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Insights into panicle trait variation and *DUF-640* gene conservation in Indonesian foxtail millets (*Setaria italica*)

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Abstract: Foxtail millet (*Setaria italica*) is a resilient yet underutilised C_4 cereal valued for its adaptability to abiotic stress and high nutritional content. While panicle traits have been linked to yield in many cereals, the spatial arrangement of foxtail millet panicles remained unexplored, especially among locally adapted genotypes. This study aimed to characterise spatial panicle architecture traits and to analyse the *DUF-640*, a gene controlling primary branch number, among Indonesian foxtail millet genotypes. Results revealed substantial variation in panicle architecture, including primary branch number, grain number, and grain density in eight Indonesian foxtail millet genotypes, suggesting potentially greater diversity across broader germplasm. In contrast to the substantial panicle trait variations, phylogenetic and structural analyses showed that *DUF-640* genes were highly conserved across *Setaria* species. Although previously associated with primary branch development, the coding sequence of *DUF-640* was not associated with branching variation in this study. However, its high sequence conservation across *Setaria* species suggests a vital and possibly conserved regulatory function. This study enhances the understanding of the morphological and genetic diversity of foxtail millet, particularly among Indonesian foxtail millet genotypes. Future research should focus on the functional characterisation of *DUF-640* and the identification of regulatory sequences governing its gene expression.

Keywords: ALOG domain; C_4 plant; panicle architecture; primary branch; single nucleotide polymorphism (SNP)

Foxtail millet (*Setaria italica* (L.) P.Beauv.) is a resilient C_4 cereal crop recognised for its exceptional adaptability to marginal and resource-poor environments. Its robust tolerance to abiotic stresses such as drought and salinity makes it increasingly relevant in the context of climate change (Yang et al. 2020; Ardie et al. 2025). In addition to its environmental resilience, foxtail millet provides substantial nutritional benefits. It is a rich source of protein, dietary fibre, and micronutrients, and has been associated with protective effects against chronic diseases, including diabetes, cancer, and cardiovascular disorders (Abedin et al. 2022; Yang et al. 2022). Despite these advantages, foxtail millet remains underutilised globally due to its low productivity. Thus, enhancing its productivity has

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become a central focus of breeding programs, particularly by selecting yield-associated traits.

Several studies showed that panicle architecture is critical in determining yield potential by directly affecting grain production in foxtail millet. Panicle length has been positively correlated with grain yield, suggesting that longer panicles contribute to higher productivity (Harish & Lavanya 2022). Longer panicles, which typically accommodate more primary branches, enhance yield potential by increasing the number of grain-bearing spikelets, as both primary branch number and length are positively correlated with grain yield (Zhi et al. 2021). These findings are supported by studies in rice, where the primary branch length, branch diameter, and grain number per primary branch have been shown to influence overall yield (Saito et al. 2023; Jiang et al. 2024). Despite substantial research, the spatial variation of panicle traits across different zones of the panicle remains insufficiently explored in foxtail millet. In rice, panicle branches exhibit considerable morphological variation based on their spatial position, with spikelets in the upper zones showing higher grain filling and weight than those in the lower zones (Chen et al. 2019). Moreover, spikelet distribution and development in rice appear to be regulated by distinct mechanisms depending on their position (Zhang et al. 2025). A deeper understanding of the spatial variation in primary branch arrangement and size across different panicle zones could guide more targeted breeding strategies to enhance grain yield in foxtail millet.

The quantitative trait locus (QTL) *qPBN9.2*, associated with the primary branch number in foxtail millet, was identified through multi-environment QTL mapping in a recombinant inbred line population across several locations in China (Zhi et al. 2021). Within this QTL, the gene *Seita.9G222400* appeared to be an orthologue of *TAW1* in rice, a key regulator of meristem activity and inflorescence development belonging to the *Arabidopsis* LIGHT-DEPENDENT SHORT HYPOCOTYLS 1 (LSH1) and *Oryza* G1 (ALOG) protein family (Yoshida et al. 2013). In wheat, *ALOG* genes were shown to play a crucial role in branch meristem regulation (Nan et al. 2018). Based on their conserved amino acid sequences, the Pfam database classifies *ALOG* family genes as *DUF-640*. Increasing evidence highlights the critical role of this gene family in floral development across land plants (Rieu et al. 2024; Wang et al. 2024; Luo et al. 2025).

This study aims to investigate the spatial variation in panicle traits among Indonesian foxtail millet genotypes and characterise the *DUF-640* gene within these genotypes. Indonesia's diverse agro-ecological zones have shaped the adaptation of local millet accessions, potentially leading to uniquely conserved genetic segments influenced by edaphic and environmental conditions (Ratnawati et al. 2024). Previous studies have shown that domestication processes and geographical distribution significantly affect the genetic diversity of foxtail millet (Fukunaga & Kawase 2024), further supporting the importance of characterising indigenous genotypes. Assessing panicle traits variation alongside candidate gene characterisation may uncover consistent genetic markers, offering valuable tools for future breeding strategies to improve productivity in foxtail millet.

