

The Genetic Variability of Indonesian Local Foxtail Millet Accession Based on Agro-morphological Traits and Early Salinity Tolerance Evaluation Utilizing *SiDREB2*-based SNAP Marker

Sri Ratnawati¹, Rika Miftakhul Jannah¹, Yuli Irvana Dewi², Ramadaniarto Rizqullah², Willy Bayuardi Suwarno³, Sintho Wahyuning Ardie^{3*}

¹Plant Breeding and Biotechnology Study Program, Faculty of Agriculture, IPB University, Dramaga Campus, Bogor 16680, Indonesia

²Agronomy and Horticulture Study Program, Faculty of Agriculture, IPB University, Dramaga Campus, Bogor 16680, Indonesia

³Department of Agronomy and Horticulture, Faculty of Agriculture, IPB University, Dramaga Campus, Bogor 16680, Indonesia

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ABSTRACT

Foxtail millet (*Setaria italica* L. Beauv.) is a grain-producing crop with high nutritional benefits and adaptability to broad environmental conditions. Indonesian local foxtail millet accessions are valuable genetic resources for crop improvement of the species. This study aims to assess the biodiversity of Indonesian local foxtail millet accessions and estimate their salinity tolerance level utilizing the *SiDREB2*-based SNAP marker and early evaluation at the seedling stage. Our results showed that eight Indonesian local foxtail millet accessions, namely Toraja, Mauliru-2, Hambapraing, NTB-1, ICERI-5, ICERI-6, Botok-4, and Botok-10, had high variability based on agro-morphological traits. The Hambapraing and Mauliru-2 were the potential accessions with high yield estimates (2.33 ton.ha⁻¹ and 1.93 ton.ha⁻¹) and early harvest time (<110 DAT). ICERI-6 was indicated as a salinity-tolerant genotype based on the *SiDREB2*-based marker and early salinity evaluation at the seedling stage, while Toraja, Mauliru-2, NTB-1, and Botok-4 were categorized as sensitive genotypes. Germplasm variability and the estimated salinity tolerance level of Indonesian local foxtail millet accessions presented in this study serve as essential information in the foxtail millet breeding for high productivity, early harvest time, and salinity tolerant variety.

1. Introduction

Millets, known as ancient crops, are a group of small-seeded cereals that play a vital role in arid and semi-arid regions (Tripathi and Vyas 2023). Nevertheless, there was a large decrease in the global cultivation of millets, in contrast to a large increase in major crops, such as wheat, soybeans, and rice, over 57 years since the Green Revolution started (John and Babu 2021). Conservation and maintenance of traditional species, such as millets, is important for sustainable food security (Salgotra and Chauhan 2023). Among the small millets, foxtail millet (*Setaria italica* (L.) P. Beauv.) ranks second in global millet production, with India as the top global millet producer, followed by African

countries and China (FAO 2021). In Indonesia, foxtail millet is known as Jewawut (Kadir *et al.* 2021). Due to its nutritional benefits and adaptability to broad environmental conditions, foxtail millet has gained significant attention (FAO 2023). The nutritional and health benefits of foxtail millet include its low glycemic index (Arora *et al.* 2023), high protein and dietary fiber content, and anti-oxidant potential (Goudar *et al.* 2023). Foxtail millet has also been reported to show comparable tolerance to salinity (Ardie *et al.* 2015; Akter *et al.* 2020) and drought (Xiao *et al.* 2021) stresses, indicating the adaptive nature of the species to adverse environmental conditions. Nahar *et al.* (2018) reported that salt-tolerant foxtail millet accession could maintain its dry matter and grain yield at the salinity level of 6 dS.m⁻¹, while Krishnamurthy *et al.* (2014) evaluated 155 foxtail millet accessions under soil saturated with 100 mM NaCl and identified 13 highly tolerant

* Corresponding Author

E-mail Address: sintho_wa@apps.ipb.ac.id

foxtail millet accessions. The salt tolerance potential of foxtail millet is associated with its efficient energy production under stress (Han *et al.* 2022). Plant salinity tolerance involves complex regulatory mechanisms, including the dehydration-responsive element binding (DREB) transcription factors in the ABA-independent pathway (Zelm *et al.* 2020). A synonymous single nucleotide polymorphism (SNP) at the 558th base pair (an A/G transition) in the *SiDREB2* gene was detected in a core set of 45 foxtail millet accessions and further associated with dehydration tolerance (Lata *et al.* 2011). Subsequently, a *SiDREB2*-based single nucleotide amplified polymorphism (SNAP) marker was developed to estimate foxtail millet's drought- or salt-tolerance level (Widyawan *et al.* 2018).

There are some challenges in superior foxtail millet development in Indonesia. Until recently, there was no clear documentation on the number of local foxtail millet accessions in Indonesia, leading to no superior variety being released. Collection and characterization of Indonesian local foxtail millet accessions are initial attempts to conserve and maintain the genetic diversity of the species, and it is also an essential step in the breeding programs of superior foxtail millet variety. Genetic diversity studies of 27 Nepalese foxtail millet landraces (Yadav *et al.* 2018) indicated tremendous diversity among the studied landraces. However, foxtail millet is considered an underutilized crop and received only limited scientific interest in Indonesia. Evizal (2020) reported that foxtail millet is cultivated as a minor crop in West Sulawesi, Buru, Sumba, and Central Java. Separate studies indicated the genetic variability potential of foxtail millet in Indonesia, including the genotyping of 26 Indonesian local foxtail millet accessions using a *SiDREB2*-based SNAP marker (Widyawan *et al.* 2018) and the characterization of six local accessions of foxtail millet in West Sulawesi (Ramlah *et al.* 2020). Local accession is one of the important plant genetic resources that harbor various beneficial traits and yet potentially improved to be a superior variety bearing desired traits through crop improvement programs (Salgotra and Chauhan 2023). This study reported the morphological and agronomical characterization and early evaluation of salinity tolerance of eight Indonesian local foxtail millet accessions. The information from this study may be useful for breeding improved foxtail millet varieties tolerant to salinity.

