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Digital phenotyping for robust seeds variability assessment in *Setaria italica* (L.) P. Beauv.

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ABSTRACT: Foxtail millet (*Setaria italica* L.) is a cereal crop with potential as a functional food due to its high nutritional value and its wide adaptability to unfavorable environmental conditions. Seed-related traits of foxtail millet are difficult to observe due to its small size. Therefore, the development of an efficient and accurate method for characterizing foxtail millet seeds using digital imaging technology is essential. This study aimed to characterize the seed morphology for variability assessment of Indonesian local foxtail millet genotypes and to develop a model to estimate the 100-seed weight using ImageJ. A total of 28 Indonesian local foxtail millet genotypes were used in this study for seed morphology characterization and the development of the 100-seed weight estimation model. Foxtail millet genotypes from different regions in Indonesia exhibited diverse seed morphologies. The 100-seed weight estimation model ($y = 0.123x - 0.0821$ ($R^2 = 0.9223$)) demonstrated a highly significant positive correlation ($r = 0.96$, $p < 0.01$) between the predicted and actual 100-seed weights. The correlation coefficient from model validation was 0.8731 ($p < 0.01$), indicating that the obtained model could estimate the 100-seed weight of foxtail millet seeds in future studies.

Index terms: 100-seed weight, foxtail millet, ImageJ, local genotype, model, seed morphology.

RESUMO: O painço (*Setaria italica* L.) é um cereal com potencial como alimento funcional devido ao seu alto valor nutricional e à sua ampla adaptabilidade a condições ambientais desfavoráveis. As características relacionadas às sementes dessa espécie são difíceis de observar devido ao seu pequeno tamanho. Portanto, o desenvolvimento de um método eficiente e preciso para caracterizar sementes de painço utilizando tecnologia de imagem digital é essencial. Este estudo teve como objetivo caracterizar a morfologia das sementes para avaliação da variabilidade de genótipos de painço locais da Indonésia e desenvolver um modelo para estimar o peso de 100 sementes usando o *software* ImageJ. Um total de 28 genótipos de painço locais da Indonésia foram utilizados neste estudo para caracterização da morfologia das sementes e o desenvolvimento do modelo de estimativa de peso de 100 sementes. As sementes dos diferentes genótipos de painço da Indonésia exibiram diversos caracteres morfológicos distintos. O modelo de estimativa de peso de 100 sementes ($y = 0,123x - 0,0821$ ($R^2 = 0,9223$)) demonstrou uma correlação positiva altamente significativa ($r = 0,96$, $p < 0,01$) entre os pesos previstos e reais de 100 sementes. O coeficiente de correlação da validação do modelo foi de 0,8731 ($p < 0,01$), indicando que o modelo obtido poderia estimar o peso de 100 sementes de painço em estudos futuros.

Termos para indexação: peso de 100 sementes, painço, ImageJ, genótipo local, modelo, morfologia de sementes.

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INTRODUCTION

Foxtail millet (*Setaria italica* L.) is a cereal crop with potential as a functional food due to its high nutritional value. It has been recommended to be consumed by people with type II diabetes due to its low glycaemic index (Ren et al., 2022). The carbohydrate content of foxtail millet is comparable to that of rice, with higher protein, fiber, and minerals (Ca and Fe) content compared to rice (Verma et al., 2020). The health benefits of foxtail millet include hypertension prevention (Hou et al., 2018), cardiovascular risk factors reduction (Jali et al., 2012), and cancer inhibition (Shan et al., 2014; Zhang and Liu, 2015). Moreover, foxtail millet possesses wide adaptability to unfavorable environmental conditions, such as drought (Wang et al., 2023), salinity (Pan et al., 2020), and limited nitrogen and phosphate availability (Nadeem et al., 2020).

Despite the emerging potential of foxtail millet, this species is considered an underutilized crop in Indonesia. The number of cultivated foxtail millet genotypes is poorly documented in the country. Based on the genetic materials studied in separate reports, some local genotypes of foxtail millet are being collected in the Indonesian Cereals Research Institute (ICERI) or being cultivated locally in West Sumatra, Belitung Island, West Sulawesi, East Nusa Tenggara, West Nusa Tenggara, and Maluku (Ardie et al., 2015; Widyawan et al., 2018; Ramlah et al., 2020). Those studies implied the rich biodiversity potential of local Indonesian foxtail millet genotypes. Characterization of these local genotypes is essential to identify particular genotypes with traits of interest. One of the most important, yet difficult to characterize, is the seed-related traits.

Six out of 29 characters listed in the UPOV (2013) descriptor for foxtail millet characterization are related to seed (grain), namely 100-grain weight, grain shape, grain color, number of grains on the primary branch, the color of dehusked grain, and endosperm type. The characterization of seed-related traits in foxtail millet is challenging due to its tiny seed. The seed size of foxtail millet varies depending on genotypes, ranging from 1-2 mm in length and 1-1.5 mm in width (Sunil et al., 2016; Ramlah et al., 2020). Observation of seed-related traits is indispensable since it not only contributes to biodiversity assessment (Jackson et al., 2010) but is also important in trait selection for yield improvement (Zhang et al., 2022).

