carried out on the

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PromethION 24 platform using MinKNOW v.24.11.10 software (Oxford Nanopore Technologies, UK). The sequencing run lasted approximately 72 hours, generating raw nanopore signal data (.pod5). The raw signal data were base called using the super-accuracy (SUP) model in Dorado v0.9.6 and exported as FASTQ format for downstream analysis. Low-quality reads and adapter sequences were filtered using Filtlong v0.3.1 to obtain high-quality reads suitable for genome assembly. The quality of the output FASTQ reads was assessed using NanoPlot v.1.41.6 (De Coster & Rademakers, 2023).

2.3. Chloroplast Genome Assembly and Annotation

Chloroplast genome assembly from the filtered long reads was performed using TIPPo v2.4 (Xian et al., 2025). Filtered long reads were mapped back to the assembled chloroplast genome to estimate genome coverage, sequencing depth and assembly quality metrics. The assembly graph was visualized and inspected using Bandage v0.8.1 (Wick et al., 2015). The assembled genome was subsequently annotated using GeSeq to generate a GenBank (GB) annotation file (Tillich et al., 2017). The final annotation results were visualized to generate circular chloroplast genome maps using OGDRAW v.1.3.1 (Greiner et al., 2019). The complete chloroplast genome sequences have been deposited in the NCBI GenBank database under accession numbers PZ344155 ('Kidang Kencana') and PZ364438 ('Bululawang').

2.4. Extraction of *rpo* Gene Family

The annotated chloroplast genome in GenBank (GB) format was imported into Unipro UGENE v53.0 (Okonechnikov et al., 2012) and all genes belonging to the *rpo* family were identified based on gene annotations. The full gene sequences, coding DNA sequences (CDS), and amino acid sequences were extracted and exported in FASTA format for downstream analysis.

2.5. Gene Structure and Predicted 3D Protein Structure Analysis

The CDS and full gene sequences of the *rpo* gene family were used to analyze intron and exon organization using the Gene Structure Display Server 2.0 (Hu et al., 2015). The analysis included sequences from the studied genome together with 15 reference sequences from the genus *Saccharum* and one outgroup sequence from *Miscanthus sinensis* (GenBank: LN896358) from the NCBI GenBank database.

Amino acid sequences of the *rpo* gene family were used to predict three-dimensional protein structures using the AlphaFold 3 Server (https://alphafoldserver.com). The predicted protein models were visualized and analyzed using PyMOL software. Structural similarity among the predicted proteins was evaluated by calculating the root mean square deviation (RMSD) values through structural alignment with reference protein structures.

2.6. Selective Pressure Analysis

Selection pressure was assessed by estimating Ka/Ks ratios for each *rpo* coding sequence (CDS). Gene sequences were aligned using the Align by MUSCLE algorithm implemented in MEGA 12, followed by trimming prior to downstream analysis. Synonymous (Ks) and nonsynonymous (Ka) substitution rates were calculated in DnaSP v6.12.03 using the Nei–Gojobori method with Jukes–Cantor correction (Rozas et al., 2017). Pairwise comparisons with Ks $\leq$0.0001 or undefined Ka/Ks values (e.g., Ks = 0) were excluded to avoid unreliable estimates resulting from extremely low synonymous substitution rates. Comparisons with Ka = 0 and Ks >0 were retained because they represent valid Ka/Ks values of zero, indicating strong purifying selection. To improve data visualization, Ka/Ks values greater than 2.0 were treated as outliers and excluded from the plots. The resulting Ka/Ks distributions were visualized for each gene using violin plots overlaid with jittered points to display individual pairwise comparisons. All visualizations were generated in Python 3 using the Plotly library.

2.7. Conserved Motif Analysis

Conserved motif analysis was performed using the MEME Suite v5.5.7 online server (https://meme-suite.org/meme/tools/meme). The amino acid sequences of the *rpo* gene family, together with reference and outgroup protein sequences, were used as input for motif discovery. The MEME algorithm was applied with the maximum number of motifs set to 30 to allow comprehensive detection of conserved motifs, including potentially rare or lineage-specific motifs within the *rpo* protein family, while other parameters were kept at their default settings.

2.8. RSCU-Based Codon Usage Analysis

Codon usage bias analysis was performed using a custom Python pipeline executed through the Anaconda Prompt (Python 3) environment. The input data consisted of a complete chloroplast genome annotation file in GenBank format (.gbk). CDS shorter than 60 nucleotides were excluded from the analysis and stop codons were

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removed before codon counting to avoid statistical bias in codon frequency estimation. The *rpo* gene family, including *rpoA*, *rpoB*, *rpoC1*, and *rpo*C2, was identified based on gene annotations in the GenBank file, while all remaining CDS were grouped as the other chloroplast genes. The pipeline generated output files, including CSV tables and heatmap visualizations.

2.9. Phylogenetic Reconstruction

Phylogenetic relationships were reconstructed using Maximum Likelihood (ML) and Bayesian Inference (BI) approaches based on a concatenated dataset of the *rpo* gene family. The coding sequences of *rpo*A, *rpo*B, *rpo*C1, and *rpo*C2 were aligned into a single alignment file. The optimal partitioning scheme for the dataset was determined by PartitionFinder v.2.1.1 (Lanfear et al., 2017) based on the Bayesian Information Criterion (BIC). ML analysis was conducted in IQ-TREE v2.3.1 (Nguyen et al., 2015) with 1000 bootstrap replicates, whereas BI analysis was performed in MrBayes v3.2.6 (Ronquist et al., 2012) using a Markov Chain Monte Carlo (MCMC) run of 4,000,000 generations. *Miscanthus sinensis* (GenBank: LN896358) was used as the outgroup. The resulting phylogenetic trees were visualized using Fig Tree v1.4.4 (Rambaut, 2018).

3. RESULTS AND DISCUSSION

3.1. Identification of *rpo* Gene Family

Long-read sequencing generated approximately 8.0 Gb of data with a read N50 of approximately 13 kb. Mapping of the filtered long reads back to the assembled chloroplast genomes yielded an average sequencing depth of 178.6 ×, indicating sufficient read support for accurate genome assembly. The complete chloroplast genome sequences generated in this study have been deposited in GenBank under accession numbers PZ344155 (‘Kidang Kencana’) and PZ364438 (‘Bululawang’) (Fig. 2). Genome annotation enabled the identification and comparative characterization of the plastid-encoded RNA polymerase (*rpo*) gene family. A total of four genes belonging to the plastid-encoded RNA polymerase (PEP) were identified in the chloroplast genomes of *Saccharum* hybrid ‘Kidang Kencana’ and ‘Bululawang’. These genes include *rpoA*, *rpoB*, *rpoC1*, and *rpoC2*, each present as a single copy within the plastome (Fig. 2). All identified *rpo* genes were located in the large single copy (LSC) region, consistent with the plastome organization reported in members of the Poaceae family (Lohmaneeratana et al., 2024; Chen et al., 2025; Li et al., 2025; Sathyamurthy et al., 2025; Kim et al., 2026). The annotated *rpo* genes varied in gene length, coding sequence (CDS) length, and predicted protein size, as summarized in Table 1.

