Evaluation of Toraja (Indonesia) local aromatic rice mutant developed using heavy-ion beam irradiation

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Abstract. *Okasa AM, Sjahril R, Riadi M, Mahendradatta M, Sato T, Toriyama K, Ishii K, Hayashi Y, Abe T. 2021. Evaluation of Toraja (Indonesia) local aromatic rice mutant developed using heavy-ion beam irradiation. Biodiversitas 22: 3474-3481.* The aromatic local Toraja rice "Pare Bau" has a good grain quality and aroma. However, it has some disadvantages, including a late heading and low yield for a modern farming system. This study aims to evaluate and select early heading as well as high yield mutant lines induced by heavy-ion beam irradiation. Furthermore, dry seeds of Pare Bau were irradiated with Argon-ion (300 keV/μm) and Carbon-ion (30 keV/μm) at RI-beam factory, RIKEN Nishina Center, Japan. The germination percentages of the M_1 seeds were 49% for Pare Bau irradiated with Argon-ion (PB-A), 53% for Pare Bau irradiated with Carbon-ion (PB-C), and 70% for the Control. The 13 PB-A and 13 PB-C M_1 plants were selected, and the seeds were sampled in the paddy field of Hasanuddin University (20 m asl.), Makassar. During the following planting season, the M_2 generation plant was examined in Enrekang District (650 m asl.), South Sulawesi, Indonesia. Based on the early heading and a larger number of panicles, the 18 PB-A and one PB-C M_3 line were selected from a total of 404 M_2 survival plants. The selected lines and control were grouped into seven clusters based on the quantitative phenotypic traits, indicating the existence of genetic variability. The plant yield was significantly correlated with plant height, the number of tillers, the number of panicles, as well as grain weight per panicle, which showed that these traits are good criteria for selection.

Keywords: Early heading, heavy-ion beam, high yield, mutant, Toraja cultivar

Abbreviations: LET: Linear energy transfer; PB-A: Pare Bau irradiated with Argon-ion; PB-C: Pare Bau irradiated with Carbon-ion

INTRODUCTION

Tana Toraja is a highland region in South Sulawesi, at an altitude of 700 m to 1425 m asl., with a rich biodiversity of local rice that has yet to be exploited. Maulana et al. (2014) examined 18 local rice varieties from Tana Toraja that showed high diversity based on Random Amplified Polymorphic DNA (RAPD) markers. One of the local varieties known as the local aromatic rice is "Pare Bau" which has unique characteristics such as pleasant aroma, better taste, and higher grain quality and has additional socio-economic aspects. However, it also has several disadvantages, including the late heading, high plant posture, and low yield.

The heading date and yield are important traits that determine the productivity of many crops. Furthermore, the heading date is a trait that is closely associated with the regional adaptability of rice varieties and is controlled by internal signals and the environment (Kobayashi and Weigel 2007). The yield was determined by sink (the number of panicles, number of grains, and grain weight) and source (Huang et al. 2013; Khush 2013). It is controlled by external environmental conditions and is governed by genes (Zhang et al. 2017). Furthermore, the primary strategy for increasing production is an increase in rice yield. The heading date has a significant impact on the agronomic traits related to the yield (Jung and Muller, 2009). Several researchers studied the relationships between heading date and yield in rice (Augustina et al. 2013; Ye et al. 2018; Hu et al. 2019). However, manipulation of the early heading date and high yield in rice is an important objective in breeding programs.

Previously, conventional breeding was used to develop new varieties, but breeders were unsatisfied with the results. One of the most effective ways to improve the traits of plants is through mutation breeding, which has been actively practiced for over half a century, with the joint FAO/IAEA Division based in Vienna helping developing countries to adopt advances in the technology quickly (Jain 2005). Mutation breeding can be advantageous in the

production of varieties with the desired trait within a defined germplasm diversity. Furthermore, several achievements have been obtained through mutation breeding regarding improving rice grain quality (Luz et al. 2020).

Plant breeding using ion beam irradiation is a unique technology. Heavy-ion beams are often used as a novel physical mutagen in plant breeding (Abe et al. 2012). Due to their high linear energy transfer (LET), the mutagenic efficiency of heavy-ion beams was significantly higher than that of all other physical mutagens such as gamma rays (Kazama et al. 2011). The ion beam irradiates the samples via an automatic irradiation system, expected to produce double-stranded DNA fractions (Abe et al. 2012). Several studies have been carried out on the mutagenic effect of ion beam irradiation on rice (Ishikawa et al. 2012, Zheng et al. 2020), such as from immature embryos (Fekih 2013), callus obtained from seeds (Mostafa et al. 2015), and cell suspension cultures (Chen et al. 2013). However, seeds are easier to handle and do not require any specialized structure, making them the most commonly used material (Hayashi et al. 2018).

Only a few studies have been conducted using heavy-ion beam irradiation in local aromatic rice, especially in Indonesia. Furthermore, screening and creating a new mutant variety with improved traits is also a primary goal for future studies. Therefore, this study aims to evaluate and select early heading as well as high yield mutant lines from local Toraja aromatic rice "Pare Bau" induced by heavy-ion beam irradiation.