MATERIAL AND METHODS

Study site and genetic materials. This research was divided into two experiments. The first experiment was conducted at the experimental field of IPB University in West Java (6.565549°S, 106.725046°E) from March to July 2024. The second experiment was conducted from June to October 2024 at the Plant Molecular and Biotechnology Laboratory (PMB-2), Department of Agronomy and Horticulture, IPB University. The list of foxtail millet genotypes used is presented in Table 1. The same set of nine genotypes was initially intended to be used in both experiments. However, Botok-10 failed to establish in the field due to poor germination and growth and was therefore excluded from Experiment 1. Additionally, Botok-4 was represented by two distinct seed lots: one used for Experiment 1 (field experiment) and the other for Experiment 2 (molecular analysis). These experiments were conducted in parallel. Upon harvest, we observed that the seed size of Botok-4 from the field experiment differed significantly from previous reports (Jannah et al. 2024), raising concerns about its genetic identity. To distinguish this variant, we designated the field-grown material as Botok-4(B), while the original Botok-4 was retained for DNA isolation. As a result, Experiment 1 included eight genotypes, while Experiment 2 included nine genotypes.

Morphological characterisation of panicle architecture on eight Indonesian foxtail millet genotypes. The climate from March to July 2024 was characterised by an average temperature of 26.5 °C, relative humidity of 84%, and monthly rainfall averaging 216 mm/month. The soil at the research site is classified as Latosol, characterised by a pH of 4.84 and low organic carbon

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Table 1. List of local foxtail millet genotypes used in the study

No.	Genotypes	Origin	Experiment 1	Experiment 2
1	ICERI-5	Indonesian Cereal Research Institute (ICERI)	included	included
2	ICERI-6		included	included
3	ICERI-7		included	included
4	Botok-4(B)	East Nusa Tenggara	included	excluded
5	Botok-4		excluded	included
6	Botok-10		excluded	included
7	Hambapraing		included	included
8	Mauliru-2		included	included
9	Buru	North Maluku	included	included
10	NTB-1	West Nusa Tenggara	included	included

content (Alghifari et al. 2023). Land preparation involved weed removal and soil tillage to optimise planting conditions, followed by the application of manure (2.5 t/ha) and dolomite (300 kg/ha) one week before planting. Seeds were sown three per hole at a spacing of 25 cm between rows and 10 cm within rows. Fertilisers were applied at two weeks post-planting (urea, 150 kg/ha; SP-36, 150 kg/ha; and KCl, 75 kg per ha), and at six weeks post-planting (urea, 150 kg/ha). Pest, disease, and weed controls were applied based on field assessments using appropriate pesticides, followed by the installation of a protective net to prevent further pest infestations.

The primary experiment was conducted using a randomised complete block design (RCBD), with foxtail millet genotype as the single factor. The experiment included three replications; each arranged as a block measuring 6 × 12 m. Within each block, genotypes were randomly assigned to plots sized 1.5 × 1.2 m. Each plot consisted of a planting bed with six rows, and each row contained 12 plants, resulting in a total of 72 plants per genotype per replication. Observations were recorded from eight sample plants in each plot, excluding border plants to minimise edge effects.

Observations were conducted on the main panicle, which was harvested at the ripening stage. The ripening stage, defined according to UPOV (2013) and indicated by panicle browning and leaf drying, occurred at varying times across genotypes: Buru (73 days after planting/DAP), ICERI-5 (88 DAP), ICERI-6 (89 DAP), ICERI-7 and Hambapraing (93 DAP), Botok-4(B) and Mauliru-2 (97 DAP), and NTB-1 (114 DAP). The foxtail millet panicle exhibits a complex branching architecture (Figure 1), consisting

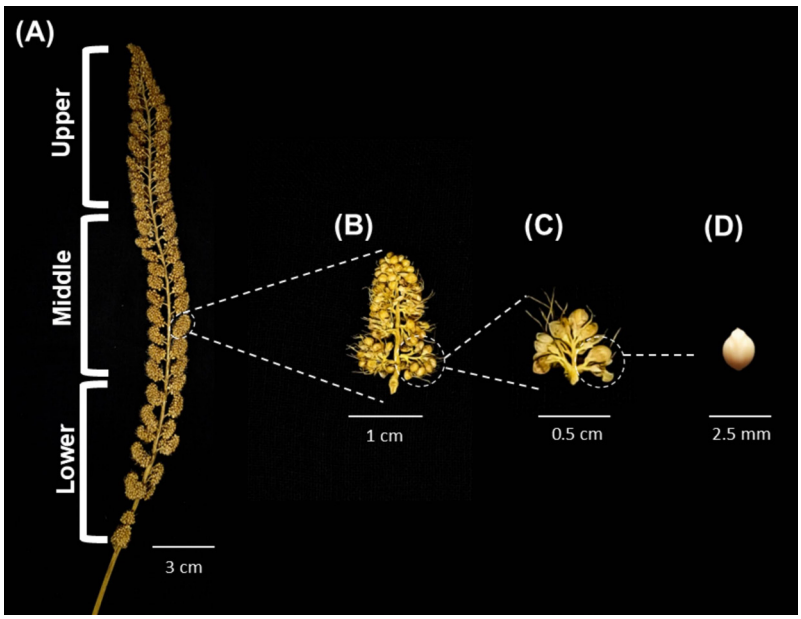

Figure 1. Panicle architecture of foxtail millet: a full view of the panicle, subdivided into upper, middle, and lower zones (A), enlarged view of a primary branch (B), a single secondary branch dissected from the primary branch (C), a mature seed derived from a secondary branch (D)

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of primary branches emerging from the central axis and supporting secondary branches. Panicle architecture traits were assessed following UPOV (2013) and Zhi et al. (2021).