2. Materials and Methods

2.1. Morphological and Agronomical Characterization

The field experiment was conducted at Cikabayan Bawah Experimental Station (240 m above sea level, m asl), IPB University, Bogor, Indonesia (6°33'24.23 "S, 73 106°43'33.4" E). The recorded agro-climates conditions during the experiment (February-July 2021) include an average temperature of 26.02°C, an average humidity of 84.78%, and an average rainfall of 279.60 mm per month (BMKG 2021). This experiment was arranged in a randomized complete block design (RCBD) with eight foxtail millet accessions as a single factor, replicated three times. Each replicate was a block consisting of 64 planting rows, with plant spacing of 75 cm × 10 cm. Each accession was planted in eight rows per replication. Each row consisted of eight plants, with three sample plants per row; thus, 24 sample plants per accession per replication. The eight foxtail millet accessions were two accessions from the Indonesian Cereals Research Institute (ICERI-5 and ICERI-6), one local accession from Sulawesi (Toraja), one local accession from West Nusa Tenggara (NTB-1), and four local accessions from East Nusa Tenggara (Hambapraing, Mauliru-2, Botok-4, and Botok-10).

Seeds were immersed in 52°C water for 20 minutes before being sown in seedling trays containing a media mixture of compost and manure (1:1, v/v) and 3% Carbofuran. The seedlings were transplanted to the field after five weeks in the nursery. Fertilizers of SP-36 (150 kg.ha⁻¹), KCl (75 kg.ha⁻¹), and urea (150 kg.ha⁻¹) were applied two weeks after transplanting (WAT). The second fertilizer was applied at 6 WAT for urea (150 kg.ha⁻¹). A plant net was installed at 2 WAT, while insecticide (1 g.L⁻¹ of 50% Pymetrozine) was applied to control the rice ear bug (*Leptocorisa oratorius*) at the grain-filling stage.

Observations were made on agro-morphological traits (nine qualitative traits and 16 quantitative traits) according to UPOV (2013). The qualitative traits consist of grain shapes, endosperm types, panicle types, tillers growth type, anthocyanin coloration of the basal leaf sheath, attitude of the leaf blade, glume anthocyanin coloration, grain color, and dehusked grain color. Among the observable qualitative traits in the UPOV descriptor, anthocyanin coloration of the basal leaf sheath, grain color, and endosperm type is agreed as useful grouping characteristics

in foxtail millet. The endosperm type of foxtail millet can be categorized into waxy and non-waxy types, depending on the starch composition in the endosperm. Waxy endosperms will be stained reddish-brown, while non-waxy endosperms will be stained dark blue following immersion in Lugol's solution (UPOV 2013). Low or nearly undetectable level of amylose leads to waxy endosperm (Bao 2019). The quantitative traits consist of plant height (cm), stem diameter (mm), flag leaf length (cm), flag leaf width (cm), number of internodes, number of tillers when harvested, number of leaves when harvested, main panicle length (cm), plant grain weight (g), plant dry weight (g), 100-grain weight (g), grain length (mm), grain width (mm), heading time (DAT), harvest time (DAT), and potential yield estimation ($\text{ton}\cdot\text{ha}^{-1}$). Potential yield estimation was calculated according to the following formula:

$$\text{Yield estimation } (\text{ton}\cdot\text{ha}^{-1}) = \frac{(\text{grain weight per plant (g)} / 10^6) \times \text{number of plants per ha} \times 60\%}{}$$

2.2. Estimation of Salinity Tolerance using *SiDREB2*-based SNAP Marker

An experiment using a *SiDREB2*-based SNAP marker to estimate the salinity tolerance of the eight foxtail millet accessions was conducted at Plant Molecular Biology-2 Laboratory, IPB University, Bogor, Indonesia. The eight foxtail millet accessions' genomic DNA was extracted from the leaves of ten days-old seedlings following the CTAB method with slight modification (Murray and Thompson 1980; Aboul-Maaty and Oraby 2019) and diluted in nuclease-free water to $12 \text{ ng}\cdot\mu\text{L}^{-1}$ concentration. Primer pairs used to amplify a 300 bp *SiDREB2* gene fragment that contains an SNP at the 558th base are listed in Table 1 (Widyawan *et al.* 2018).

The amplification of the DNA fragments was performed in 10 μL final volume of the PCR reaction

Table 1. *SiDREB2*-based SNAP marker used in this experiment

Primer name	Nucleotide sequence (5'-3')	T _m (°C)	Primer type
SD2-558-SNP-G	GCAAGTCCGTGGAGGTACTACAG	58.8	Forward
SD2-558-SNP-A	AAGTCCGTGGAGGTACTGCAA	58.3	Forward
SD2-558-SNP-Rev	AGGAACTCAACACACAGGACAAC	57.9	Reverse

Source: Widyawan *et al.* (2018)

using Esco's Swift Maxi Thermal Cycler (Esco Technologies, Singapore). The final reaction mixture consisted of 2.5 μL of genomic DNA ($12 \text{ ng}\cdot\mu\text{L}^{-1}$), 2.5 μL of forward (SD2-558-SNP-A or SD2-558-SNP-G) and reverse primer (SD2-558-SNP-Rev) (10 pmol each), 5.0 μL of 2 \times PCR mix (KAPA2G Fast HotStart ReadyMix, Sigma-Aldrich, Germany). The PCR profile was referred to Widyawan *et al.* (2018) with slight modification as follows: denaturation for 5 minutes (94°C), 25 cycles of denaturation for 5 seconds (94°C), annealing for 1 minute (55°C), and extension for 30 seconds (72°C). The final extension was performed for 7 minutes at 72°C after the end of the cycles. PCR products were analyzed by electrophoresis at 90 volts for 40 minutes in 1 \times TAE buffer on 1.5% (w/v) agarose gel. PCR results were stained in $0.5 \mu\text{g}\cdot\text{mL}^{-1}$ ethidium bromide solution and were visualized using a UV transilluminator (AlphaImager[®] Mini).