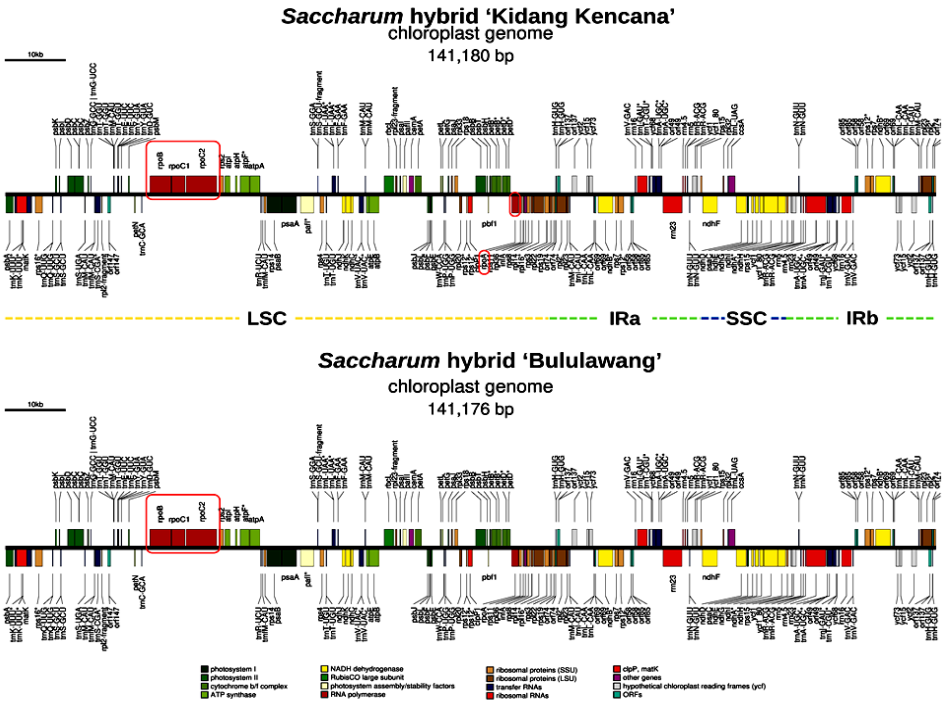

Fig. 2: Linear maps of the complete chloroplast genomes of *Saccharum* hybrid 'Kidang Kencana' and 'Bululawang'. LSC, large single-copy region; SSC, small single-copy region; IRa and IRb, inverted repeat regions. Gene colors indicate different functional categories. The *rpo* gene family (*rpoA*, *rpoB*, *rpoC1*, and *rpoC2*) is highlighted in red.

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The *rpo* genes identified in both cultivars exhibited identical gene lengths and predicted protein lengths, indicating a highly conserved genomic organization of the plastid-encoded RNA polymerase (PEP) system in sugarcane. Among the four genes, *rpo*C2 was the largest gene (4605 bp; ~1535 amino acids), whereas *rpo*A was the smallest (1020 bp; 339 amino acids). This size variation reflects the structural complexity and functional specialization of the encoded PEP subunits rather than simple differences in coding capacity. Specifically, the *rpoA* gene encodes the α subunit, which forms a homodimer that provides the structural scaffold for polymerase assembly, whereas *rpoB*, *rpoC1*, and *rpoC2* encode the β, β′, and β″ subunits, respectively, that together constitute the conserved bacterial-type RNA polymerase responsible for plastid gene transcription (Prado et al., 2024).

Table 1: Basic features of *rpo* genes identified in the chloroplast genomes of *Saccharum* hybrid 'Kidang Kencana' and 'Bululawang'

Sugarcane cultivar	Gene	Start (bp)	End (bp)	Gene Length (bp)	CDS Length (bp)	Protein Length (aa)	Plastome Region
Kidang Kencana	*rpoA*	76817	77836	1020	1020	339	LSC
	rpoB	21918	25145	3228	3228	1075	LSC
	rpoC1	25183	27234	2052	2052	683	LSC
	rpoC2	27434	32038	4605	4605	1535	LSC
Bululawang	*rpoA*	5205	6224	1020	1020	339	LSC
	rpoB	57900	61127	3228	3228	1075	LSC
	rpoC1	55811	57862	2052	2052	683	LSC
	rpoC2	51007	55611	4605	4605	1535	LSC

The PEP catalytic core is evolutionarily derived from the cyanobacterial ancestor of chloroplasts and serves as the primary RNA polymerase responsible for transcribing most photosynthesis-related genes, together with numerous rRNA and tRNA genes required for chloroplast biogenesis and plastid function (Tadini et al., 2020). Consequently, the conservation of all four plastid-encoded *rpo* genes reflects their indispensable role in maintaining plastid gene expression and photosynthetic competence. The comparatively large size of *rpoC2* is consistent with its role as the largest catalytic subunit of the PEP complex, whereas the smaller α subunit primarily functions in polymerase assembly and structural stabilization. Similar gene size distributions have been reported in other *Saccharum* plastomes, supporting the structural conservation of PEP-related genes across cultivated sugarcane (Palanivelu, 2019; Tadini et al., 2020). The identical lengths of all four *rpo* genes observed in both cultivars further suggest strong functional conservation of the PEP complex, reflecting the evolutionary constraints imposed on chloroplast transcription machinery.

The genomic organization revealed relatively short intergenic spacer (IGS) regions between adjacent genes, particularly 37 bp between *rpoB* and *rpoC1* and 199 bp between *rpoC1* and *rpoC2*. The close proximity of these genes supports a compact gene cluster within the LSC region. In chloroplast genomes, functionally related genes are frequently organized into operons or operon-like transcriptional units and transcribed as polycistronic precursor RNAs prior to RNA processing (Stern et al., 2010; Ping et al., 2023). The conserved clustering of *rpoB*, *rpoC1*, and *rpoC2* observed in both cultivars is therefore consistent with the conserved transcriptional organization of plastid genomes and is expected to support coordinated polycistronic transcription, thereby facilitating efficient assembly and function of the plastid-encoded RNA polymerase (PEP) complex (Ping et al., 2023; Wang et al., 2024; Ahrens et al., 2025). However, this inference is based solely on genomic organization, and experimental validation, including transcript mapping or RNA sequencing, would be required to confirm coordinated polycistronic transcription of the *rpo* gene cluster. The conserved gene order, compact intergenic regions, and identical gene architecture observed in both Indonesian sugarcane cultivars indicate a highly stable plastid transcriptional organization maintained under strong functional and evolutionary constraints. This conserved genomic organization provided the structural basis for subsequent analyses of exon–intron architecture, sequence evolution, and protein conservation within the *rpo* gene family.

3.2. Gene Structure Analysis

The exon–intron structure of the *rpo* genes was analyzed using GSDS by comparing genomic sequences with their coding DNA sequences (CDS) across the two studied cultivars, 15 additional *Saccharum* accessions, and one outgroup species to evaluate structural conservation among related taxa (Fig. 3). All four genes exhibited intron-less structure consisting of a single exon. The gene lengths differed among the *rpo* genes, with *rpoA*, *rpoB*, *rpoC1*, and *rpoC2* measuring 1020 bp, 3228 bp, 2052 bp, and 4605 bp, respectively. This intron-less organization is commonly observed in plastid genes and reflects a conserved structural pattern in chloroplast genomes. The absence of introns may support a streamlined transcriptional process without the requirement for RNA splicing, potentially enabling efficient expression of genes involved in plastid transcription (Aviña-Padilla et al., 2021; Liu et al., 2021).