Twenty panicle-related traits were observed and categorised into whole panicle traits and segmented panicle traits. Whole panicle traits included five traits, namely panicle length, panicle density, primary branch number, grain number per panicle, and grain density per panicle. Panicle length was measured from base to tip using a ruler, while panicle density was calculated as the number of primary branches per unit length. The total number of primary branches was counted based on those emerging from the central axis. Grain number per panicle was recorded as the total grain count, and grain density was determined by dividing the grain number by panicle length.

For segmented panicle traits, each panicle was divided into three equal-length zones – upper, middle, and lower – by dividing the total panicle length by three to ensure consistent sectioning. Traits assessed within each zone included primary branch length, primary branch diameter, primary branch number, grain number per zone, and grain density per zone, resulting in a total of 15 traits. Primary branch length was measured using a ruler, and branch diameter was assessed at the midpoint using digital callipers. The number of primary branches was manually counted per zone. Grain number per zone was recorded by counting all grains attached to primary branches within each zone, and grain density was calculated by dividing the total grain number per zone by the corresponding zone length.

DNA isolation, *DUF-640* amplification, and sequencing. Genomic DNA of nine foxtail millet genotypes was isolated from the leaf tissues following the CTAB method described by Aboul-Maaty and Oraby (2019) with slight modification. The isolated DNA was diluted with nuclease-free water to a final concentration of 12 ng/µL. Primers targeting the *DUF-640* gene were manually designed based on the sequence of *Seita.9G222400* in the *Setaria italica* v2.2 genomic data from Phytozome, located at the flanking sequence of approximately 100 bp before and after the coding DNA sequence. Forward primer (5'-GGTTGCTTTCGCAGATGGACCTG-3') and reverse primer (5'-AGCTCCGGCGGCCATCAC-3') were used to amplify the 675 bp target amplicon. PCR was performed in a 50 µL volume, containing DNA template (4 ng/µL), forward and reverse primers (0.2 µM, each), and My*Taq* HS Redmix (1×). My*Taq* HS Red Mix (Bioline, US) was used due to its high-fidelity and proofreading activity of an efficient 3'-to-5' exonuclease, which ensures amplification accuracy. The PCR cycle included initial denaturation at 95 °C for 3 min; 35 cycles of 95 °C for 25 s, 63 °C for 25 s, and 72 °C for 10 s; and a final extension at 72 °C for 10 min. Amplified products were analysed on a 1.5% agarose gel in 1× TAE buffer at 100 V for 35 minutes. Sanger sequencing was conducted by 1st BASE (Singapore) and performed on an ABI PRISM 3730xl Genetic Analyzer using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Thermo Fisher Scientific Inc., USA) according to the manufacturer's instructions. The primers used for sequencing were the same as those used to amplify the *DUF-640* gene.

Data analysis. The five whole panicle traits were analysed using a randomised complete block design, while the 15 segmented panicle traits were evaluated using a nested design to investigate the influence of panicle zones on panicle architecture. In the nested design, panicle zones (upper, middle, and lower) were nested within the main factor, genotype, allowing for zone-specific trait analysis. Analysis of variance (ANOVA) was performed for all traits, followed by the least significant difference (*LSD*) test at the 5% level, using SAS OnDemand for Academics and Microsoft Excel.

A dendrogram illustrating genotype dissimilarity based on panicle traits was constructed using the "dendextend" package in RStudio (R 4.4.1) with Euclidean distance as the dissimilarity metric. The data were normalised using the Min-Max scaling method, which adjusts the values within the dataset to a uniform range. This normalisation ensures that all features contribute equally to the machine learning model, thereby minimising bias resulting from differences in variable scales (Sulistya et al. 2024). The normalisation method was described by Zhang et al. (2022), with the formula $[y = 2 \times ((x - x \text{ min})/(x \text{ max} - x \text{ min})) - 1]$. A *t*-test analysis was conducted using SAS OnDemand for Academics to identify discriminant traits.