2.3. Salinity Tolerance Evaluation at Seedling Stage

This greenhouse experiment was conducted at Cikabayan Bawah Experimental Station (240 m asl) from December 2022 to February 2023. The greenhouse's temperature and relative humidity (RH) were recorded daily at 07.30 AM, 1.30 PM, and 5.30 PM using the Elitech GSP-6 data logger and calculated according to Handoko (1994). The average greenhouse temperature for 21 days of the experiment was 27.5°C (max. temperature of 34.9°C , min. temperature of 24.0°C), while the average RH was 88.7% (max. RH of 100%, min. RH of 60.3%). The greenhouse experiment was arranged in an RCBD with two factors and three replications. The first factor was five Indonesian local foxtail millet accessions with different alleles at the 558th base of *SiDREB2*, namely ICERI-6 (A/A), Botok-4 (G/G), Mauliru-2 (G/G), NTB-1 (G/G), and Toraja (G/G). The second factor was NaCl concentration, which consisted of 0 and 75 mM. The NaCl concentration of 75 mM was expected to simulate moderate salinity stress. Foxtail millet was reported to have good salinity tolerance in the moderate range (4-8 $\text{dS}\cdot\text{m}^{-1}$) (Nahar *et al.* 2018; Mushtaq *et al.* 2021). Each experimental unit comprises eight polypots (1.7 L) containing a media mixture of soil and sand (1:1, v/v), with one seedling per polypot.

Calcium carbonate (CaCO_3) was applied at a rate of 10 g per polypot one week before planting to

adjust the pH of the growing media to 6.5. Fertilizers of urea, SP-36, and KCl with a rate of 0.33:0.25:0.33 g per polypot were applied three days before planting, while carbofuran (3%) was applied during planting. Initial watering with either 0 or 75 mM NaCl solution was conducted with a volume of 490 ml during planting, and the subsequent treatments were applied every three days with a volume of 260 ml per polypot until 21 days after planting (DAP). The condition of the growing media was monitored every six days following the PourThru method (Cavins *et al.* 2008). The pH of the growing media solution was measured using pH paper (Q/3211821AB001-2002), while the electrical conductivity (EC) was measured using TDS and EC meter EZ-2. Based on USDA (1954), EC in this experiment for 0 mM NaCl was in non-saline condition (1.01-2.44 dS.m⁻¹), and for 75 mM NaCl was moderate (3.81-6.23 dS.m⁻¹). pH observed for 21 days of the experiment was <8.5.

Observations were done for shoot dry weight and total dry weights at 21 DAP to calculate the salt tolerance indices. Observation values under salinity stress (Y_s), observation values under control condition (Y_p), the average observation values under salinity stress (\bar{Y}_s), and the average observation values under control conditions (\bar{Y}_p) were used to calculate the following tolerance indices:

1. Geometric Mean Productivity (GMP) = $(Y_s \times Y_p)^{1/2}$ (Fernandez 1992)
2. Stress Tolerance Index (STI) = $(Y_s \times Y_p) / (\bar{Y}_p)^2$ (Fernandez 1992)
3. Yield Index (YI) = Y_s / \bar{Y}_s (Gavuzzi *et al.* 1997)
4. Harmonic Mean (HM) = $(2 \times (Y_s \times Y_p)) / (Y_s + Y_p)$ (Schneider *et al.* 1997)
5. Tolerance Index (TOL) = $Y_p - Y_s$ (Rosielle and Hamblin 1981)

Plant biomass could be an efficient observed trait in the salt tolerance assessment of genotypes at various growth stages. However, the performance of the genotypes under stress and non-stress conditions might vary. Thus, stress tolerance indices are commonly used to evaluate genotypes' performance in stress conditions relative to non-stress conditions (Mubushar *et al.* 2022). Genotypes with good performance under stress and non-stress conditions can be identified satisfactorily using STI (Fernandez 1992), while genotypes with good performance under non-stress conditions and reasonable performance under stress conditions can be captured by the GMP (Fernandez 1992). The YI could identify genotypes

with good performance under only stress conditions but failed to detect genotypes with good performance under non-stress conditions (Gavuzzi *et al.* 1997). The HM could distinguish the stability of genotypes under stress and non-stress conditions (Schneider *et al.* 1997). Meanwhile, TOL figured genotypes with the least biomass reduction under stress conditions compared to the non-stress conditions (Rosielle and Hamblin 1981).

2.4. Data Analysis

Quantitative data from agro-morphological characterization and salinity tolerance evaluation at the seedling stage were analyzed using the F test, followed by Duncan's Multiple Range Test (DMRT) at $\alpha = 5\%$ using SAS On Demand for Academics (welcome.oda.sas.com). Scatter plots of eight Indonesian local foxtail millet accessions were built using Microsoft Excel based on accession means of yield estimates (Y-axis) and harvesting time (X-axis). The dendrogram of eight Indonesian local foxtail millet accessions was built based on agro-morphological traits (eight qualitative traits and 15 quantitative traits) using the modified Gower dissimilarity and Neighbor-joining methods on PBSTAT-CL (www.pbstat.com). The endosperm type and yield estimate were excluded from traits used to build the dendrogram for the following reasons. There was no variability in the endosperm type among accessions, while the yield estimate was already represented by grain weight per plant. The salinity tolerance of eight foxtail millet local accessions was estimated using *SiDREB2*-based SNAP markers. A 300 bp band was produced by the SD2-558-SNP-A and SD2-558-SNP-rev primer pair, indicating a tolerant genotype. In contrast, A 300 bp band was produced by the SD2-558-SNP-G and SD2-558-SNP-rev primer pair, indicating a sensitive genotype.