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The identical exon and intron organization observed across all *Saccharum* accessions and the outgroup further indicates that the genomic architecture of the plastid-encoded RNA polymerase genes has remained remarkably stable throughout the evolutionary history of the genus. No structural variation, such as intron gain, intron loss, or exon rearrangement, was detected among the analyzed taxa despite their phylogenetic divergence. This extensive structural conservation is consistent with the strong purifying selection inferred from the Ka/Ks analysis (Fig. 4), in which all four *rpo* genes exhibited Ka/Ks ratios below one. The concordance between conserved gene architecture and low nonsynonymous substitution rates suggests that both gene structure and protein-coding sequences have been evolutionarily constrained to preserve the essential functions of the plastid-encoded RNA polymerase complex involved in chloroplast transcription. The structural and functional conservation of the plastid-encoded RNA polymerase (PEP) complex is well documented in plant biology, reflecting its essential role in chloroplast biogenesis and maintenance (Ahrens et al., 2025). Accordingly, the remarkable stability in the genomic architecture of the *rpo* genes (*rpoA*, *rpoB*, *rpoC1*, and *rpoC2*) observed in this study is likely to contribute to preserving the catalytic machinery required for transcription of photosynthesis-related genes (Vergara-Cruces et al., 2024).

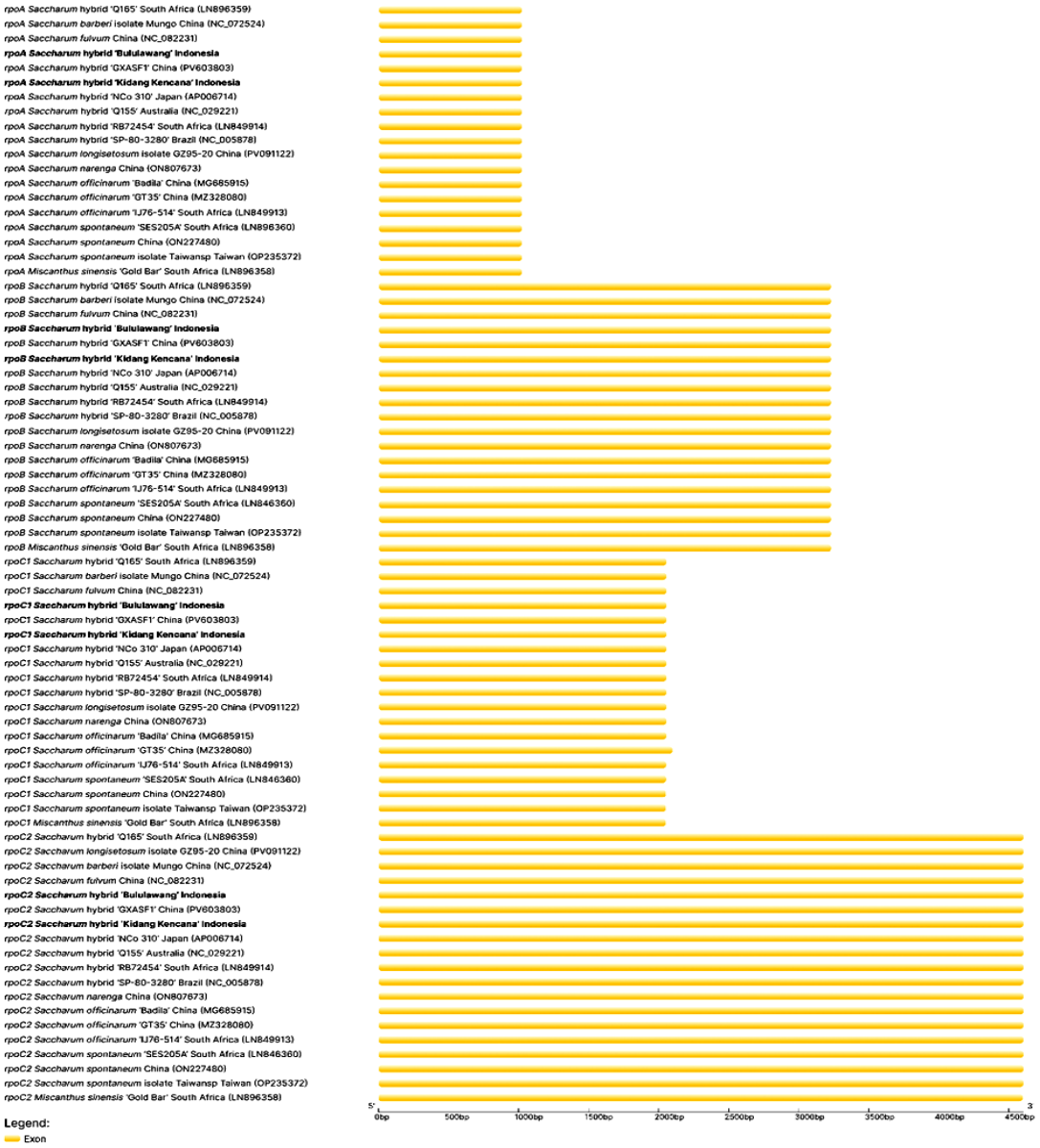

Fig. 3: Gene structure of the *rpo* genes. The structure of *rpo* genes was visualized using the online tool GSDS by submitting their genomic and CDS sequences. The yellow boxes indicate exons (coding sequences) and horizontal scale represents gene length in base pairs (bp).

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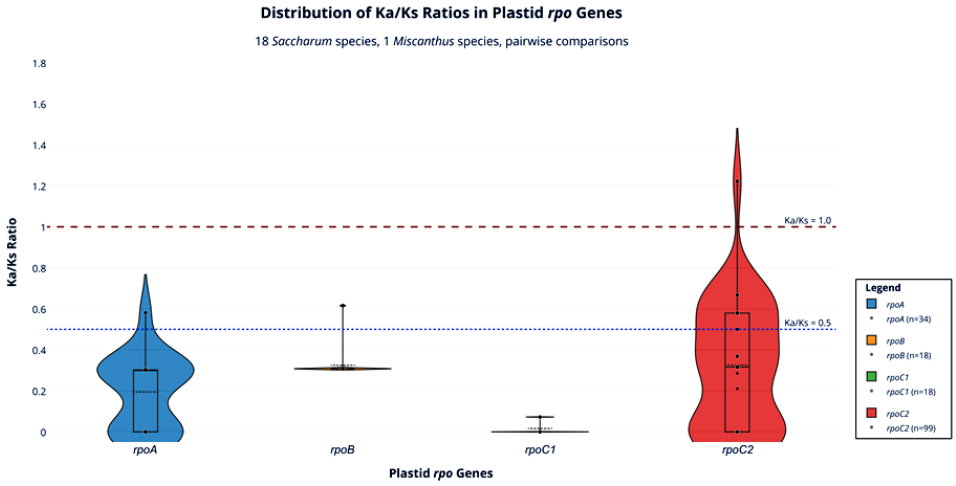

Fig. 4: Distribution of pairwise Ka/Ks ratios among chloroplast *rpo* genes in 18 *Saccharum* accessions and one *Miscanthus* species. Violin plots depict the density and full distribution of pairwise Ka/Ks values for *rpoA*, *rpoB*, *rpoC1*, and *rpoC2*. Black dots represent individual pairwise comparisons. Sample sizes (n) indicate the number of valid pairwise comparisons retained after filtering (Ks > 0.0001, Ka > 0, Ka/Ks < 0.2).