Nucleotide sequence assembly and alignment were performed using BioEdit 7.2 software with the MUSCLE alignment program. The gene structure was developed using WormWeb version 4 (Bhatla 2012). FinchTV was used to visualise and verify the quality and trend of the nucleotide sequences obtained from sequencing. Genetic variation within the *DUF-640* gene was queried using the variation (single nucleotide polymorphism (SNP), InDel, and SV) feature of the

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SetariaDB portal (http://111.203.21.71:8000/), with Yugu1_v2.2 as the reference genome across a global dataset of 1 844 foxtail millet accessions. The search parameters targeted chromosome 9, spanning positions 16 557 984 to 16 558 631, and included all types of variation. Phylogenetic analysis based on amino acid sequences was performed using the Neighbor-Joining method in MEGA11 (Tamura et al. 2021), with bootstrap values calculated from 1 000 replicates. Sequences were obtained via BLASTp using an Indonesian foxtail millet protein sequence as the query. Only sequences meeting the following criteria were selected: query coverage above 70%, per cent identity exceeding 70%, exclusion of putative and hypothetical proteins, and inclusion of only one representative protein per species (Table S1 in the Electronic Supplementary Material (ESM)). The substitution model used for phylogenetic reconstruction was the Jones-Taylor-Thornton (JTT) model, with rate variation among sites modelled using a gamma distribution (G).

RESULT AND DISCUSSION

Variation in panicle architecture among Indonesian foxtail millet genotypes. The evaluation of eight Indonesian foxtail millet genotypes revealed three distinct panicle architecture types: long-dense panicle, short-dense panicle, and short-loose panicle, each reflecting different reproductive strategies and yield potentials (Table 2, Table S2, Figure S1 in ESM). Genotypes such as Mauliru-2, Hambapraing, and NTB-1 exhibited long-dense panicles characterised by extended panicle length (≥ 23 cm), high primary branch numbers, and substantial grain number and density. These traits suggest efficient reproductive allocation and strong yield potential. ICERI-7 and Buru were classified into the short-dense panicle group, characterised by short panicle length but relatively high panicle and grain density. Its compact structure likely results from reduced panicle length rather than enhanced reproductive capacity, as its total grain number was lower than that of the long-dense group. Meanwhile, ICERI-5, ICERI-6, and Botok-4(B) formed the short-loose panicle group, marked by shorter panicles (~15–18 cm), fewer branches, and lower grain number and density. These genotypes demonstrated limited reproductive efficiency, highlighting the variability in panicle architecture and its implications for foxtail millet breeding strategies aimed at yield improvement. Although our phenotypic observations were limited to eight genotypes, broader studies have confirmed extensive variation in panicle traits across foxtail millet germplasm. For example, panicle length, main panicle diameter, panicle weight, grain weight per panicle, and thousand-grain weight were greatly varied among 407 foxtail millet accessions (Liu et al. 2022).

The yield-associated panicle traits observed in our genotypes are consistent with prior research, which identified Mauliru-2, Hambapraing, and NTB-1 as high-yielding foxtail millet genotypes, whereas ICERI-5 and ICERI-6 were associated with lower

Table 2. Mean of panicle density (PD), primary branch number per panicle (PBN), panicle length (PL), grain number per panicle (GNP), and grain density per panicle (GDP) of eight local foxtail millet genotypes

Genotype	PD (PBN/cm)	PBN	PL (cm)	GNP	GDP (GN/cm)
ICERI-5	8.64^{b}	124.36^{cd}	15.06^{b}	1 636.1^{e}	111.53^{c}
ICERI-6	6.60^{c}	111.79^{d}	17.05^{b}	2 445.4^{cde}	142.53^{c}
ICERI-7	12.70^{a}	133.04^{cd}	10.81^{c}	3 391.0^{cd}	309.72^{a}
Botok-4(B)	6.97^{bc}	127.34^{cd}	18.89^{b}	2 327.0^{de}	120.32^{c}
Buru	8.09^{bc}	135.46^{c}	17.26^{b}	4 099.7^{bc}	232.77^{ab}
Hambapraing	8.26^{b}	222.09^{b}	27.11^{a}	5 754.3^{ab}	210.15^{b}
Mauliru-2	12.60^{a}	290.21^{a}	23.78^{a}	8 115.5^{a}	325.74^{a}
NTB-1	8.45^{b}	194.18^{b}	23.90^{a}	3 539.4^{bcd}	146.46^{c}
F-test	**	**	**	**	**
CV (%)	5.40	6.49	11.56	3.50	4.04

Numbers followed by the same letter on the same column indicate no significant difference based on *LSD* test at $P < 0.05$; **indicates significance of the *F*-test at the 0.01 level

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yield potential (Ratnawati et al. 2024). Among key yield-determining traits, panicle length plays a central role in both rice and foxtail millet, primarily through its positive correlation with grain yield per plant due to the increased spikelet-bearing surface provided by longer panicles (Saketh et al. 2023; Harsha et al. 2025). In wheat, elevated spikelet number has been strongly linked to yield enhancement (Bigyan et al. 2025). Similarly, studies in rice have shown that an increased primary branch number expands grain-bearing sites and improves reproductive output (Agalya et al. 2024). As observed in wheat genotypes, variations in spikelet branching reflect differences in assimilate production and partitioning (Abbai et al. 2024).

Panicle density also plays an important role in shaping yield outcomes. In quinoa, optimal panicle compactness was found to balance structural and reproductive efficiency (Habib et al. 2024). Rice studies have indicated that genotypes with higher grain filling tend to show increased grain number and panicle density (Das et al. 2018), while dense rice panicles enhance nutrient allocation and reproductive performance (Gong et al. 2025). However, when coupled with reduced grain volume, higher grain density may lead to decreased grain weight (Li et al. 2022). Given these findings, targeting panicle architecture traits – including panicle length, primary branch number, and panicle density – could be a key strategy for improving yield potential in foxtail millet through enhanced grain-bearing capacity, reproductive efficiency, and resource allocation.