3. Results

3.1. Morphological and Agronomical Characterization of Indonesian Local Foxtail Millet (*Setaria italica* L. Beauv) Accessions

Strong anthocyanin coloration of basal leaf sheath was observed in Toraja and ICERI-6 accessions. At the same time, ICERI-5 showed only weak anthocyanin coloration, and the five other accessions showed no anthocyanin coloration of the basal leaf (data not shown). The grain, endosperm, and panicle types of the eight Indonesian local foxtail millet accessions

are shown in Figure 1. Variability was observed in the grain color and grain shape. The grain color varied from red for the Toraja accession, brown for the Botok-10 accession, and yellow for the other six accessions (Figure 1A). Despite the variability in their grain color, the dehusked grain color of all observed accessions was uniformly yellow (data not shown). The grain shape of the ICERI-5 and ICERI-6 accessions was medium ovate, while the other six had circular grain shapes (Figure 1A). All of the observed Indonesian local foxtail millet accessions in this study have non-waxy endosperms, indicated by the dark blue color of the endosperms (Figure 1B). The panicle type of foxtail millet is one of the easiest observable characteristics. There are seven categories for panicle type in foxtail millet, but only four panicle types could be observed in this study. The panicle types observed were conical (Toraja, NTB-1, ICERI-5, and ICERI-6 accessions), cat foot (Mauliru-2 and Hambapraing accessions), cylindrical (Botok-4 accession), and duck mouth (Botok-10) as shown in Figure 1C.

The mean value of plant height, heading time, and several important agronomical traits of the eight foxtail

millet accessions are shown in Table 2. Plant height greatly varied from the shortest of 57.28 cm (Toraja accession) to the tallest of 207 cm (Botok-10 accession). The main panicle weight observed has a variability from 2.45 to 17.03 g. High variability was also observed in heading time, which ranged from 48 DAT (Toraja accession) to 140 DAT (Botok-10 accession). We observed a significant positive correlation between plant height ($r = 0.847^{**}$), main panicle weight (0.699^{**}), heading time ($r = 0.881^{**}$), and harvest time ($r = 0.811^{**}$) with grain weight per plant. Consequently, the Toraja accession showed the lowest grain weight per plant. Potential accessions based on yield estimation and harvest time were visualized using a scatter plot (Figure 2). The Hambapraing and Mauliru-2 were the potential accessions with high yield estimation ($2.33 \text{ ton}\cdot\text{ha}^{-1}$ and $1.93 \text{ ton}\cdot\text{ha}^{-1}$, respectively) and early harvest time ($<110 \text{ DAT}$).

The Neighbor-joining method evaluated genetic distance and similarity between accessions based on eight qualitative traits (excluding endosperm type) and 15 quantitative traits (excluding yield estimate). They were visualized in a phylogenetic tree, as shown in

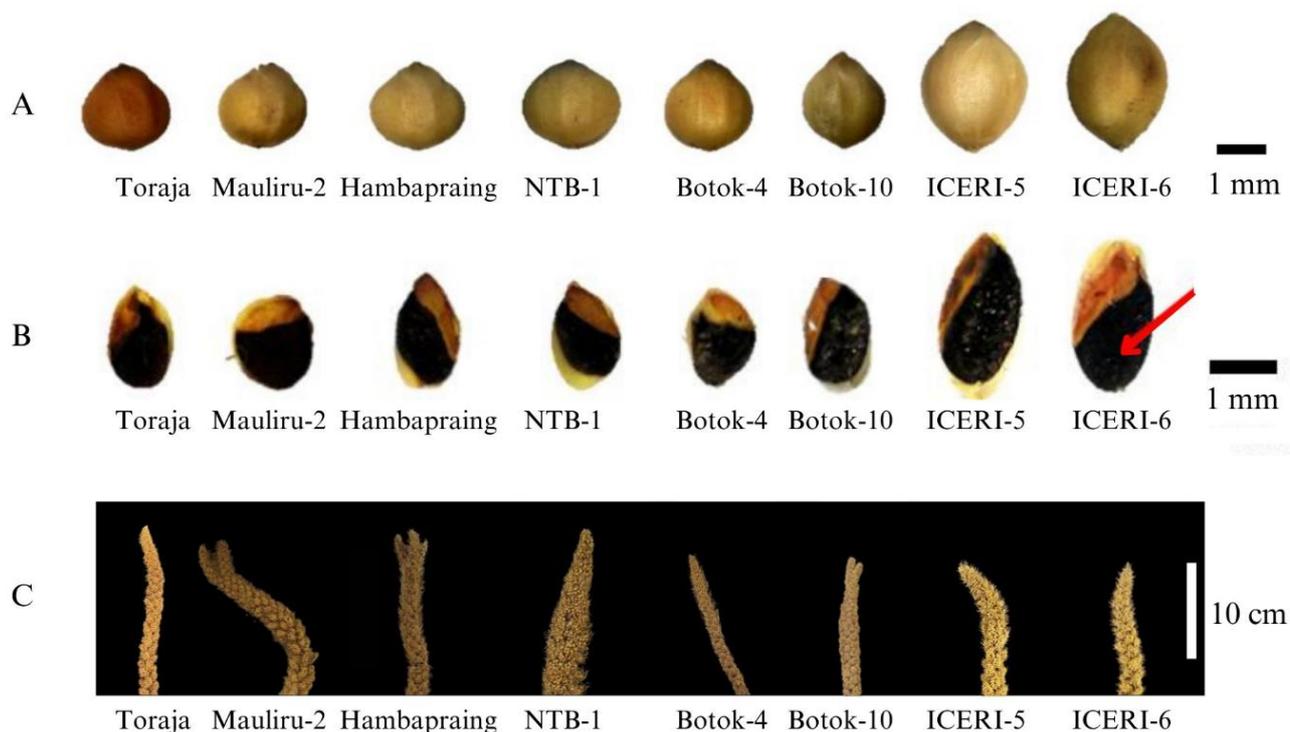


Figure 1. Variability in the grain shape and color (A), endosperm type (B), and panicle type (C) of eight Indonesian local foxtail millet accessions. The red arrow in (B) indicates dark blue coloration in the endosperm following Lugol's solution staining

Table 2. The agronomical mean value of eight Indonesian local foxtail millet accessions