Manual phenotyping of small seeds requires high accuracy, tends to be subjective, and is time-consuming (Zhang et al., 2018). Therefore, it is necessary to develop an efficient and accurate phenotyping method to observe seed-related traits in foxtail millet by utilizing digital imaging technology. The advancement of digital imaging technology has been extensively used for the precise and efficient evaluation of phenotypic traits in plants (Omari et al., 2020). Analyzing images captured under controlled conditions can greatly assist in accurate characterization, including the characterization of seed traits (Kapadia et al., 2017; Hemender et al., 2018). The utilization of digital imaging in seed characterization has been documented for various crops, including tomato (Borges et al., 2019), soybean (Franca-Silva et al., 2023), rice (Santos et al., 2019), and melon (Medeiros et al., 2020). Several software programs have been reported to be useful for seed characterization, such as ImageTool (Behtari et al., 2014), SmartGrain (Tanabata et al., 2012), PhenoSeeder (Jahnke et al., 2016), and ImageJ (Severini et al., 2011). Among the mentioned software programs, ImageJ is considered among the most user-friendly since it does not require sophisticated instruments and is free (Cervantes et al., 2016). Yet it still offers a wide range of built-in features for image processing, including logical and arithmetic operations between images, contour detection, and mathematical morphology (Gonzalez and Woods, 2013). ImageJ facilitates the conversion of qualitative image data into quantitative measurements in terms of numbers and shapes (Schneider et al., 2012).

The utilization of digital image technology is expected to accelerate the characterization of foxtail millet seeds for biodiversity assessment and for developing high-yielding foxtail millet varieties through a breeding program. The objectives of this study were to characterize the seed morphology for variability assessment of Indonesian local foxtail millet genotypes and to develop a model to estimate the weight of 100 seeds using digital image technology and ImageJ v1.53 software.

MATERIAL AND METHODS

Plant material

Two consecutive experiments were conducted using different genetic materials. The first experiment aimed to assess the biodiversity of Indonesian local foxtail millet genotypes based on seed characters and to establish a model for seed weight estimation using ImageJ v1.53 software, while the objective of the second experiment was to validate the model obtained in the first experiment. The first experiment utilized seeds from 28 foxtail millet genotypes as a training set that can be categorized into three groups based on their origin, namely Botok, ICERI, and local groups (Figure 1). Botok group originated from East Nusa Tenggara and consists of nine genotypes. ICERI group originated from the collection of the Indonesian Cereals Research Institute and consists of eight genotypes. The remaining 11 genotypes were categorized as local groups as they originated from various areas in Indonesia, including West Sumatera (Padang), South Sulawesi (Toraja), West Nusa Tenggara (Bima and NTB-1), East Nusa Tenggara (Labapu-2, Mauliru-2, Sanc Loe Nagekeo, and Wete Nagekeo), East Sumba (Hambapraing), Belitung Island (Belitung), and Maluku (Buru). The prediction model developed in the first experiment was validated using F_3 foxtail millet families derived from Botok-4 x ICERI-6 cross (30 F_3 families) and from ICERI-6 x Botok-4 (30 F_3 families) cross as the validation set.

Procedures

Image acquisition for data creation

The image was taken in a Mini LED Studio measuring 30 cm x 40 cm x 30 cm (w x l x h) equipped with 1 LED light (brightness 6000 K, 40 cm strip) and a paper background (21.0 cm x 29.7 cm). Seed impurities, such as unfilled grains, seed coats, and gravel, were removed manually prior to image acquisition. Seeds were taken using a 0.5 mL measuring spoon (approximately 100-200 seeds, depending on the size of the seed for a particular genotype), and were spread on a paper background in the Mini LED Studio from a distance of 10 cm. Seeds were arranged so no overlapping objects were in the

Figure 1. Seed appearance of 28 foxtail millet genotypes used in the first experiment (bar = 1 mm).

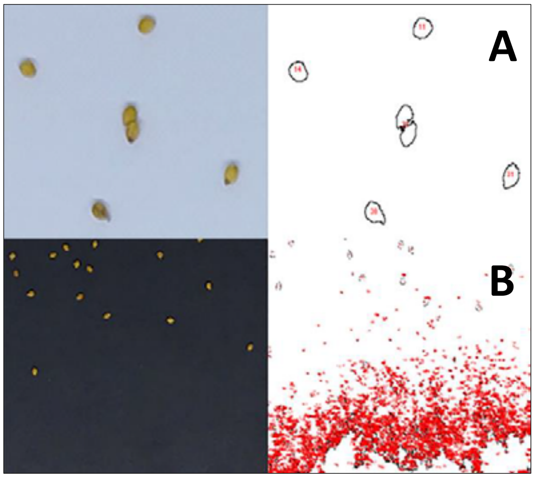

Figure 2. Optimization in the image acquisition for digital phenotyping of foxtail millet seeds. A. Overlapping seeds caused analysis error as the overlapping objects were detected as one object in the particle analysis, B. Noise – showed by red dots – caused by the light reflection on the porous textured black background.

image-capturing area, as overlapping objects could cause analysis errors (Figure 2A). A ruler was placed next to the seeds as a measuring scale. The digital image of the seeds was captured using a cellular phone camera (64 MP) with a distance of 30 cm from the camera to the object, a fixed shooting angle, and the proper lens focus. Each genotype was photographed with ten replicates, and no recovery was performed on seed collection. A total of 280 images in the training set and 600 images in the validation set were produced and were further analyzed using ImageJ v1.53.