MATERIALS AND METHODS

Plant materials and irradiation treatment

In the present study, M_0 seeds of local aromatic rice varieties (*Oryza sativa* L. var 'Pare Bau') were obtained from Toraja Agriculture Office, South Sulawesi, Indonesia. Dry rice seeds were placed in a plastic box in a single layer due to the limited range of the Argon-ion beam. Furthermore, a sponge was used as a lower surface in the plastic rectangular box (75 x 50 x 18 mm). The seeds in a single layer were pressed between the sponge and the cover plastic of the box. The seeds were then irradiated with Argon-ion at a dose of 10 Gy and Carbon-ion at a dose of 150 Gy at the RI-beam factory, RIKEN Nishina Center, Japan. The average LET of each ion was calculated to be 300 keV/μm for Argon-ion and 30 keV/μm for Carbon-ion.

Experimental site and crop cultivation

The experiment was carried out in the paddy field of Hasanuddin University, Makassar, Indonesia (S: 5°7'53.50" E: 119°28'57.97"; 20 m asl.). While the M_2 generation was carried out in the paddy field of mountainous Enrekang District, South Sulawesi, Indonesia (S: 3°19'47.44" E: 119°50'1.57"; 650 m asl.) neighboring to Tana Toraja District, South Sulawesi, Indonesia.

Evaluation of M_1 generation

To measure the germination percentage, 1000 seeds from each treatment were sown in small plastic seedling pots and grown in a screen house. The germination percentage was calculated based on the following formula:

$$Germination\ percentage\ (\%) = \frac{No.\,of\ seeds\ germinated}{No.\,of\ seeds\ sown} \times 100$$

All mutant and 100 control seedlings were transplanted in the paddy field. Plant survival was recorded at the maturity stage. M_2 seeds were harvested from individual M_1 plants on a panicle. The 13 PB-A and 13 PB-C M_1 plants were selected, which showed 50 larger numbers of grain per panicle, and then sampled.

Evaluation of M_2 generation

During the following planting season, fifty seedlings per each 13 PB-A, 13 PB-C M_2 lines, and control were transplanted using a single plant per hill in paddy field. The distance between hills was 30 cm x 30 cm. The number of transplanted M_2 mutant seedlings was 650. The panicles on the plants were cut and placed in individual paper bags, where they were dried for several days to produce M_3 seeds.

Data collection and statistical analysis

The data collection was carried out on the plant height (cm), heading date (day), the number of tillers, the number of panicles, grain weight per panicle (g), the number of fertile grains, the number of sterile grains, 100-grain weight (g), and plant yield (g). The early heading and a larger number of panicles were used as selection criteria. To avoid the scaling problem, the selected traits were normalized and mapped in two dimensions. The mutant frequency was calculated using the following formula:

$$Mutant\ frequency\ (\%) = \frac{No.\,of\ mutant\ plants}{No.\,of\ population} \times 100$$

A dissimilarity matrix was generated based on the Squared-Euclidean distance across nine quantitative phenotypic traits between the lines and control. This matrix was used with a hierarchical clustering technique of the Wards Minimum variance method, and results were presented graphically in the dendrograms (Ward 1963). Furthermore, the phenotypic correlation was analyzed to see the correlation among traits. A dissimilarity matrix, hierarchical clustering, and correlation analysis were performed using SPSS software.

RESULTS AND DISCUSSION

Generation of the mutant population

In the current study, the germination percentage and the number of survival plants among 2000 mutagenized seeds and control during M_1 generation were observed. The germination percentages of M_1 seeds were 49% for PB-A, 53% for PB-C, and 70% for control (Figure 1). The germination percentages above the control decreased for PB-A by 21% and PB-C by 17%. This may be due to the harmful effects of the ion beam on the M_0 seeds, which weakened the embryo. The seeds can absorb the mutagen, which then penetrates the meristematic zone and affects the germ cell, leading to a reduction in germination (Ke et al. 2019). Similarly, the reduction in germs can be attributed

to damage to the cell components (Kumar et al. 2013). Physiological and biological processes are slowed or inhibited due to changes in enzyme activity (Talebi et al. 2012). In addition, due to field conditions, which include heavy rain for the first few days after planting, some seedlings even grew until the early vegetative stage but died before the pod set. The normal grown-up seedlings were 94 PB-A, 79 PB-C, and 57 for control. In the M_1 generation, the condition was heterozygous, in such a way that most mutations were recessive and are not expressed in the first generation. The plants with seeds larger than 50 were selected and ensuring the survival of the M_1 generation plant is beneficial. The possibility of phenotypic mutation was extremely low during M_1 generation, and only dominant mutations can be detected genetically (Roychowdhury and Tah 2013). The use of a heavy-ion beam has been shown to reduce seed germination.

Evaluation of M_2 generation

Survival plants in the M_2 generation were 196 PB-A and 208 PB-C, and 42 for control. The plant height of PB-A ranges from 60 cm to 175 cm, while PB-C ranges from 111 cm to 183 cm. PB-A produced 5 dwarf phenotype plants with a frequency of 2.6% and 79 semi-dwarf plants with a frequency of 40.3%, while PB-C and the control did not produce dwarf plants (Table 1). Dwarf rice plants caused by ion beam, as observed in the present study was previously reported by Oono et al. (2020). Zheng et al. (2020) showed that heavy-ion beam irradiation was effective in inducing mutations in rice, and revealed the characteristics of mutations at the genome level.