3.3. Selective Pressure Analysis of *rpo* Genes

Selective pressure acting on the plastid-encoded *rpo* genes was evaluated using pairwise nonsynonymous-to-synonymous substitution rate ratios (Ka/Ks) across all analyzed *Saccharum* accessions (Fig. 4).The number of valid pairwise comparisons ranged from 18 for *rpoB* and *rpoC1* to 99 for *rpoC2*, with the lower counts mainly resulting from the exclusion of comparisons with Ka = 0 or values that failed the filtering criteria. Among the four genes, *rpoC1* had the lowest mean Ka/Ks ratio (0.016) and the narrowest distribution, indicating strong evolutionary conservation. The mean Ka/Ks ratio increased to 0.194 in *rpoA*, whereas *rpoB* and *rpoC2* showed nearly identical mean values (0.325). Despite their similar means, the two genes differed markedly in their distributions. Most *rpoB* comparisons clustered within a narrow range (SD = 0.072), whereas *rpoC2* showed much greater variation (SD = 0.305), with several pairwise comparisons exceeding Ka/Ks = 1.0 and a maximum value of 1.222. Even so, the average Ka/Ks ratio of *rpoC2* remained well below 1.0, indicating that purifying selection remained the predominant evolutionary force acting on this gene (Table 3).

The Ka/Ks patterns observed in the *rpo* genes were comparable to those reported from plastome-wide analyses of *Bidens*. Xue et al. (2025) found that approximately 94.7% of plastid protein-coding genes had Ka/Ks values below 0.5, indicating that purifying selection dominates plastid genome evolution. A similar pattern was observed here, as all four *rpo* genes had mean Ka/Ks ratios below 1.0. However, the strength of selection differed among individual genes. *rpoC1* was the most conserved, with a mean Ka/Ks ratio of only 0.016, whereas *rpoA*, *rpoB*, and *rpoC2* accumulated a greater proportion of amino acid-changing substitutions. The broader distribution of *rpoC2* suggests that selective constraints varied more among species than in the other *rpo* genes. Although a few pairwise comparisons produced Ka/Ks values greater than 1.0, these represented only a small fraction of the dataset and did not alter the overall pattern of purifying selection. This agrees with Xue et al. (2025), who proposed that elevated Ka/Ks values in a limited number of plastid genes are more consistent with relaxed purifying selection or lineage-specific adaptive episodes than with persistent positive selection across the entire gene. This interpretation is further supported by broader comparative studies demonstrating that purifying selection is the predominant evolutionary force acting on plastid-encoded protein-coding genes (Robbins & Kelly, 2023). Together with the conserved exon–intron organization described in the preceding section, the present results indicate that both the genomic architecture and coding sequences of plastid-encoded *rpo* genes have remained under strong evolutionary constraint throughout *Saccharum* diversification. Such selective pressure is likely to preserve the functional integrity of the PEP complex by limiting amino acid substitutions that could compromise chloroplast transcription.

3.4. Conserved Motif Analysis

The sugarcane *rpo* proteins vary in size from 339 amino acids (*rpoA*) to 1,535 amino acids (*rpoC2*), reflecting their distinct structural and functional roles within the plastid-encoded RNA polymerase (PEP) complex. Analysis using the MEME Suite v5.5.7 online server identified 30 conserved motifs across the *rpo* gene family proteins, as shown in Fig. 5, demonstrating substantial structural conservation. The statistical significance of motif conservation

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is strongly supported by extremely low p-values. The *rpoA* protein in both 'Kidang Kencana' and 'Bululawang' exhibited a p-value of 1.64×10^{-158}, which is comparable to those observed in several *Saccharum* spp. hybrids. Slightly lower yet still highly significant p-values (2.12×10^{-158}) were detected in species such as *Saccharum spontaneum*, *S. fulvum*, *S. narenga*, *S. barberi*, *S. officinarum*, and Taiwanese hybrids, suggesting strong evolutionary constraint across the genus.

In contrast, *rpoB*, *rpoC1*, and *rpoC2* exhibited p-values of 0.00e+0, indicating statistical significance beyond the computational precision limit of the MEME algorithm. This pattern reflects exceptional conservation of functional motifs, consistent with the essential roles of these proteins in forming the catalytic core of the plastid-encoded RNA polymerase (PEP) complex responsible for chloroplast transcription. This pattern is also consistent with the strong purifying selection inferred from the Ka/Ks analysis, indicating that functionally important protein regions have been maintained throughout evolutionary timescales. This is consistent with the general pattern in protein-coding genes, where purifying selection eliminates deleterious non-synonymous mutations, thereby preserving conserved functional motifs across different species (Douglas & Shapiro, 2024; Chen et al., 2025).

Beyond the *rpo* gene family, the preservation of conserved motif architecture is a common characteristic of evolutionarily constrained gene families in plants. A comparable pattern has been reported for the MYB gene family in pearl millet (*Pennisetum glaucum*), where conserved motif architecture among homologous proteins was associated with the maintenance of essential functional domains (Lin et al., 2023). Likewise, the highly conserved motif composition observed in the *Saccharum rpo* gene family suggests that evolutionary constraints have preserved protein regions essential for maintaining the structural and functional integrity of the plastid-encoded RNA polymerase complex. Moreover, the order and distribution of conserved motifs remained identical across all analyzed *Saccharum* accessions, indicating that the overall domain architecture of the *rpo* proteins has been evolutionarily maintained. Such structural conservation likely contributes to maintaining the coordinated interactions among PEP subunits that are essential for efficient chloroplast transcription.

3.5. Predicted 3D Protein Structure

The predicted three-dimensional (3D) structures of *rpo* proteins in *Saccharum* hybrid 'Kidang Kencana' and 'Bululawang' were generated to evaluate their structural organization and stability (Fig. 6). All predicted *rpo* proteins exhibited well-defined folded conformations with high-confidence regions predominantly represented in blue (pLDDT >90), indicating reliable structural predictions. Lower-confidence regions (yellow to orange) were mainly located at the terminal ends and loop regions, suggesting intrinsic flexibility commonly observed in protein structures (Hossen et al., 2025; Sriram et al., 2025). Among the four proteins, *rpoB* and *rpoC2* displayed more complex and extended architectures, consistent with their larger protein sizes and roles as core catalytic subunits of the plastid-encoded RNA polymerase (PEP). The *rpoA* protein exhibited a more compact structure, reflecting its structural role in stabilizing the PEP core, whereas *rpoC1* showed an intermediate structural complexity, consistent with its contribution to the catalytic machinery (Palanivelu, 2019; Tadini et al., 2020). Previous structural studies further demonstrated that the polymerase core, comprising *rpoA*, *rpoB*, *rpoC1*, and *rpoC2* together with PAP12, adopts a conserved crab claw-like architecture characteristic of multisubunit RNA polymerases, highlighting the importance of maintaining structural integrity for efficient chloroplast transcription (Prado et al., 2024). In particular, the *rpoC2* protein exhibited not only a more extended architecture but also predominantly light blue regions, indicating slightly lower yet still high confidence scores compared to other *rpo* proteins. This structural pattern is consistent with the role of *rpoC2* as the β″ subunit of the PEP complex, which contains the evolutionarily conserved bridge helix, a key structural element of multisubunit RNA polymerases that participates in nucleotide addition and transcription elongation, thereby contributing to the conformational dynamics of the catalytic center. The relatively lower confidence observed in several terminal and loop regions may therefore reflect intrinsic conformational flexibility that could facilitate dynamic structural rearrangements and protein-protein interactions within the PEP complex (Prado et al., 2024).