During optimization, different color of the paper background was used depending on the seed color. Light-colored seeds (yellow to brown) were captured on a black background, while dark-colored seeds (dark brown to black) were captured on white background. However, our optimization in the image acquisition showed that a white background could also be used for dark-colored seeds by adjusting the light and threshold values. Moreover, the black background used in our study caused light reflection due to its porous texture and led to the miss-detection of the reflection as objects by the software as shown by red dots in Figure 2B. Thus, a white background was used instead of a black background during image acquisition.

Image processing in ImageJ

Image processing in ImageJ v1.53 was conducted following the software manual. In brief, the steps include size calibration, image area selection, and binary image generation. The size calibration was performed by drawing a straight line on the ruler in the image, measuring 1 cm long. Subsequently, in the available distance column listed in the scale section, the known distance was changed to '1' and the unit was adjusted to 'cm'. This allowed the software to calibrate any distance in the image according to the scale on the captured ruler. Image area selection was performed by applying the rectangle area selection tool to select the closest area to the seed, and cropping out the outer areas by duplicating the image. Generation of binary image started by converting the image type from 8-bit RGB (red-green-blue color model) to 8-bit and changing the color type to grayscale. Severini et al. (2011) stated that image conversion from 8-bit RGB to grayscale is necessary to facilitate the distinction between objects and the background. The image was further transformed into a binary image using a threshold. The threshold value was set according to the analyzed image to separate the background from the object (Mussadiq et al., 2015). The threshold value was manually determined as in the study conducted by Kimura et al. (1999), and the threshold values used in this study ranged from 0 to 255. During the analysis, a minimum size was set to "0.009-Infinity" based on the best detection results in this study. Mussadiq et al. (2015) mentioned that ImageJ requires a minimum size to accurately identify objects and their sizes while removing noise from the background. The output of the analyzed quantitative data is then presented in ImageJ's "Analyze

Particles” feature, including seed numbers and seed morphological characters, such as seed areas, perimeter, width, height, circularity, aspect ratio, round, and solidity (Table 1).

Experimental data generation and data analysis

The seed morphological data generated by ImageJ v1.53 from the captured images was analyzed to evaluate the genetic variability among foxtail millet genotypes. An analysis of variance was conducted to examine the variation in seed morphological traits among genotypes followed by a Tukey’s post hoc test to determine the differences between genotypes. A correlation analysis was conducted to assess the strength of the relationships among the morphological traits. Cluster analysis was performed using the Neighbor-joining method based on Euclidean distances to examine the grouping of the genotypes based on seed traits studied, utilizing the PBSTAT-CL application (www.pbstat.com).

In order to develop the seed weight estimation model, two types of data were generated in the first experiment namely software-generated data and data generated from manual observation. Seeds were counted and weighed manually to develop the prediction model together with the software-generated data in the training set (28 foxtail millet genotypes). The developed model was then validated in the validation set (60 F_3 foxtail millet families). A linear regression analysis was performed using the weight of 100 seeds obtained through manual calculations (y) and the seed area obtained from digital image analysis (x) to develop a model to estimate the weight of 100 seeds based on seed size generated by ImageJ. Manual calculation of 100 seed weight was performed on the same seeds used for image acquisition. Seeds that were taken using a 0.5 mL measuring spoon were manually counted after the removal of seed impurities (approximately 100-200 seeds, depending on the size of the seed for a particular genotype) and subsequently weighed using a digital analytical scale with a 0.001 g accuracy. The weight of the seeds was then divided by the number of seeds and multiplied by 100 to obtain the 100 seed weight. Microsoft Excel 2019 and SAS® OnDemand for Academics were the software tools used for this analysis.

Table 1. Definition of the measured variables by ImageJ for morphological characterization of foxtail millet seeds.

Variable	Definition
Area	Seed area
Perimeter	The length of the outside boundary of the selection
Width	Seed width (Minor axis)
Height	Seed length (Major axis)
Circularity	The ratio of area to a measured perimeter as: Cir. = $4\pi \times \frac{Area}{Perimeter^2}$ A value of 1.0 showed a perfect circle Value approaches 0.0: increasingly elongated polygon
Aspect ratio (AR)	The proportional relationship between its height and its width = $\frac{Height}{Width}$
Round	Seed roundness rate = $\frac{4 \, x \, Area}{\pi \, x \, Height^2}$ A value of 1.0 showed a perfect circle Value approaches 0.0: more oval shape
Solidity	The texture of the seed surface $\frac{Area}{Convex\ area}$