Accessions	PH (cm)	SD (mm)	PH:SD ratio	MPW (g)	GW (g)	HeT (DAT)	HaT (DAT)
Toraja	57.28d	2.45d	24.28d	3.21d	3.64d	48.33e	64.33h
Mauliru-2	164.19b	5.51ab	30.15c	11.27bc	27.89a	78.67c	108.33d
Hambapraing	155.14b	5.00b	31.37bc	8.28c	24.18ab	77.67c	105.33e
NTB-1	166.59b	5.28b	32.26abc	17.03a	21.95ab	83.00b	112.00c
ICERI-5	101.93c	3.29c	32.44abc	3.71d	10.93c	58.33d	74.00g
ICERI-6	93.77c	2.79cd	34.97ab	2.45d	10.04c	55.33d	71.00f
Botok-4	166.04b	4.89b	34.66abc	10.41bc	19.41b	79.67bc	113.33b
Botok-10	207.00a	5.97a	36.28a	13.96ab	23.99ab	87.33a	139.67a
F-test	**	**	**	**	**	**	**
CV (%)	6.41	8.52	7.46	28.29	18.17	3.20	0.69

Numbers followed by the same letter in the same column differ based on DMRT at α : 5%

**significant at α : 1%. PH: plant height, SD: stem diameter, PH:SD ratio: plant height: stem diameter ratio, MPW: main panicle weight, GW: grain weight per plant, HeT: heading time, and HaT: harvest time, CV: coefficient of variation

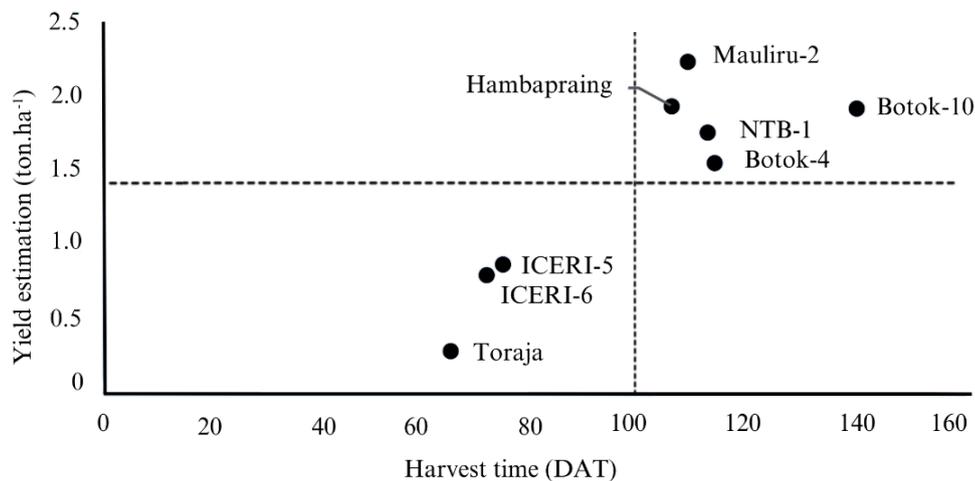


Figure 2. Scatter plot of eight Indonesian local foxtail millet accessions based on yield estimation and harvest time

Figure 3. The phylogenetic tree indicates that ICERI-6, ICERI-5, and Toraja accessions were closely related. Mauliru-2 accession was closely related to Hambapraing, while Botok-4, NTB-1, and Botok-10 were closely related.

3.2. Estimation of Salinity Tolerance using *SiDREB2*-based SNAP Marker

The salinity tolerance level of the eight Indonesian foxtail millet local accessions was estimated using *SiDREB2*-based SNAP markers, as shown in Figure 4. The 300 bp bands amplified by the SD2-558-SNP-G primer indicate sensitive genotypes, while bands amplified by the SD2-558-SNP-A primer indicate tolerant genotypes. ICERI-5 and ICERI-6 accessions were predicted to be tolerant to salinity stress, while Toraja, Mauliru-2, Hambapraing, NTB-1, Botok-4, and Botok-10 were predicted to be sensitive to salinity stress.

3.3. Salinity Tolerance Evaluation at Seedling Stage

To confirm the predicted salinity tolerance level by the *SiDREB2*-based SNAP marker, the salinity tolerance of five accessions from different clusters in the phylogenetic tree was evaluated at the seedling stage by subjecting them to either 0 or 75 mM NaCl (Figure 3). This experiment did not evaluate three accessions (Botok-10, ICERI-5, and Hambapraing), considering their close relatedness with Botok-4, ICERI-6, and Mauliru-2, respectively. Botok-10 and Botok-4 originated from the same area in East Nusa Tenggara. ICERI-5 is closely related to ICERI-6, while Hambapraing is closely related to Mauliru-2 since they were clustered in the same group based on the agro-morphological traits (Figure 3). The 75 mM NaCl concentration showed the EC range at 3.81-6.23 dS.m⁻¹ and greatly inhibited