Comparative structural analysis based on RMSD values (Table 2) further supported the overall conservation of *rpo* proteins across *Saccharum* species. Most *rpoB* and *rpoC1* proteins exhibited consistently low RMSD values, generally ranging from approximately 0.275 to 1.224 Å, indicating a high degree of structural similarity. The *rpoA* protein also showed relatively low RMSD values in most accessions, although moderate variation was observed in several species. However, markedly higher RMSD values were observed in specific cases. Notably, *rpoA* exhibited extreme values of 12.583 Å in *Saccharum barberi* ('Kidang Kencana' comparison) and 22.966 Å in the 'Bululawang' comparison, indicating substantial structural deviation. Elevated RMSD values were observed for *rpoC2* in comparisons with Bululawang, including 15.357 Å in *Saccharum* hybrid 'RB72454' and 10.107 Å in *Saccharum* hybrid 'SP-80-3280'.

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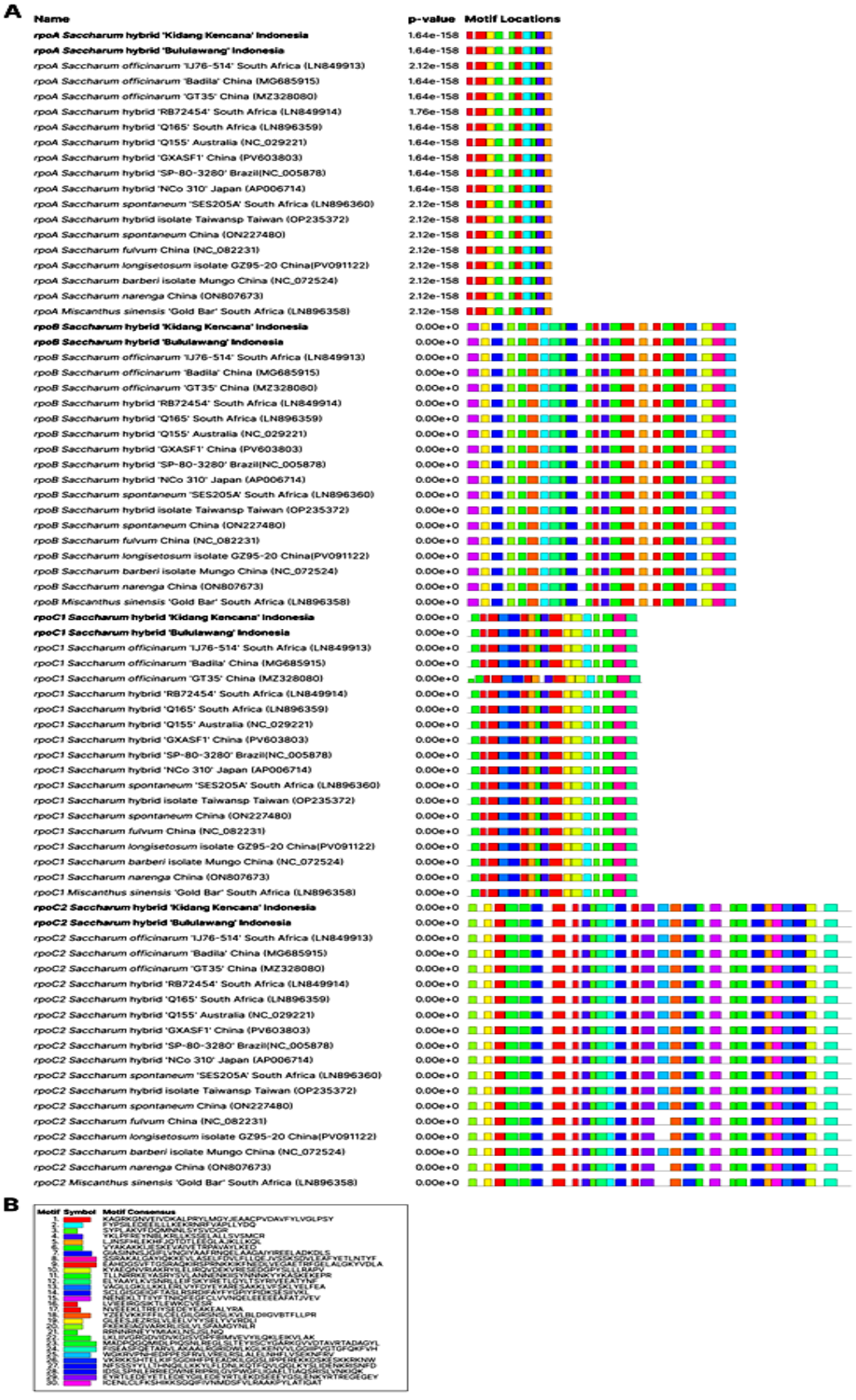

Fig. 5: *rpo* gene family protein motifs. Thirty motifs were detected in *rpo* proteins using online MEME tool. (A) Motifs and (B) motif sequences.

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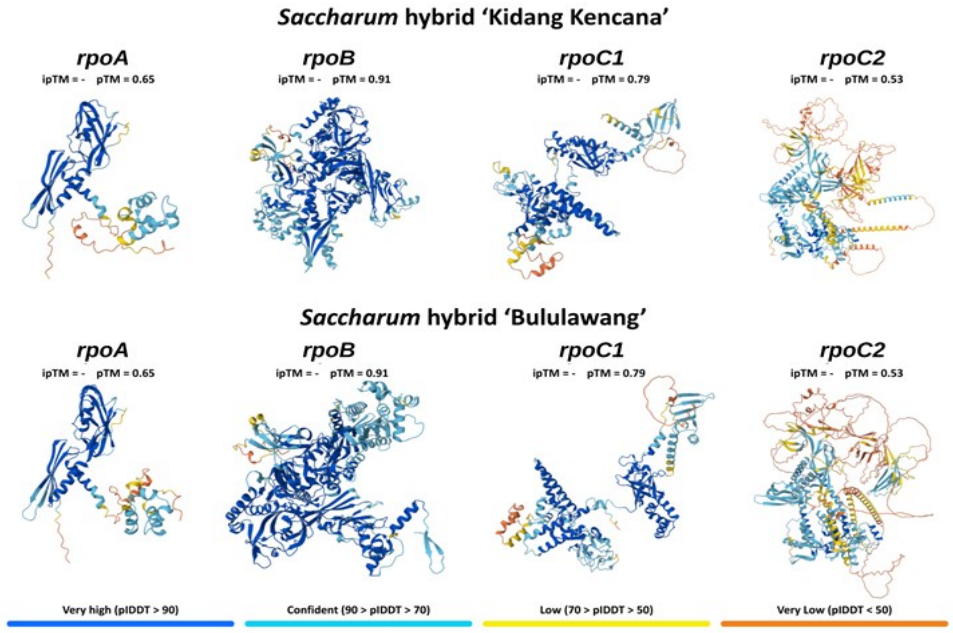

Fig. 6: Predicted 3D structures of *rpo* proteins in *Saccharum* hybrid 'Kidang Kencana' and 'Bululawang'.