Grain development patterns, reproductive efficiency, and total yield potential are all influenced by the spatial arrangement of primary branches within different panicle zones (Mohapatra & Sahu 2022). Understanding how these branches are distributed across the upper, middle, and lower zones provides insight into structural adaptations that optimise resource allocation and grain production. Structural traits, particularly primary branch length (PBL) and diameter (PBD), significantly increased from the upper to the lower panicle zones. In contrast, the primary branch number (PBN) decreased accordingly (Table 3, Table S3 in ESM). This contrasting pattern suggests a compensatory architectural strategy in which fewer but longer and thicker branches in the lower zones may support similar grain-carrying capacity. Consequently, grain number (GN) and grain density (GD) did not differ significantly between zones (Table 4), indicating relatively uniform grain distributions along the panicle axis regardless of structural variations.

Among the genotypes, ICERI-6, Hambapraing, and ICERI-7 exhibited the highest PBL, with ICERI-6 and ICERI-7 also showing the greatest PBD. However, these structural traits varied across genotypes and did not consistently correspond to specific panicle types. Genotypes classified under the long-dense panicle group – Mauliru-2, Hambapraing, and NTB-1 – consistently exhibited the highest PBN across all zones, which appears to underpin their high grain production. ICERI-5 and ICERI-6 produced the largest seeds among 28 Indonesian foxtail millet genotypes (Jannah et al. 2024), suggesting a reproductive

Table 3. Mean primary branch traits across panicle zones in eight local foxtail millet genotypes

Genotype	PBL (mm)				PBD (mm)				PBN			
	U	M	L	mean	U	M	L	mean	U	M	L	mean
ICERI-5	4.93	7.61	10.80	7.78^{c}	5.60	6.94	8.01	6.85^{bc}	61.98	36.90	25.48	41.45^{cd}
ICERI-6	7.06	9.78	13.57	10.14^{a}	6.81	7.85	8.95	7.87^{a}	58.46	31.58	21.75	37.26^{e}
ICERI-7	5.92	8.94	11.33	8.73^{b}	6.21	7.68	9.21	7.70^{a}	62.29	40.58	30.17	44.35^{c}
Botok-4(B)	4.17	7.07	10.06	7.10^{d}	5.71	7.27	8.19	7.06^{b}	70.38	35.71	21.25	42.45^{de}
Buru	4.52	7.37	9.87	7.25^{cd}	5.19	6.27	6.90	6.12^{d}	80.79	38.00	16.67	45.15^{de}
Hambapraing	7.90	8.70	11.38	9.33^{ab}	6.20	6.35	7.08	6.54^{cd}	111.29	77.17	33.63	74.03^{b}
Mauliru-2	5.63	7.07	8.67	7.12^{cd}	6.12	6.75	7.43	6.77^{bc}	184.54	73.71	31.96	96.74^{a}
NTB-1	4.64	7.13	10.51	7.43^{cd}	5.09	6.39	7.82	6.43^{cd}	102.63	52.42	39.13	64.73^{b}
Mean	5.60^{c}	7.96^{b}	10.77^{a}	8.11	5.87^{c}	6.94^{b}	7.95^{a}	6.92	91.55^{a}	48.26^{b}	27.51^{c}	55.77

PBL – primary branch length; PBD – primary branch diameter; PBN – primary branch number; U – upper zone; M – middle zone; L – lower zone; numbers followed by the same letter on the same column or row indicate no significant difference based on LSD test at $P < 0.05$

https://doi.org/10.17221/86/2025-CJGPB

Table 4. Mean grain number and grain density per panicle zone of eight local foxtail millet genotypes

Genotype	GN				GD (GN/cm)			
	U	M	L	mean	U	M	L	mean
ICERI-5	471.3	608.9	555.9	545.4^{f}	92.77	126.48	115.35	111.53^{e}
ICERI-6	898.5	745.7	801.2	815.1^{de}	156.94	130.18	140.46	142.53^{d}
ICERI-7	959.5	1 137.7	1 293.8	1 130.3^{cd}	261.51	315.14	352.50	309.72^{a}
Botok-4(B)	671.6	851.7	803.7	775.7^{e}	104.34	132.67	123.96	120.32^{de}
Buru	1 558.5	1 528.4	1 012.8	1 366.6^{bc}	268.62	259.93	169.77	232.77^{bc}
Hambapraing	2 337.1	2 144.5	1 272.7	1 918.1^{ab}	256.63	237.14	136.69	210.15^{c}
Mauliru-2	4 567.7	2 243.0	1 304.9	2 705.2^{a}	540.40	275.00	161.81	325.74^{ab}
NTB-1	996.0	1 115.4	1 428.0	1 179.8^{cd}	124.23	139.55	175.60	146.46^{de}
Mean	1 557.5	1 296.9	1 059.1	1 304.5	225.68	202.01	172.02	199.90

GN – grain number; GD – grain density; U – upper zone; M – middle zone; L – lower zone; numbers followed by the same letter on the same column or row indicate no significant difference based on LSD test at $P < 0.05$

strategy favouring fewer but larger grains. However, both genotypes showed lower overall productivity than Mauliru-2, Hambapraing, and NTB-1 (Ratnawati et al. 2024), indicating that seed size alone does not compensate for lower PBN. These findings highlight the importance of panicle architecture, particularly PBN, in determining the productivity of foxtail millet genotypes.