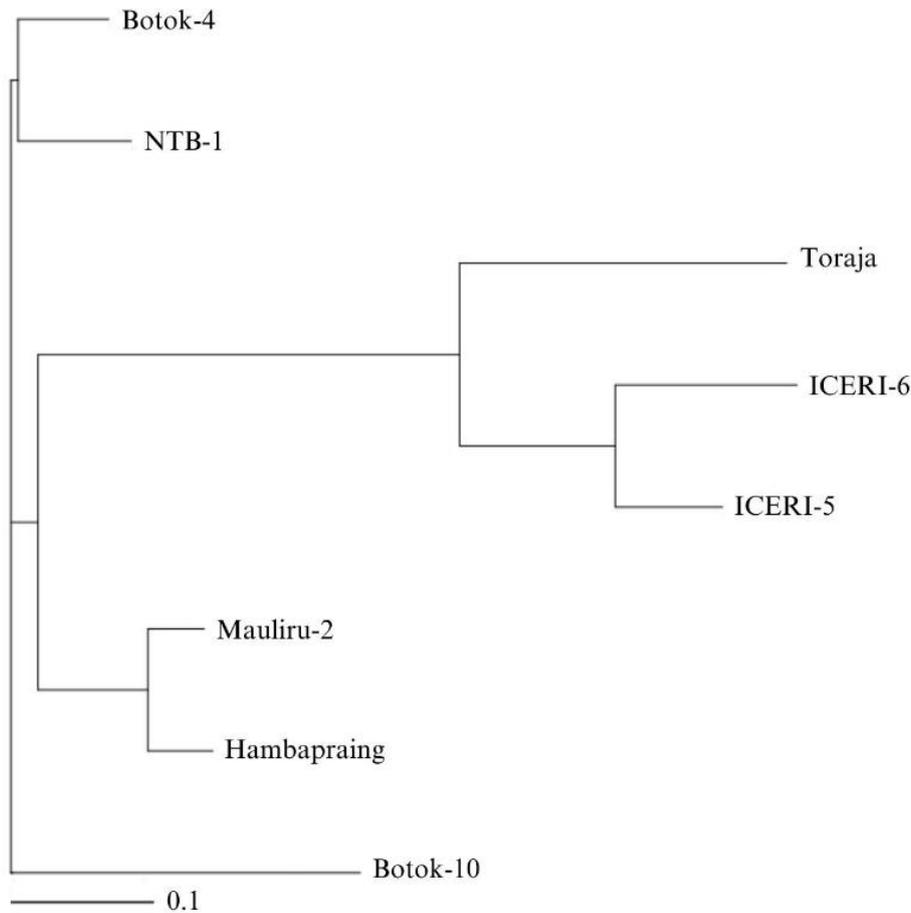


Figure 3. Dendrogram of eight Indonesian local foxtail millet accessions based on agro-morphological traits using the Neighbor-joining method

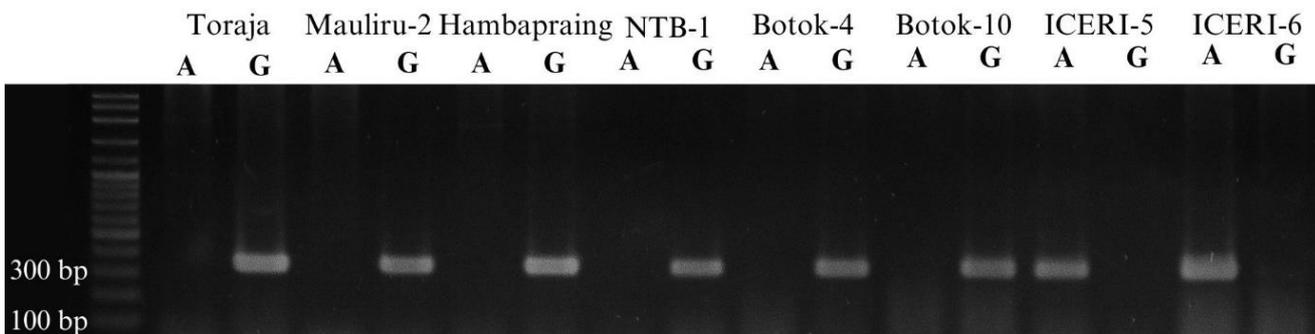


Figure 4. Gel electrophoresis result of eight Indonesian local foxtail millet accessions using the *SiDREB2*-based SNAP marker

seedlings' growth at 21 DAP of all five foxtail millet accessions evaluated, as shown in Figure 5. ICERI-6 accession showed the tallest seedlings (10.65 cm), while Toraja showed the shortest (2.50 cm) under salinity stress conditions. Moreover, the salinity of 75 mM NaCl in this study caused a total dry weight reduction of

18.22-39.77% (Figure 5B). The STI, GMP, YI, HM, and TOL indices were calculated based on the shoot- and total dry weight (Table 3). ICERI-6 accession showed significantly higher GMP, STI, YI, and HM values than the other four accessions, indicating its tolerance to salinity stress.