Reference: www.imagej.nih.gov/ij

RESULTS AND DISCUSSION

Digital phenotyping requires some considerations to ensure data accuracy, including high image quality during image acquisition. Hartig (2013) highlighted the significance of image quality in image processing and analysis using ImageJ. In our study, high-quality images were produced by controlling factors during image acquisition including the consistency in camera distance, focus, exposure time, uniform lighting, and suitable background properties. The arrangement of objects in the image-capturing area is also an important factor in ensuring data accuracy. We have removed impurities from the seed lot prior to image acquisition and arranged the seeds in the image-capturing area since attached or overlapping objects could decrease data accuracy as reported by Komyshev et al. (2017). Potential noise can be minimized by using a proper background. Wang et al. (2022) reported that a textured background leads to a decrease in accuracy since the software might falsely detect the background texture as objects. We used a white background rather than a black background in our study since the material of the black background was textured and led to false-object detection. We have conducted all necessary technical considerations to ensure data accuracy for further seed morphological characterization.

The seed morphological characteristics generated by ImageJ in this study can be categorized into seed size-related characters and seed shape-related characters. Seed size-related characters include seed width, height, area, and perimeter, while seed shape-related characters include aspect ratio (AR), roundness, circularity, and solidity. Seed size-related characters showed a significant positive correlation among each other (Table 2) and higher values indicate larger seed size. Seed shape-related characters can be further divided into two-dimensional shape characters, consisting of AR, circularity, and roundness; and a three-dimensional shape character consisting of solidity. The AR is the ratio of seed width to seed height, where a higher AR value indicates seeds tend to be more ovate and an AR value of 1.0 indicates a perfect circle. Circularity is the ratio of area to a measured perimeter, while seed roundness is the ratio of area to a measured width. A value of 1.0 for circularity and roundness showed a perfect circle. Seed roundness showed a significant positive correlation with circularity (r = 0.78**), and a negative correlation with the AR (r = -0.53*) as shown in Table 2. A significant negative correlation between AR and seed roundness was also reported by Kim et al. (2022) for soybean seeds, thus explaining the seed shape. The solidity or surface texture of the seed is the ratio of the seed area to the convex area of the seed (Schneider et al., 2012) which indicates the level of seed density (Perez and Pascau, 2013). All seven seed morphological characters generated by ImageJ were further utilized to assess the variability of Indonesian local foxtail millet genotypes.

Our results showed that the seed morphological characteristics of the 28 foxtail millet genotypes are varied, with seed area being the most diverse character (CV 20.08%) and solidity being the least diverse (CV = 1.44%) (Table 3). Kassout et

Table 2. Correlation among foxtail millet seed morphological characteristics generated by ImageJ.

Seed characteristics	Area	Weight[1]	Perimeter	Width	Height	Circularity	Aspect ratio	Roundness
Weight[1]	**0.96****							
Perimeter	0.93**	0.90**						
Width	0.94**	0.89**	0.92**					
Height	0.97**	0.94**	0.97**	0.91**				
Circularity	-0.20^{ns}	-0.20^{ns}	-0.52*	-0.29^{ns}	-0.37^{ns}			
Aspect ratio	-0.22^{ns}	-0.27^{ns}	-0.28^{ns}	-0.07^{ns}	0.34^{ns}	-0.22^{ns}		
Round	-0.16^{ns}	0.17^{ns}	0.39*	-0.17^{ns}	-0.39*	0.78**	-0.53*	
Solidity	0.22^{ns}	0.19^{ns}	-0.13^{ns}	0.09^{ns}	0.05^{ns}	0.90**	-0.08^{ns}	0.65**

[1]100-seed weight based on manual measurement; **: Significant at $p < 0.01$, *: Significant at $p < 0.05$, ns: not significant.

al. (2022) also reported the seed area as the most varied character compared to other seed morphological characters generated by ImageJ in *Ceratonia siliqua* L. The size of the 28 foxtail millet genotypes varied as indicated by their width, height, and area which ranged from 1.41 to 1.99 mm, 1.47 to 2.15 mm, and 1.51 to 2.98 mm^2, respectively. The ICERI-1 genotype had the largest seed, while the ICERI-10 genotype had the smallest seed. The seed size of the ICERI-1 genotype was potentially larger than the superior foxtail millet variety ‘Yugu1’ in China which has an average seed width of 1.63 mm and seed height of 1.76 mm as reported by Xiang et al. (2017). Ramlah et al. (2020) reported the range of seed width (1-1.5 mm) and seed height (1.5-2 mm) of six local foxtail millet genotypes from West Sulawesi. The only genotype originating from Sulawesi in this study, Toraja, had an average seed width of 1.48 mm and a seed height of 1.53 mm.

Table 3. The seed morphological characteristics of 28 foxtail millet genotypes generated by ImageJ.