Table 2: Comparative root mean square deviation (RMSD) values of *rpo* proteins among *Saccharum* species and selected outgroup (Å)

Species	*Saccharum* hybrid 'Kidang Kencana'				*Saccharum* hybrid 'Bululawang'			
	rpoA	*rpoB*	*rpoC1*	*rpoC2*	*rpoA*	*rpoB*	*rpoC1*	*rpoC2*
Saccharum officinarum 'IJ76-514'	0.922	0.370	0.642	4.902	0.506	0.310	1.224	3.824
Saccharum officinarum 'Badila'	0.567	0.434	0.500	4.141	0.472	0.300	0.642	4.992
Saccharum officinarum 'GT35'	0.473	0.320	0.348	4.792	0.298	0.378	0.690	4.059
Saccharum hybrid 'RB72454'	0.534	0.306	0.456	8.293	0.639	0.285	0.651	15.357
Saccharum hybrid 'Q165'	0.995	0.419	0.694	4.484	2.060	0.367	1.034	8.788
Saccharum hybrid 'Q155'	0.698	0.358	0.487	3.144	1.189	0.351	0.854	6.406
Saccharum hybrid 'GXASF1'	1.049	0.327	0.421	3.819	1.333	0.346	0.668	5.039
Saccharum hybrid 'SP-80-3280'	4.463	0.382	0.450	3.997	3.109	0.343	0.814	10.107
Saccharum hybrid 'NCo 310'	0.373	0.297	0.449	6.532	0.387	0.278	0.951	3.350
Saccharum spontaneum 'SES205A'	0.781	0.342	0.404	3.516	1.319	0.396	0.888	7.348
Saccharum spontaneum isolate Taiwansp	2.977	0.451	0.441	3.421	0.801	0.301	0.384	4.413
Saccharum spontaneum	0.446	0.367	0.386	3.787	0.833	0.275	0.631	6.376
Saccharum fulvum	0.686	0.333	0.349	3.648	1.069	0.315	0.726	5.269
Saccharum longisetosum	0.685	0.379	0.541	4.168	1.806	0.329	0.736	8.073
Saccharum barberi	12.583	0.393	0.420	5.567	22.966	0.414	0.753	4.753
Saccharum narenga	3.134	0.346	0.866	6.541	0.677	0.339	0.527	6.167
Miscanthus sinensis 'Gold Bar'	0.785	0.478	0.606	6.218	0.337	0.366	0.917	7.640

Table 3: Ka/Ks ratios differed among the four *rpo* gene

Gene	N	Mean	Median	SD	Min	Max
rpoA	34	0.194	0.302	0.177	0.000	0.581
rpoB	18	0.325	0.307	0.072	0.308	0.615
rpoC1	18	0.016	0.000	0.031	0.000	0.072
rpoC2	99	0.325	0.316	0.305	0.000	1.222

Notes: N, number of valid pairwise comparisons included in the analysis; Mean, arithmetic mean of Ka/Ks ratios; Median, median Ka/Ks ratio; SD, standard deviation; Min, minimum Ka/Ks ratio; Max, maximum Ka/Ks ratio.

These pronounced deviations are likely associated with structural variability in flexible or poorly aligned regions, particularly in larger or more complex proteins. In *rpoC2*, this pattern is consistent with its predominantly light blue confidence distribution in the predicted structures, suggesting increased flexibility and variation in domain orientation. Conformational differences in flexible terminal segments or loop regions can disproportionately increase the overall RMSD without substantially altering the conserved structural core of the protein because RMSD is highly sensitive to the spatial orientation of aligned atoms. This interpretation is supported by the AlphaFold predictions, in which the regions exhibiting lower confidence scores were primarily confined to terminal and loop regions rather than the well-folded core domains.

Importantly, such high RMSD values do not necessarily indicate functional divergence, as the conserved core regions and motif architecture remain largely preserved. The occurrence of extreme RMSD values in certain species, particularly in *S. barberi*, may also reflect greater evolutionary divergence relative to other *Saccharum* accessions. For *rpoC2*, the elevated RMSD values are further consistent with previous structural studies showing

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that this β″ subunit contains the bridge helix and other conformationally dynamic regions within the plastid-encoded RNA polymerase (PEP) complex, where limited structural flexibility is required to accommodate transcription-associated conformational changes while maintaining the overall polymerase architecture (Prado et al., 2024). The combination of low RMSD values across most proteins and localized structural deviations indicates that the *rpo* proteins remain structurally conserved across *Saccharum* species, while limited flexibility in specific regions may facilitate conformational changes required for plastid-encoded RNA polymerase (PEP) function without compromising the integrity of the catalytic core (Palomar et al., 2022; Vergara-Cruces et al., 2024; Hossen et al., 2025; Sriram et al., 2025).

3.6. Codon Usage Bias of *rpo* Genes

The comparative analysis of codon usage between *rpo* genes and other chloroplast genes revealed a highly consistent pattern in both cultivars, Kidang Kencana (KK) and Bululawang (BL) (Fig. 7). The deviation values for most codons were nearly identical, with differences generally less than 0.02, indicating that codon usage bias in *rpo* genes is highly conserved across cultivars. Several synonymous codon pairs exhibited opposite deviation values, indicating strong codon preference in *rpo* genes. The lysine codons AAA and AAG displayed contrasting patterns, with AAA being overrepresented (0.07 in KK; 0.08 in BL) and AAG underrepresented (−0.07 in KK; −0.08 in BL). Similarly, the glutamine codons CAA and CAG showed opposing deviations, with CAA having a positive deviation (0.16 in both KK and BL) and CAG a negative deviation (−0.16 in both KK and BL). These consistent opposing patterns among synonymous codons suggest selective codon usage, potentially associated with tRNA availability and translational efficiency in chloroplasts (Geng et al., 2026).

Several codons exhibited extreme deviations, indicating that codon usage in *rpo* genes is non-random. The arginine codon CGT showed the strongest negative deviation (−0.57 in KK; −0.59 in BL), reflecting strong avoidance, whereas GCG (alanine) was highly preferred (0.36 in KK; 0.37 in BL). Glycine codons followed a similar trend, with GGA overrepresented (0.36 in KK; 0.34 in BL) and GGT underrepresented (−0.35 in both KK and BL). Additional codons displayed mirrored patterns: CTT (leucine) was underrepresented (−0.17 in both KK and BL) while CTG was overrepresented (0.13 in KK; 0.12 in BL), and TGC (cysteine) was preferred (0.26 in KK; 0.28 in BL) relative to TGT (−0.26 in KK; −0.28 in BL). These trends indicate strong codon-specific selection rather than random mutational bias. The preferential usage of several A/T-ending synonymous codons is consistent with the AT-rich composition that characterizes most chloroplast genomes. Such codon preference has been widely reported in plastid genomes of Poaceae and other angiosperms, where low GC content, particularly at the third codon position, contributes to conserved codon usage patterns during chloroplast genome evolution (Geng et al., 2026). These observations suggest that both underlying nucleotide composition and translational selection have shaped codon usage in the chloroplast-encoded *rpo* genes while maintaining efficient protein synthesis (Parvathy et al., 2022; Wang et al., 2024).