Clustering analysis enables the classification of genotypes based on multiple trait performances, facilitating the identification of distinct groups with superior characteristics (Ceasar et al. 2020). The dendrogram, constructed using Euclidean distance based on five whole panicle architecture traits and 15 zone-specific panicle architecture traits, revealed two distinct clusters among the eight foxtail millet genotypes (Figure 2). The first cluster consisted of long-dense panicle genotypes, namely Hambapraing, Mauliru, and NTB-1. Interestingly, although classified as a short-dense panicle type, the Buru and ICERI-7 genotypes were clustered with the long-dense panicle group in Cluster I, suggesting structural similarities in panicle morphology that may contribute to comparable productivity patterns. The second cluster grouped the remaining genotypes, including ICERI-5, ICERI-6, and Botok-4(B), representing the short-loose panicle type. Our results showed that genotypes grouped in Cluster I may serve as promising genotypes for breeding programs focused on yield improvement. The independent *t*-test analysis of 20 panicle traits identified PBN, GNP, and GDP as key discriminant panicle traits distinguishing the two clusters. Spatially, discriminant traits included PBN in the middle zone, GN, and GD in the upper and

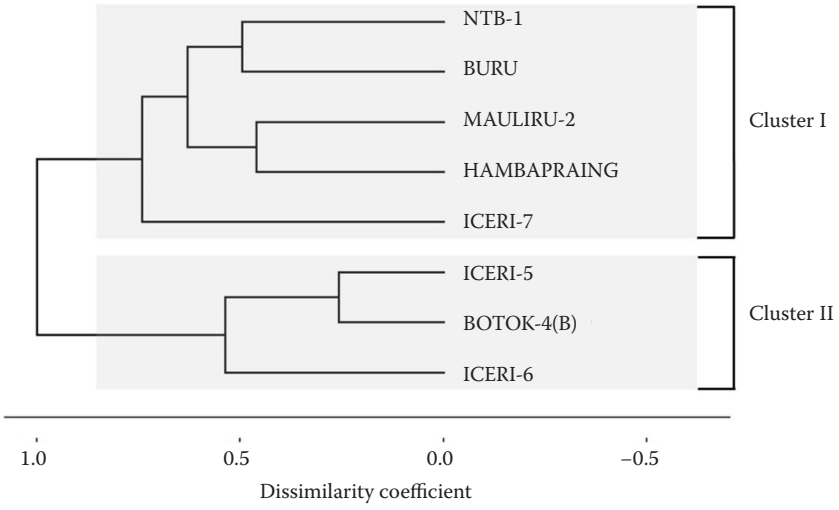

Figure 2. Dendrogram depicting clustering of foxtail millet genotypes by panicle characteristics

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middle zones (Table S4 in ESM). Given that genotypes grouped in Cluster I showed higher productivity, the analysis further highlights that PBN is one of the important traits contributing to productivity.

Variation and phylogenetic classification of *DUF-640* from nine Indonesian foxtail millet genotypes. Given the substantial variation in panicle architecture – particularly in PBN – among the foxtail millet genotypes, we investigated potential molecular variation in the *DUF-640* gene, previously identified as a candidate regulating this trait via QTL analysis (Zhi et al. 2021). The *DUF-640* gene (675 bp) was successfully amplified from nine Indonesian foxtail millet genotypes (Figure S2 in ESM). The resulting sequences have been submitted to the NCBI GenBank database under accession numbers PV006696–PV006704. The *DUF-640* gene comprises two exons (147 and 474 bp) separated by a 26 bp intron (Figure 3).

Variation in PBN among eight Indonesian foxtail millet genotypes contrasts with the complete conservation of the *DUF-640* gene, which shows no non-synonymous mutations and identical nucleotide sequences across genotypes. Comparative alignment with reference genotypes Yugu1 (*Seita.9G222400*) identified only minor variations: a synonymous SNP (T/G) at position 169 bp and two intronic changes – a two-base deletion at positions 248–249 bp and a single-base substitution (G/C) at position 250 bp, none of which affected the coding sequence (Figure S3 in ESM). Given that our analysis was based on a limited set of only nine genotypes, we sought to determine whether the *DUF-640* gene is similarly conserved across a broader and more diverse panel of foxtail millet accessions. Therefore, we queried SetariaDB, which includes genomic data from 1 844 foxtail millet accessions representing at least 13 countries. Using the Yugu1_v2.2 reference, only two synonymous SNPs were identified within exon 2 of the *DUF-640* gene, with a low putative impact level (Table S5 in ESM). These variants were annotated directly by SetariaDB and classified as low impact based on their synonymous nature and predicted lack of functional consequence. These findings indicate that *DUF-640* is highly conserved across diverse foxtail millet germplasms, reinforcing its potential functional importance and suggesting that observed phenotypic variation in PBN is unlikely to be driven by coding sequence variation in this gene.