Foxtail millet genotype	Area (mm^2)	Perimeter (mm)	Height (mm)	Width (mm)	Circularity	Aspect ratio	Roundness	Solidity
Botok-2	1.66^{i-m}	5.03^{g-j}	1.62^{e-h}	1.45^{klm}	0.83^{d-g}	1.12^{b}	0.80^{k-n}	0.93^{b-h}
Botok-4	1.52^{mn}	4.79^{j}	1.50^{lmn}	1.41^{m}	0.85^{a-g}	1.06^{b-f}	0.86^{e-i}	0.92^{d-h}
Botok-6	1.74^{ij}	5.97^{b}	1.69^{def}	1.59^{f-i}	0.65^{j}	1.06^{b-f}	0.78^{n}	0.88^{j}
Botok-7	1.52^{mn}	4.82^{ij}	1.53^{k-n}	1.41^{m}	0.83^{d-g}	1.08^{b-f}	0.83^{h-m}	0.92^{fgh}
Botok-8	1.68^{i-l}	5.46^{de}	1.62^{e-h}	1.52^{h-l}	0.74^{i}	1.07^{b-f}	0.81^{j-n}	0.90^{i}
Botok-10	1.66^{i-m}	5.41^{d-g}	1.63^{e-h}	1.50^{i-m}	0.73^{i}	1.09^{bc}	0.79^{lmn}	0.90^{i}
Botok-15	1.71^{ij}	5.04^{f-j}	1.57^{h-l}	1.50^{h-m}	0.84^{a-g}	1.04^{cdef}	0.89^{b-g}	0.93^{a-g}
Botok-19	1.64^{j-n}	4.98^{hij}	1.55^{i-m}	1.50^{h-m}	0.84^{a-g}	1.03^{ef}	0.87^{c-h}	0.93^{b-h}
Botok-20	1.79^{hi}	5.20^{e-i}	1.60^{g-j}	1.56^{g-j}	0.84^{b-g}	1.03^{ef}	0.89^{c-g}	0.93^{c-h}
ICERI-1	2.98^{a}	6.84^{a}	2.15^{a}	1.99^{a}	0.82^{gh}	1.08^{b-e}	0.82^{i-n}	0.94^{ab}
ICERI-2	2.24^{cd}	5.91^{bc}	1.83^{c}	1.72^{de}	0.82^{fgh}	1.06^{b-f}	0.85^{g-k}	0.92^{c-h}
ICERI-4	2.36^{c}	5.97^{b}	1.86^{c}	1.78^{cd}	0.84^{b-g}	1.04^{c-f}	0.87^{c-h}	0.93^{a-d}
ICERI-5	2.73^{b}	6.78^{a}	2.11^{a}	1.93^{ab}	0.76^{hi}	1.09^{bc}	0.78^{mn}	0.92^{fgh}
ICERI-6	2.66^{b}	6.74^{a}	1.95^{b}	1.87^{bc}	0.75^{i}	1.04^{c-f}	0.89^{b-g}	0.92^{h}
ICERI-7	2.25^{cd}	5.85^{bc}	1.84^{c}	1.45^{klm}	0.86^{c-g}	1.28^{a}	0.84^{g-l}	0.93^{a-f}
ICERI-9	2.28^{c}	5.97^{b}	1.84^{c}	1.70^{de}	0.82^{efg}	1.09^{bcd}	0.86^{f-j}	0.93^{b-h}
ICERI-10	1.51^{n}	4.68^{j}	1.49^{mn}	1.42^{lm}	0.87^{a-e}	1.05^{c-f}	0.87^{d-i}	0.93^{b-h}
Belitung	1.54^{lmn}	4.82^{ij}	1.48^{mn}	1.43^{lm}	0.85^{a-g}	1.03^{def}	0.90^{a-f}	0.92^{e-h}
Bima	2.08^{ef}	5.52^{cde}	1.70^{de}	1.65^{efg}	0.86^{a-f}	1.03^{ef}	0.92^{abc}	0.93^{a-d}
Buru	2.12^{de}	5.64^{bcd}	1.72^{d}	1.69^{def}	0.85^{a-g}	1.02^{f}	0.91^{a-e}	0.93^{a-e}
Hambapraing	1.69^{ijk}	4.94^{hij}	1.53^{k-n}	1.48^{j-m}	0.88^{abc}	1.03^{def}	0.92^{abc}	0.93^{abc}
Labapu-2	1.90^{gh}	5.44^{de}	1.65^{d-g}	1.60^{fgh}	0.81^{fgh}	1.03^{def}	0.89^{c-g}	0.92^{h}
Mauliru-2	1.78^{hij}	5.18^{e-i}	1.58^{g-k}	1.54^{h-k}	0.85^{a-g}	1.03^{def}	0.91^{a-f}	0.93^{c-h}
NTB-1	1.91^{gh}	5.44^{def}	1.65^{d-g}	1.60^{f-i}	0.83^{d-g}	1.04^{c-f}	0.89^{b-g}	0.92^{gh}
Padang	1.55^{k-n}	4.70^{j}	1.47^{n}	1.41^{m}	0.88^{a-d}	1.04^{c-f}	0.92^{a-d}	0.93^{a-e}
Sanc Loe Nagekeo	1.94^{fg}	5.23^{e-h}	1.62^{f-i}	1.58^{ghi}	0.89^{a}	1.02^{ef}	0.94^{a}	0.94^{a}
Toraja	1.65^{i-n}	4.97^{hij}	1.53^{j-n}	1.48^{j-m}	0.85^{a-g}	1.04^{c-f}	0.89^{a-g}	0.93^{b-h}
Wete Nagekeo	1.94^{efg}	5.28^{d-h}	1.64^{e-h}	1.59^{f-i}	0.89^{ab}	1.03^{ef}	0.94^{ab}	0.94^{a}
CV(%)	20.08	11.07	10.38	9.86	6.37	4.66	5.35	1.44

Means followed by the same letter within each column are not significantly different based on Tukey’s HSD test ($p < 0.05$).