Minor quantitative differences between cultivars were observed, such as AGG (0.21 in KK; 0.22 in BL) and TGC (0.26 in KK; 0.28 in BL), which are unlikely to be biologically significant. The conserved and non-random codon usage observed in *rpo* genes likely optimizes the translational efficiency of plastid-encoded RNA polymerase subunits, essential for chloroplast gene expression. This pronounced codon bias may reflect the critical role of *rpo* genes in plastid transcription, requiring high translational accuracy and efficiency. The highly conserved codon usage profiles observed in both cultivars are also consistent with the strong purifying selection, conserved motif composition, and structural conservation identified in the present study, indicating that evolutionary constraints act at multiple molecular levels to preserve *rpo* gene function. Efficient translation of PEP subunits is expected to support stable chloroplast transcription, thereby contributing to the maintenance of photosynthetic gene expression and chloroplast function. Together, these findings suggest that codon usage bias represents an additional evolutionary mechanism reinforcing the functional conservation of plastid-encoded RNA polymerase during chloroplast genome evolution in *Saccharum*. This interpretation is consistent with previous studies demonstrating that codon usage bias represents an evolutionary balance between optimizing translational efficiency and preserving genomic integrity in functionally important genes (Li et al., 2026). Collectively, these results support translational selection as a major force shaping codon usage in these essential genes (Yao et al., 2023; Li et al., 2024).

3.7. Phylogenetic Reconstruction of *rpo* Genes

The phylogenetic tree was constructed based on concatenated sequences of four plastid-encoded RNA polymerase genes (*rpoA, rpoB, rpoC1,* and *rpoC2*), including the two studied cultivars (*Saccharum* hybrid 'Kidang Kencana' and 'Bululawang'), as well as related *Saccharum* species and an outgroup. The tree was inferred using both Maximum Likelihood (ML) and Bayesian Inference (BI) methods, which produced a consistent topology with strong statistical support across most nodes (Fig. 8). The phylogenetic tree robustly resolved all *Saccharum*

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accessions into a well-supported monophyletic clade, indicating a shared evolutionary origin of the *rpo* gene family within the genus. Within this clade, the two studied cultivars, *Saccharum* hybrid 'Kidang Kencana' and 'Bululawang', were clearly positioned within the modern hybrid lineage. Both cultivars clustered tightly with other commercial hybrids, including 'GXASF1', 'NCo 310', 'Q155', and 'Q165', forming a strongly supported subclade (ML = 100%; BI = 1.00). The short branch lengths observed within this group indicate minimal sequence divergence among hybrid accessions, reflecting a high degree of conservation in *rpo* genes. This pattern is consistent with the shared genetic background and relatively narrow diversity of modern sugarcane cultivars derived from interspecific hybridization (Wang et al., 2022).

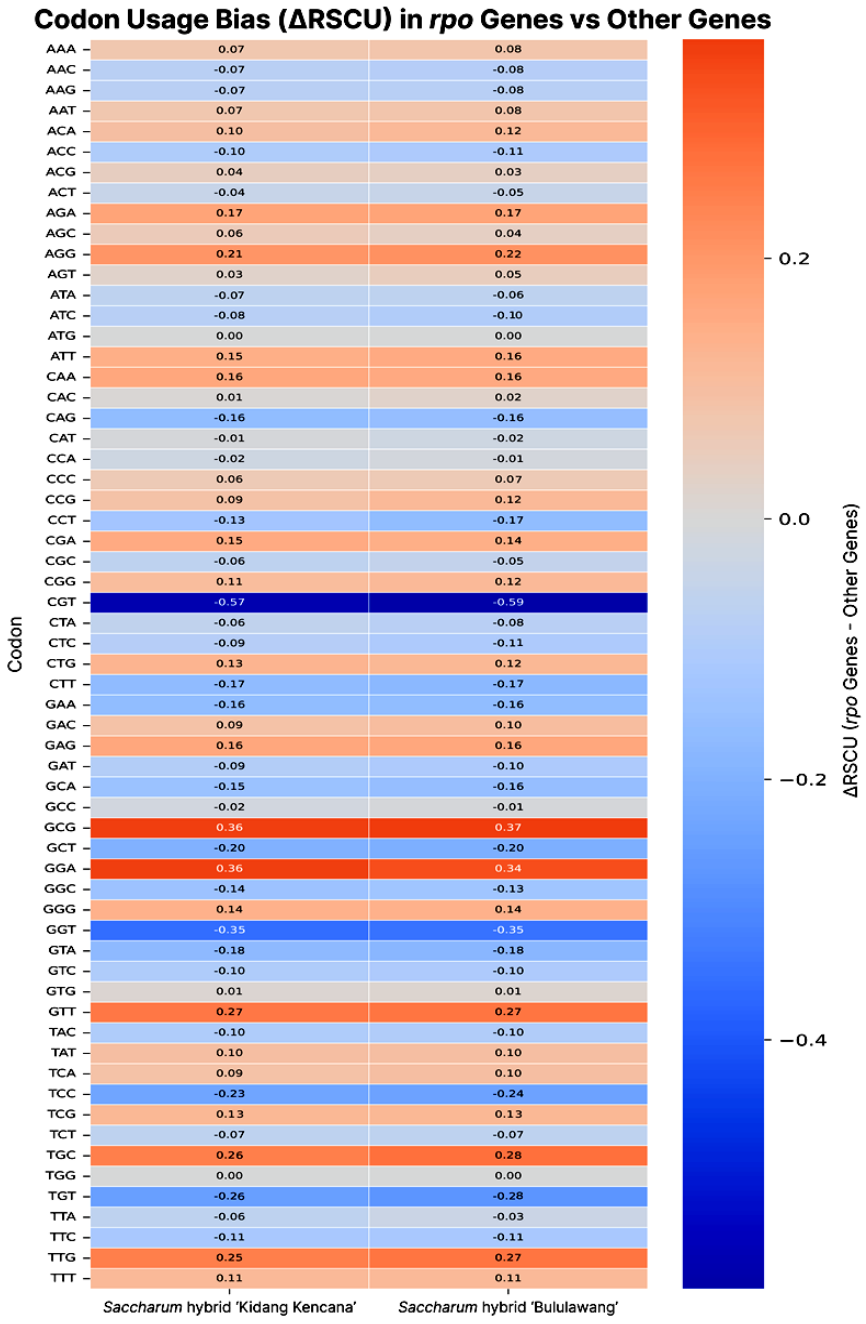

Fig. 7: Comparative codon usage bias of *rpo* genes vs other chloroplast genes in *Saccharum* hybrid 'Kidang Kencana' and 'Bululawang'.