To further explore the evolutionary conservation of *DUF-640*, a phylogenetic tree was constructed based on amino acid sequence similarity across diverse plant species (Figure 4). In the tree, *Setaria italica*

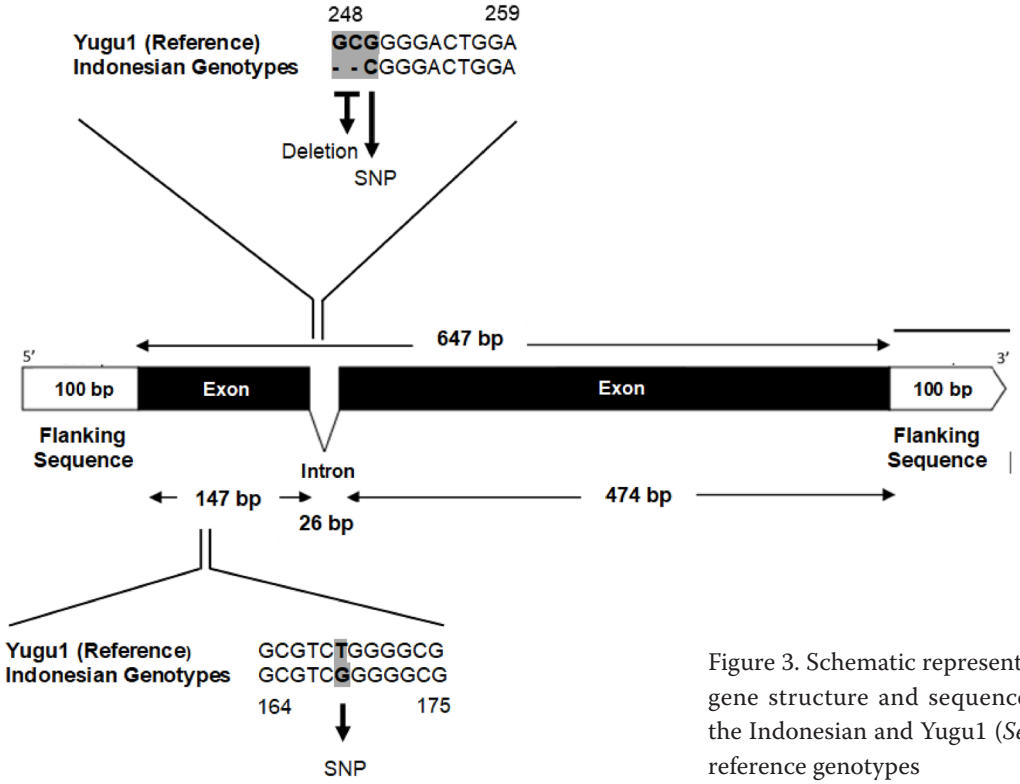

Figure 3. Schematic representation of the *DUF-640* gene structure and sequence variations between the Indonesian and Yugu1 (*Seita.9G222400*) as the reference genotypes