The seed of foxtail millet is a caryopsis-type of fruit with an ovate shape (Hermuth et al., 2015). UPOV (2013) categorized the seed shape of foxtail millet into narrow ovate, medium ovate, and circular, with seed pictures representing each category as phenotyping guidelines. ImageJ produced quantitative data to determine seed shape. Our size quantification of seed pictures provided in the UPOV (2013) descriptor showed that the AR value is approximately 2.0, 1.5, and 1.0 for narrow ovate, medium ovate, and circular grain shapes, respectively. The 28 Indonesian local foxtail millet genotypes showed the AR value ranging from 1.02 to 1.27, and seed roundness ranging from 0.78 to 0.94, indicating that all genotypes exhibited circular grain shape. Manual observation of grain shape tends to be subjective, thus grain shape determination based on quantitative values will be more reliable. The seed solidity of the observed foxtail millet genotypes in this study ranged from 0.87 to 0.94 indicating that all genotypes have high seed density. Sunil et al. (2016) mentioned seed density as one seed engineering property of foxtail millet which is important to be characterized for proper consideration in the machinery design for seed processing.

A dendrogram was constructed based on the seed's morphological characteristics generated by ImageJ to depict the similarity among foxtail millet genotypes based on seed traits studied (Figure 3). The cophenetic correlation coefficient of the constructed dendrogram was 0.97. Mohammadi and Prasanna (2003) mentioned that the cophenetic correlation coefficient reflected the relationship between dissimilarity-similarity shown in a dendrogram as the result of the analysis and the distance-similarity matrix as input for cluster analysis, and a value of more than 0.90 can be considered a very good value. The length of branches in a dendrogram represents the distance of relatedness between genotypes, with longer branches indicating larger genetic divergence (Labbe et al., 2022). The 28 Indonesian local foxtail millet genotypes were grouped into three main clusters in the dendrogram. Most genotypes originated from the Indonesian Cereals Research Institute (ICERI-1, ICERI-2, ICERI-4, ICERI-5, ICERI-6, ICERI-7, and ICERI-9) exhibited larger seeds (seed area ranging from 2.24 to 2.98 mm^2) and were grouped into the same cluster. The ICERI-10 genotype exhibited distinct seed morphological characteristics from those of the other ICERI genotypes and was grouped together with genotypes from West Sumatera (Padang), East Nusa Tenggara (Botok-4, and Botok-7), East Sumba (Hambapraing), and Belitung Island (Belitung). Ardie et al. (2017) reported molecular characterization of the ten foxtail millet genotypes originating from ICERI by RAPD markers and showed that the ICERI-10 genotype was distantly related to the other ICERI genotypes. The dendrogram also indicates that foxtail millet genotypes from the same region may exhibit diverse seed morphology. Botok genotypes which originated from the same region in East Nusa Tenggara fell into different clusters, and two genotypes from West Nusa Tenggara (Bima and NTB-1) were also located in different clusters. The diversity of

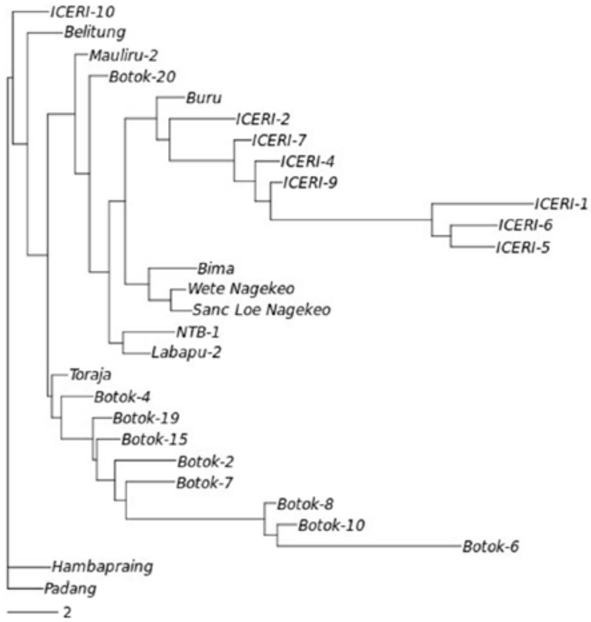

Figure 3. Dendrogram of 28 foxtail millet genotypes based on ImageJ-generated seed morphological characters.

seed morphological variations within a region may occur due to natural and artificial selection. This includes adaptation of genotypes to new cultivation systems and intended or unintended selection by farmers, resulting in similarity among local genotypes (Casanas et al., 2017).