Within the broader *Saccharum* clade, several *Saccharum officinarum* accessions were positioned in close proximity to, or intermixed with, the hybrid clade. This pattern is consistent with the breeding history of modern sugarcane, in which interspecific hybrids derived from *S. officinarum* and *Saccharum spontaneum* have undergone

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repeated backcrossing to *S. officinarum* to recover desirable agronomic traits. As a result, modern cultivars retain a substantial proportion of the *S. officinarum* genome, which likely contributes to their close phylogenetic affinity and partial clustering within the same clade. In contrast, *Saccharum spontaneum* accessions were resolved as a distinct sister clade, indicating a more divergent evolutionary lineage while remaining an important genetic contributor to hybrid cultivars. The separation between *S. officinarum* and *S. spontaneum* lineages is supported by moderate to high node support values, suggesting clear phylogenetic differentiation despite their shared role in hybrid formation (Oliveira et al., 2023).

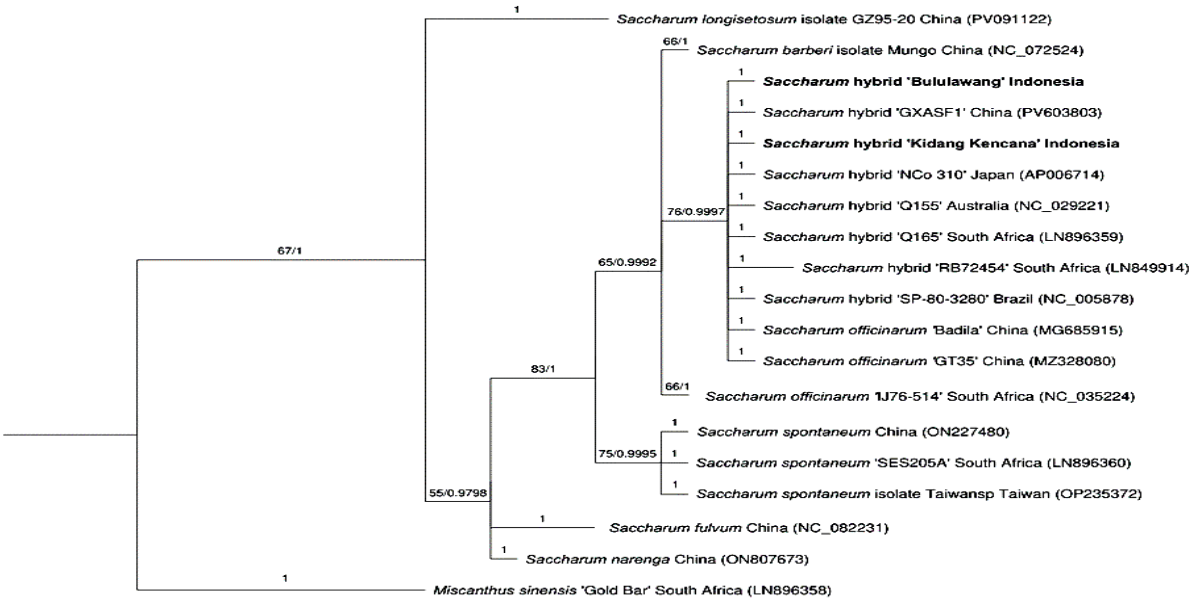

Fig. 8: Phylogenetic reconstruction of the *rpo* gene family in *Saccharum* hybrid 'Kidang Kencana' and 'Bululawang', including related *Saccharum* species and an outgroup, inferred using Maximum Likelihood (ML) and Bayesian Inference (BI) methods. Node support values are presented as ML bootstrap percentages followed by BI posterior probabilities (ML/BI).

More distantly related taxa, including *Saccharum barberi*, *Saccharum longisetosum*, *Saccharum fulvum*, and *Saccharum narenga*, were positioned outside the main hybrid progenitor cluster and exhibited relatively longer branch lengths, indicative of greater evolutionary divergence. Notably, *S. barberi* displayed a markedly extended branch, consistent with the elevated structural variation observed in the RMSD analysis, particularly for the *rpoA* protein. The outgroup, *Miscanthus sinensis*, was clearly separated from all *Saccharum* taxa with strong support, confirming appropriate tree rooting and the robustness of the inferred topology.

These results demonstrate that the *rpo* gene family is highly conserved across *Saccharum*, with limited sequence divergence among modern hybrids. The close phylogenetic association between 'Kidang Kencana', 'Bululawang', and *S. officinarum* accessions reflects the impact of repeated backcrossing during sugarcane domestication, while the distinct placement of *S. spontaneum* highlights its contribution as a genetically divergent progenitor. Collectively, these phylogenetic relationships are consistent with the conserved codon usage patterns, motif composition, and protein structural characteristics identified in the present study, indicating that strong purifying selection has maintained the evolutionary and functional stability of plastid-encoded *rpo* genes throughout *Saccharum* diversification. This high level of conservation supports the potential utility of *rpo* genes as reliable chloroplast markers for phylogenetic inference and provides a foundation for future studies investigating chloroplast genome evolution and the development of chloroplast molecular markers in sugarcane and related Poaceae species (Douglas & Shapiro, 2024; Chen et al., 2025).

Although these findings provide valuable insights into the sequence evolution, structural conservation, and phylogenetic relationships of plastid-encoded RNA polymerase (*rpo*) genes in *Saccharum*, several aspects warrant further investigation. The structural analyses were based on computational predictions and therefore would benefit from experimental validation to confirm protein conformations and intermolecular interactions within the plastid-encoded RNA polymerase (PEP) complex. In addition, the coordinated regulation of chloroplast transcription by nuclear-encoded sigma factors and PEP-associated proteins (PAPs), as well as the expression patterns of *rpo* genes under different developmental stages or environmental conditions, remain to be elucidated. Integrating comparative

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chloroplast genomics with transcriptomic, proteomic, and functional analyses across a broader range of cultivated and wild *Saccharum* accessions would provide a more comprehensive understanding of *rpo* gene evolution and regulation. Despite these limitations, this study provides the first comprehensive characterization of the plastid-encoded *rpo* gene family in elite Indonesian sugarcane cultivars. These findings expand the available genomic resources for *Saccharum* and provide a foundation for future functional studies, chloroplast marker development, evolutionary analyses, and plastome-assisted breeding in sugarcane.

4. CONCLUSION

The present study provides the first comprehensive characterization of the plastid-encoded *rpo* gene family in the elite Indonesian sugarcane cultivars 'Kidang Kencana' and 'Bululawang', thereby expanding the genomic resources available for *Saccharum*. The high evolutionary conservation of the *rpo* gene family underscores its fundamental role in plastid transcription and chloroplast function, while the conserved sequence, motif, and structural features highlight its potential as a valuable molecular resource for germplasm characterization, phylogenetic inference, and future plastome-assisted breeding. Future studies integrating functional validation, gene expression profiling, and protein interaction analyses across diverse sugarcane germplasm will further elucidate the biological roles of *rpo* genes and facilitate their application in sugarcane molecular breeding programs.

Declarations

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Conflict of Interest: None.

Data Availability: The data generated during the study are available within the article.

Ethics Statement: Not applicable.

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Generative AI Statement: The authors declare that no Gen AI/DeepSeek was used in the writing/creation of this manuscript.

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