https://doi.org/10.17221/86/2025-CJGPB

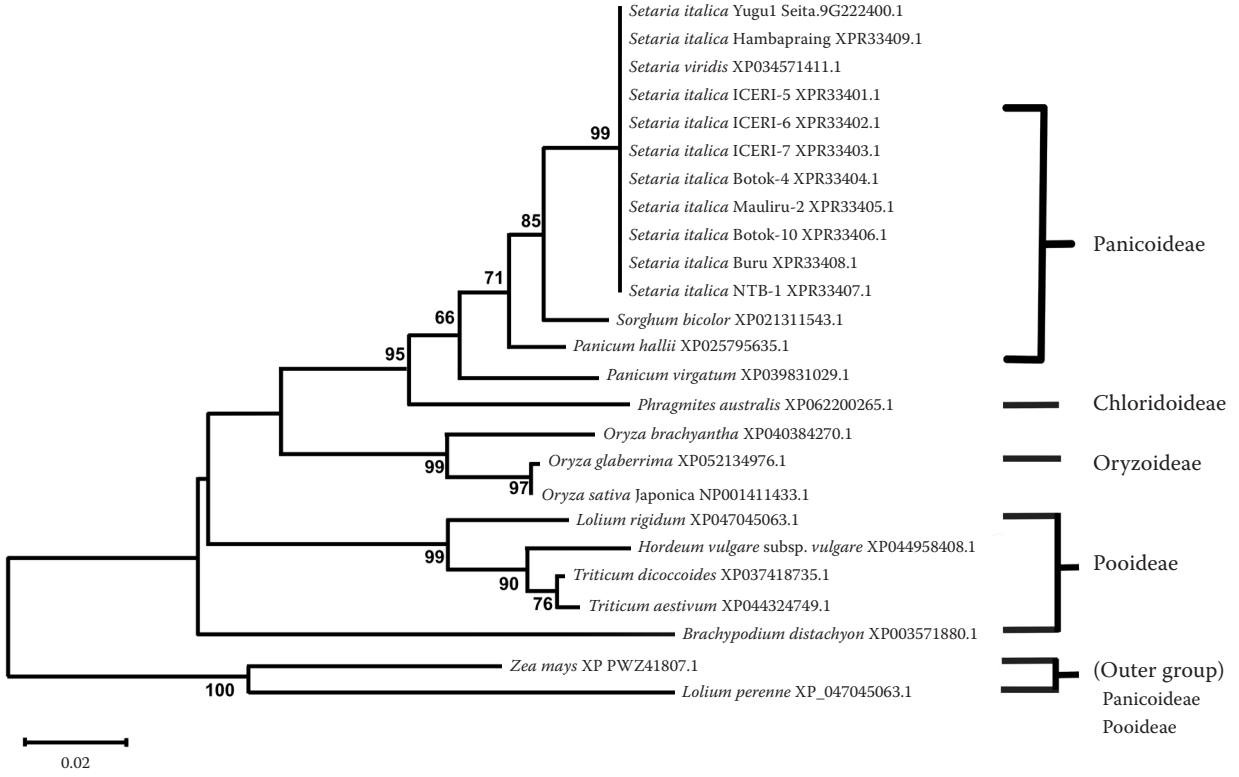

Figure 4. The phylogenetic tree based on the alignment of the DUF-640 protein sequence from nine Indonesian foxtail millet genotypes with other species

accessions, including both Yugu1 and Indonesian genotypes, form a tightly grouped cluster with *Setaria viridis*. This clustering is likely due to the high genomic similarity and close synteny between foxtail millet and *S. viridis*, as previously reported by Bennetzen et al. (2012). *Setaria* species formed a cluster with *Sorghum bicolor* and *Panicum* species in the Panicoidae subfamily. Species from the Oryzoideae subfamily and species from the Pooideae subfamily form a separate cluster from the Panicoidae subfamily. Although *Zea mays* remains within the Panicoideae subfamily, it is placed in a separate cluster from the *Setaria* group. The grouping of these species indicates a level of similarity in their protein sequences, which may reflect conserved structural or regulatory features. The conserved sequences of each species were examined using the NCBI-CDD to support the identification of the domain, which confirmed that all sequences corresponded to the ALOG domain.

The high sequence conservation of the ALOG domain implies its essential role in panicle development and meristem regulation across grass species. The ALOG domain is found in plant-specific regulatory proteins that modulate gene expression, particularly in developmental processes such as inflorescence formation (Li et al. 2019; Luo et al. 2025). Beretta et al. (2023) reported that the ALOG genes *OsG1L1* and *OsG1L2* likely regulate inflorescence development through pathways related to *TAW1*, as suggested by their similar expression patterns and mutant phenotypes in rice. In sorghum, Olatoye et al. (2020) reported that the QTL *qSbRL1.2067* colocalises with *Sobic.001G219400*, which is the sorghum ortholog of the rice *TAW1* gene. Given that the *DUF-640* (*Seita.9G222400*) from foxtail millet is located within the *qPBN9.2* QTL region linked to primary branch number (Zhi et al. 2021) and is an orthologue of the rice *TAW1* gene that controls panicle branching (Yoshida et al. 2013), *DUF-640* likely plays a similarly essential role in foxtail millet panicle development. Such critical regulatory functions may underlie the strong purifying selection acting on its coding sequence. Conserved gene sequences are critical for maintaining stable developmental processes across plant species. Nonetheless, the substantial phenotypic variation in primary branch

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number among Indonesian foxtail millet genotypes suggests that divergence may arise from regulatory differences that influence gene expression rather than protein structure. A relevant example is the *UNUSUAL FLORAL ORGANS* (*UFO*) gene, which remains highly conserved across species; yet, variation within its *cis*-regulatory elements can lead to striking differences in floral morphology (Lanctot et al. 2025). By analogy, the differential expression and phenotypic outcomes associated with primary branch number in foxtail millet may similarly result from underlying regulatory variation. This insight highlights the importance of targeting expression-level variation and regulatory elements such as cis-regulatory polymorphisms influencing *DUF-640* activity as a promising pathway for breeding strategies aimed at optimizing panicle architecture. Further investigation will be essential to elucidate these potential associations and clarify the regulatory mechanisms involved.

CONCLUSION

Indonesian foxtail millet genotypes exhibit variations in panicle architecture, with panicle branch number, grain number per panicle, and grain density as key discriminant traits. Despite these variations, structural and phylogenetic analyses showed that the amino acid sequences of *DUF-640* from Indonesian foxtail millet genotypes were highly conserved compared to those of other *Setaria* species (Yugu1 and *Setaria viridis*) and three species from the same subfamily (Panicoideae). Grouping of proteins containing the ALOG domain indicated that these genes play similar roles in regulating plant growth and inflorescence development. Future investigations should focus on elucidating the functional role of *DUF-640* and identifying the regulatory elements that control its gene expression.

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