The number of grains on the primary branch is one of the seed characteristics determining productivity in foxtail millet (Xiang et al., 2017), thus characterizing seed number is essential. However, manual counting of seed numbers for small-sized seeds tends to be inaccurate and inefficient. Felix et al. (2021) reported that species with smaller seeds required more time for manual counting. Our results showed that the number of seeds identified by manual calculation and by ImageJ showed excellent accuracy (correlation between actual and ImageJ predicted seed counts $R^2 = 1$). Therefore, ImageJ can be utilized for counting foxtail millet seeds in the future and accelerate the phenotyping process for this character.

The 100-seed weight is another important seed character determining foxtail millet productivity (Xiang et al., 2017). Manual observation of 100-seed weight for small-sized seeds, such as foxtail millet, is challenging and error-prone. We developed a model for rapid observation of 100-seed weight for foxtail millet seeds using the 28 Indonesian local foxtail millet genotypes as the training set. High variability in the training set is essential to develop a representative model (Gomez and Gomez, 1984), and our result showed that the seed area was the most diverse character (Table 3). Kim et al. (2022) reported a significant positive correlation between seed solidity value generated by ImageJ and 100 seed weight in soybeans, indicating the potential utilization of seed solidity in 100-seed weight estimation. However, seed solidity was not significantly correlated with the 100-seed weight of foxtail millet (Table 2) and it was the least diverse character (Table 3) implying the necessity of other seed size-related characters to estimate the 100-seed weight. Higher seed weight can be expected from bigger seeds (higher value of seed area) as indicated by a significant positive correlation between seed area and 100-seed weight (Table 2). A significant positive correlation between seed area generated by ImageJ and seed weight was also reported in mustard seeds (Raney, 2007), soybean seeds (Kim et al., 2022), and sunflower seeds (Polat et al., 2023). Therefore, this study developed a model equation to predict the weight of 100 foxtail millet seeds based on the seed area obtained from the digital image analysis using ImageJ.

There was significant variation in the seed area between foxtail millet groups ($p < 0.05$), with the Botok group having the smallest seed area and the ICERI group having the highest seed area (Figure 4A). As expected, the Botok group showed the lowest 100-seed weight while the ICERI group exhibited the highest 100-seed weight (Figure 4B). Thus, the model was built based on the regression analysis between seed area obtained from ImageJ analysis (x) as the independent variable and the manually-weighed 100 seeds (y) as the dependent variable. The resulting regression equation was $y = 0.123x - 0.0821$, with a coefficient of determination (R^2) value of 0.9223, indicating

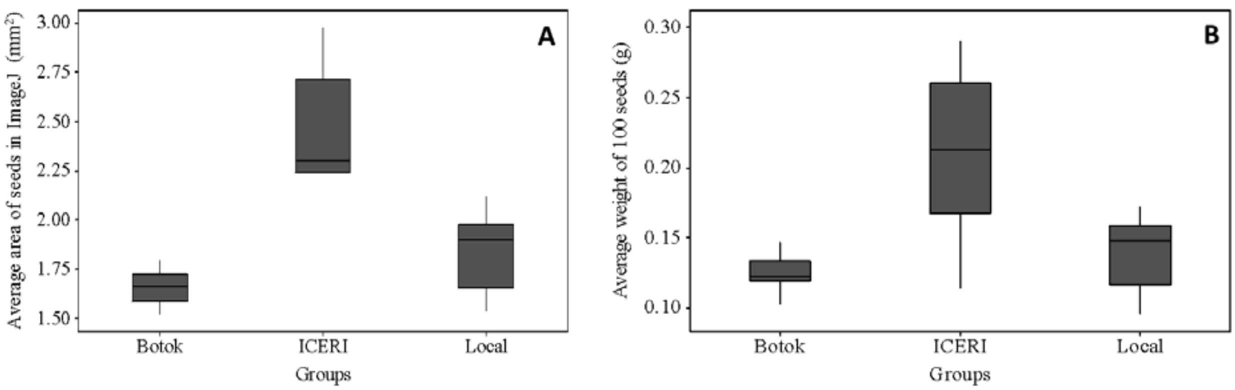

Figure 4. Variability in seed area and 100-seed weight between foxtail millet groups. A. Box plot of ImageJ-generated seed area of different foxtail millet groups. B. Box plot of 100-seed weight of different foxtail millet groups based on manual counting.