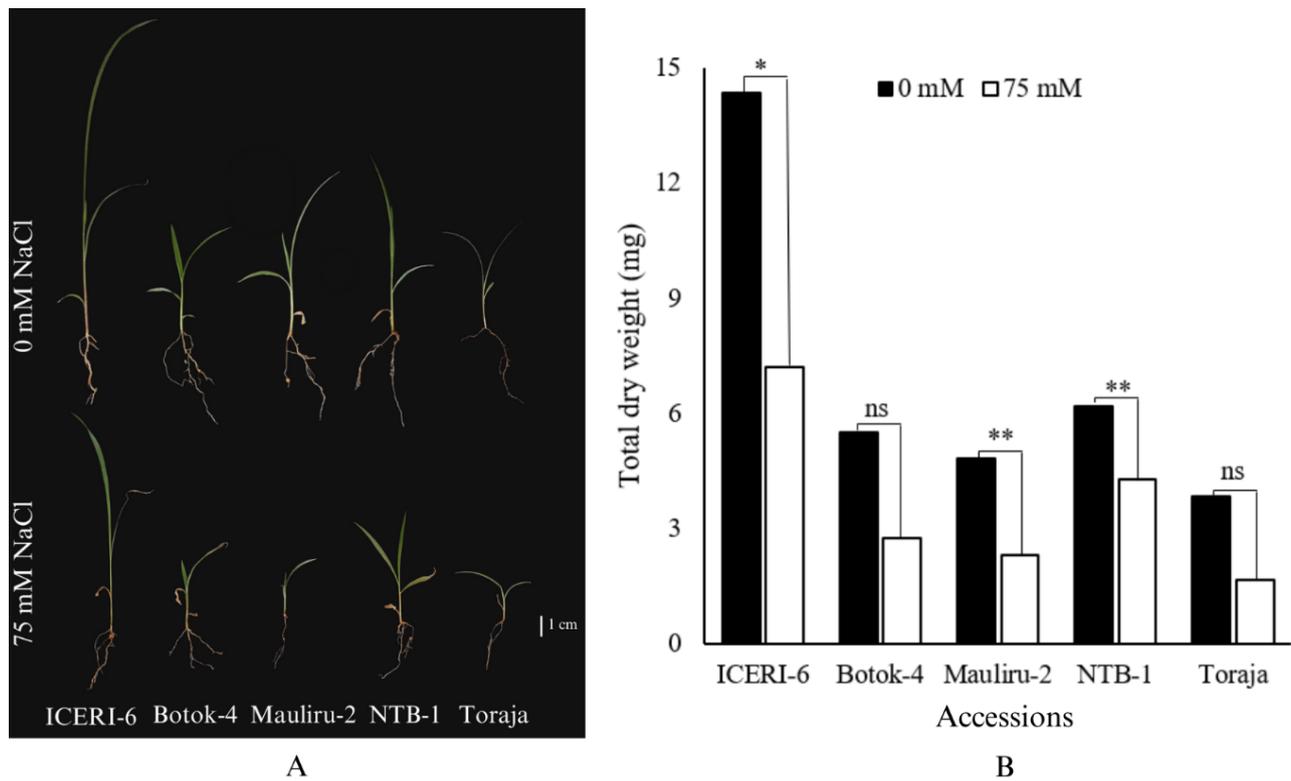


Figure 5. (A) Plant performance at seedling stage (21DAP) under control and salinity condition, and (B) total dry weight for five accessions under control and salinity condition. TDW: total dry weight, **: significant at α :1%, *: significant at α : 5%, ns: not significant

Table 3. Stress tolerance indices based on the shoot- and total-dry weight of five Indonesian local foxtail millet accessions

Accessions	Shoot dry weight						
	Yp	Ys	TOL [#]	GMP	STI [#]	YI	HM
ICERI-6	10.77a	5.10a	5.70a	7.43a	2.42a	1.99a	6.87a
Botok-4	3.73b	2.10bc	1.60b	2.67bc	0.32b	0.82bc	2.43bc
Mauliru-2	3.33b	1.63bc	1.73b	2.33bc	0.23b	0.63bc	2.17bc
NTB-1	4.13b	2.80b	1.33b	3.37b	0.49b	1.97b	3.33b
Toraja	2.27b	1.20c	1.10b	1.60c	0.12b	0.46c	1.50c
F-test	**	**	**	**	**	**	**
CV (%)	24.78	27.99	0.11	20.61	12.97	28.18	21.88
Accessions	Total dry weight						
	Yp	Ys	TOL [#]	GMP	STI [#]	YI	HM
ICERI-6	14.33a	7.23a	7.17a	10.13a	2.22a	1.98a	9.53a
Botok-4	5.53b	2.77bc	2.77b	3.80bc	0.30b	0.76bc	3.43bc
Mauliru-2	4.80b	2.30c	2.50b	3.33bc	0.24b	0.63c	3.13bc
NTB-1	6.20b	4.30b	1.93b	5.10b	0.55b	1.17b	5.03b
Toraja	3.80b	1.63c	2.17b	2.50c	0.13b	0.45c	2.27c
F-test	**	**	*	**	**	**	**
CV (%)	23.58	25.69	0.17	20.08	12.88	25.23	21.41

Numbers followed by the same letter in the same column are not different based on DMRT at α : 5%, **: significant at α :1%, *: significant at α : 5%, ns: not significant, #: data was transformed using $(x + 0.5)^{1/2}$, Yp: dry weight (mg) under non-stress condition, Ys: dry weight (mg) under stress condition, TOL: tolerance index, GMP: geometric mean productivity, STI: stress tolerance index, YI: yield index, HM: harmonic mean, CV: coefficient of variance

4. Discussion

Domestication, modern agricultural practices, and conventional farming, which led to genetic diversity loss and yield reduction under stressful environments, have increased the importance of cereal landraces as a genetic resource for breeding (Marone *et al.* 2022). Our study provides germplasm variability and early evaluation of salinity tolerance information of Indonesian local foxtail millet accessions, which will be useful for breeding superior varieties of foxtail millet in the country.

Moderate plant height, early heading time, and high productivity are some targeted traits in the foxtail millet breeding program (Sintia *et al.* 2023). Short-statured plants are more advantageous since taller plants might be prone to lodging (Shah *et al.* 2019), while early heading time is preferred so foxtail millet can be cultivated as a catch crop. Studies of 27 (Yadav *et al.* 2018) and 41 (Ghimire *et al.* 2018) Nepalese foxtail millet accessions showed a narrower range of plant height than the eight Indonesian local accessions in this study. The eight Indonesian foxtail millet local accessions in this study also showed a wider range of heading times. The earliest heading time reported in the evaluation of 27 Nepalese foxtail millet landraces was 78 days (Yadav *et al.* 2018), while the range of heading time observed by Ghimire *et al.* (2018) in their Nepalese foxtail millet accessions was 45-71 days. Compared to this study, a similar productivity range (1.5-2.5 ton.ha⁻¹) was reported in 17 foxtail millet varieties released in India during 1989-2012 (ICAR 2012). However, Kandel *et al.* (2020) observed a narrower productivity range (1.5-2.5 ton.ha⁻¹) compared to our study in 30 Nepalese foxtail millet germplasm. These results indicate the eight Indonesian local accessions highly varied in the three breeding-targeted traits.

In addition to the breeding-targeted traits, a wide range of agro-morphological traits variability among local accessions is pivotal in a successful breeding program. The eight Indonesian local foxtail millet accessions revealed high variability in qualitative and quantitative agro-morphological traits. Azam *et al.* (2023) mentioned that accession clustering based on quantitative and qualitative trait similarity helps in the effective selection of parents in plant breeding. All foxtail millet accessions studied showed non-waxy endosperm (Figure 1B);

thus, this trait was excluded from the phylogenetic analysis. Firdaus *et al.* (2020) reported that among 23 Indonesian foxtail millet accessions evaluated, only two showed waxy endosperm, namely ICERI-2 and Padang accessions. In this study, Indonesian local foxtail millet accessions were predicted to be derived from the same clan (monophyletic), meaning they were all derived from the same ancestor (Mishler 2021). The ICERI-6 has a longer clade size, indicating that these accessions appear earlier than other local accessions (Fitmawati and Hartana 2010). Three accessions, namely Toraja, ICERI-5, and ICERI-6, showed similarities in many traits; thus, they were grouped in one cluster (Figure 3). These three accessions were also located in the same quadrant of the scatter plot (Figure 2), indicating their high genetic similarity. Toraja accession was slightly distant from ICERI-5 and ICERI-6 in the phylogenetic tree (Figure 3) due to its different grain shape (Figure 1). These three accessions also shared similar important agronomical features, such as short-statured (57.28-101.93 cm) and early heading time (48-58 DAT), but with low grain weight per plant (3.64-10.93 g) (Table 2). In contrast, the other five accessions showed taller plants (155.14-207.00 cm) and longer heading time (77-87 DAT) but with higher grain weight per plant (19.41-27.89 g).