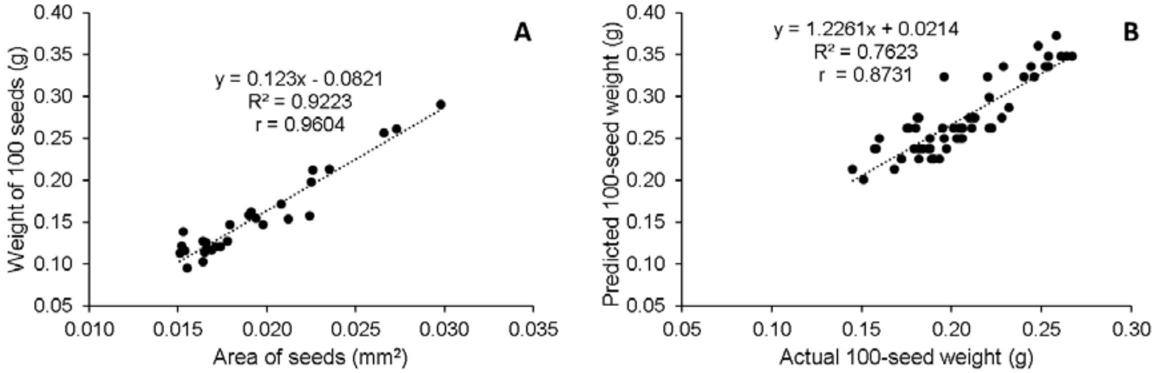

Figure 5. Model development and validation for 100-seed weight estimation in foxtail millet. A. Linear regression between the 100-seed weight from manual counting and ImageJ-generated seed area in the training set. B. Linear regression between the predicted and actual 100-seed weight in the validation set.

that 92.23% of the variation in the weight of 100 seeds could be explained by the area of the seeds obtained from ImageJ analysis (Figure 5A). Additionally, the correlation analysis revealed a strong positive relationship between the predicted weight of 100 seeds and the actual weight of 100 seeds with a correlation coefficient of 0.96 ($p < 0.01$).

In order to validate the obtained model, a validation set consisting of seeds from 30 F_3 families derived from Botok-4 x ICERI-6 cross and seeds from F_3 families derived from ICERI-6 x Botok-4 cross were used as the validation set. ICERI-6 genotype had a significantly higher seed area compared to the Botok-4 genotype (Table 3), thus their progenies are expected to exhibit high variability in the seed area and 100-seed weight characters. Using this validation set, the estimation model $y = 0.123x - 0.0821$ was used to predict the 100-seed weight. The predicted 100-seed weight was then compared to the actual 100-seed weight, resulting in a correlation coefficient of 0.8731 ($p < 0.01$) (Figure 5B).

Manual observation of 100-seed weight for foxtail millet in this study typically requires 2-4 minutes, including the seed counting and weighing the seeds. In contrast, the total time required for the acquisition of an image, including impurities removal and seeds arrangement to prevent overlapping objects, requires only approximately one minute. Further digital image analysis on ImageJ required about two minutes, resulting in a total of three minutes from image acquisition until data generation. The total 2-4 minutes for manual observation produced only one data, namely 100-seed weight. Meanwhile, a total of three minutes of ImageJ-assisted digital phenotyping produced various morphological data (Table 3), seed numbers, and the estimation of 100-seed weight with a relatively high accuracy ($r = 0.8731$). One limitation of the current model is that the predicted values of 100-seed weight tended to be biased upwards, as indicated by the regression coefficient (b) greater than 1.0 (Figure 5B). Nevertheless, our results showed that ImageJ-assisted digital phenotyping for seed characteristics is potentially more time-efficient than manual calculations, especially for larger samples. Felix et al. (2021) reported that digital seed phenotyping of 16 forest species assisted by ImageJ could reduce the average observation time by 62% compared to manual phenotyping.

Our study implies that digital phenotyping such as ImageJ can be a tool to quickly and efficiently characterize the phenotype of small-sized seeds such as foxtail millet. In this study, the digital phenotyping technology was used to assess the genetic diversity of foxtail millet based on seed characters and to estimate the 100-seed weight. The developed model to estimate the 100-seed weight of foxtail millet in this study should potentially accelerate the observation of this trait in the breeding program, particularly when large samples are involved. Xiang et al. (2017) and Zhi et al. (2021) reported seed size as one of the main traits contributing to yield in foxtail millet, emphasizing the importance of robust phenotyping tools for this trait. However, to our knowledge, no digital phenotyping platform has been reported to be utilized in the phenotyping of foxtail millet seeds to date. An ImageJ plugin was developed by Polder et al. (2012) to facilitate plant variety testing, including flax seeds, and is publicly available. A similar approach can be developed in the future for small-seeded species such as foxtail millet. Moreover, the potential of digital phenotyping in seed characterization of foxtail millet shall not be limited to diversity analyses and genetic improvement. Many studies reported the utilization of digital phenotyping technology in the evaluation of seed purity and viability as well as in the seed health assessment as reviewed by Liu et al. (2023). Considering the growing importance of foxtail millet and other minor cereals, future studies are necessary to widen the application of digital phenotyping in small-seeded crops.

CONCLUSIONS

The 28 Indonesian local foxtail millet genotypes showed high variability in seed morphological characteristics. The number of seeds can be counted with excellent accuracy and a model to estimate 100-seed weight based on the seed area showed a high correlation coefficient in the validation set. ImageJ-assisted digital phenotyping was shown to be useful in accelerating observation for small-sized seeds such as foxtail millet.

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