The high variability of Indonesian local foxtail millet accessions observed in this study would serve as important genetic resources for further plant improvement and to discover novel QTLs/genes/alleles related to the targeted traits. For example, Gao *et al.* (2022) utilized foxtail millet varieties contrasting in their plant height to identify a total of three QTLs and nine candidate genes related to plant height by bulk segregation analysis sequencing (BSA-seq) and RNA sequencing (RNA-seq). Utilization of contrasting genotypes in the identification of QTLs/genes/alleles related to a particular trait can also be performed by developing recombinant inbred lines (RIL), as reported by Zhang *et al.* (2017) who successfully identified six QTLs controlling plant height in foxtail millet. A RIL population developed from foxtail millet varieties with contrasting characteristics, namely Longgu 7 (shorter growth duration, lower plant height, lower biomass, and grain yield per plant) and Yugu1 (longer growth duration, higher plant height, higher biomass, and grain yield per plant), was also utilized to identify QTLs regulating panicle weight on chromosomes 2,

3, 5, 6, 7, and 8 (Liu *et al.* 2020). The F2 population derived from the foxtail millet varieties, Longgu 7 and Yugu1, was used to identify QTLs controlling panicle weight and grain weight per plant in chromosome 1 (Fang *et al.* 2016). An F2 population of foxtail millet generated from different parents, Aininghuang and Jingu 21, was used to identify QTLs controlling grain weight assisted by restriction site-associated DNA sequencing (RAD-seq) (Wang *et al.* 2019). These studies require genetic variability in the targeted traits to discover QTLs/genes/alleles related to the targeted traits. Similarly, the high variability observed in our study's eight Indonesian local foxtail millet accessions can be utilized further to discover beneficial QTL/genes/alleles that could be developed into applicable molecular markers.

Developing trait-related molecular markers is advantageous to accelerate breeding programs and increase selection accuracy, especially for traits that are difficult to assess (Hasan *et al.* 2021). Field evaluation of salinity tolerance is challenging due to salinity level variability (Dadshani *et al.* 2019) and interactions with other environmental factors, including soil and climatic conditions (Trusca *et al.* 2023). Moreover, salinity tolerance assessment requires replications for liable results; thus, it is impossible to perform a phenotypic selection for salinity tolerance in the early generation of a crossing population (Khan *et al.* 2016). Developing the *SiDREB2*-based SNAP marker was an example of how molecular markers will assist a breeding program. The identification of a synonymous SNP (an A/G transition) at the 558th base pair in the *SiDREB2* gene and its association with dehydration tolerance was possible due to the availability of foxtail millet accessions differing in their tolerance to drought (Lata *et al.* 2011). The further development of *SiDREB2*-based SNAP utilized four foxtail millet accessions with contrast tolerance to drought and salinity stress (Ardie *et al.* 2015; Widyawan *et al.* 2018). Widyawan *et al.* (2018) reported the application of dot-blot SNP based on the allele variation at the 558th position in the *SiDREB2* gene on 26 foxtail millet accessions, including ICERI-5, ICERI-6, Botok-4, and Botok-10.

In our study, the estimated salinity tolerance of the Indonesian local foxtail millet accessions using the *SiDREB2*-based SNAP marker (Figure 4) agreed with the tolerance level at the seedling stage based on stress tolerance indices (Table 3). The salinity

tolerance level of ICERI-5, ICERI-6, Botok-4, and Botok-10 accessions evaluated in this study also showed consistent results with Widyawan *et al.* (2018). Salinity induced-plant height reduction in this study ranged from 16.16-34.05%, while Akter *et al.* (2020) reported a salinity level of 60 mM NaCl caused 12.91-15.12% plant height reduction in the seedlings of Indian foxtail millet accession at 60 mM. Total dry weight reduction in Indian foxtail millet accession caused by a salinity level of 6 dS.m⁻¹ was in the range of 6.74-22.66% (Nahar *et al.* 2020), indicating that the salinity tolerance of foxtail millet greatly varied between accessions. Salinity tolerance evaluation at the seedling stage showed that ICERI-6 accession was the only tolerant genotype based on GMP, STI, YI, and HM tolerance indices (Table 3). Mubushar *et al.* (2022) reported that GMP, STI, YI, and HM were useful indices to identify the salt-tolerant genotypes in spring wheat. Nahar *et al.* (2008) utilized STI to select salt-tolerant genotypes of Indian foxtail millets under moderate- and high-salinity stresses. The STI was also used to identify salt-tolerant genotypes in other crop plants, such as wheat (Tao *et al.* 2021) and pearl millet (Jha *et al.* 2021). Lapuimakuni *et al.* (2018) showed YI and HM as the best indices in determining the drought-tolerance level of foxtail millet in field conditions. Utilization of tolerance indices is useful to evaluate genotypes' performances under non-stress and stress conditions (Afrooz *et al.* 2021). Combining molecular markers related to stress tolerance traits and tolerance indices might increase the tolerance estimation.

Together, germplasm diversity and early evaluation of salinity tolerance information of Indonesian local foxtail millet accessions presented in this study will serve as essential information for further breeding programs of foxtail millet in Indonesia. This diversity can be further employed to exploit QTL/genes/alleles related to traits of interest, which could be developed into beneficial molecular markers. These results indicate that the *SiDREB2*-based SNAP marker is a beneficial tool in identifying salinity tolerance genotypes in foxtail millet and the breeding acceleration through marker-assisted selection (MAS). Foxtail millet accessions bearing the most desirable traits, such as Mauliru-2, are potentially improved to be a foxtail millet superior variety.

Conflict of Interest

The authors declare no conflict of interest.

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