The heading date can be determined by the period of the vegetative growth phase, from seedling to panicle primordium initiation, and reproductive growth phase, from panicle initiation to heading (Vergara and Chang 1985). The vegetative growth phase consists of the basic vegetative phase (BVP), and photoperiod sensitive phase (PSP) (Takahashi 1984). Indonesian local rice varieties grown in mountain areas have a shorter PSP (41 days) and longer BVP (87 days) (Vergara and Chang 1985). The heading date of PB-A M_2 plants varied between 112 days to 144 days, PB-C varied between 104 days to 149 days, while that of control treatment varied between 121 days to 143 days. The 82 PB-A with a frequency of 41.8% and 26 PB-C M_2 plants with a frequency of 12.5% was nominated as the putative early heading mutants.

The number of panicles, defined as the number of tillers that produce spikes and seeds, is an important component of grain yield in rice. The number of panicles of PB-A varied between 2 to 33, while PB-C varied between 1 to 23. The highest number of panicles was in PB-A (41 plants) with a frequency of 20.9%, PB-C reached 22 plants with a frequency of 10.58%, and control reached 5 plants with the frequency of 11.9%. This was illustrated by the finding of Sjahril et al. (2020).

There are 101 plants of PB-A, and 47 of PB-C have earlier heading and a larger number of panicles compared to the control (Figure 2 and 3). However, there are only 18 PB-A and one PB-C have both categorized, i.e. more than 17 panicles and early heading date ($\leq$ 118 days). Those

lines were selected for M_3 generation. This selection uses the concept of normalization to control the variance of the two characters in such a way that both traits have the same relative variance. This selection method was previously reported by Peternelli et al. (2017) in sugarcane, Anshori et al. (2019) in rice, and Fadhli et al. (2020) in corn.

Further studies are required on the selected early heading and high yield putative mutant lines obtained from Toraja local aromatic rice. To correctly assess the early heading date and high yield traits, putative mutant lines and wild type should be crossed. Segregating populations could be derived from crosses between near-isogenic lines which exhibited phenotypic characteristics (Sun et al. 2019). They are much easier to analyze than those derived from remote crosses. Near-isogenic lines have proven to be very useful for genetic studies of quantitative traits such as heading date, which was confirmed by several previous findings (Ding et al. 2011, Fujita et al. 2011, Zhang et al. 2012).

There is an urgent need to study the isolation of mutant genes. The availability of the DNA markers and genome analysis of rice provides a more direct and faster technique for determining genes of interest. Due to this character, it is necessary to find a novel gene related to unique characters. Several genes that cause mutant phenotypes were successfully cloned by map-based cloning from ion beam-induced rice mutants, such as Morita et al. (2019) reported that long grain1 (lin1) mutant contributes to increasing grain weight. Several studies identified early heading date genes (Fujino et al. 2013, Yang et al. 2020), while Ichitani et al. (2014) reported genes related to late heading date in rice. Fujita et al. (2013) argued that the naturally varying SPIKE gene, located in the QTL cluster region of chromosome 4, had a significant impact on yield. However, due to the relatively large rice genome size, the characterization of ion beam-induced mutations at the genomic level was not achieved but has only recently been carried out (Ichida et al. 2019, Li et al. 2019).

Dissimilarity matrix and cluster analysis

The dissimilarity matrix based on their nine phenotypic traits was used to group the lines and control populations presented in Table 2. The dissimilarity matrix showed that the highest mean distance was obtained between Control and PB-A.12.2.34, indicating that these are the most divergent lines regarding phenotypic traits evaluated. In contrast, the lowest between PB-A.7.1.9 and PB-A.14.3.1 showed the least divergence and was considered the most similar to the phenotypic traits evaluated.
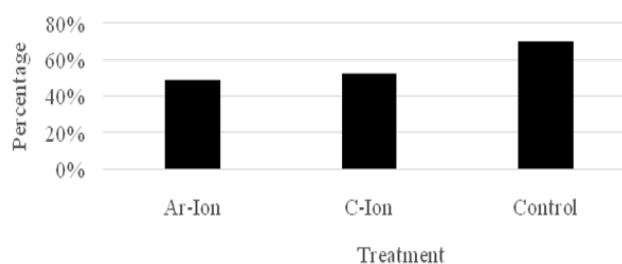

**Figure 1.** Germination percentage in M1 generation

Table 1. Growth pattern observed in the M_2 generation

| Class	Sub-Class	Description	Control			Argon-ion			Carbon-ion		
			Range	Survival plant	Frequency (%)	Range	Survival plant	Frequency (%)	Range	Survival plant	Frequency (%)
Plant Height	Dwarf	≤ 100	108-166 cm	0	0.0	60 -175 cm	5	2.6	111 -183 cm	0	0.00
	Semi-dwarf	101 - 141		13	31.0		79	40.3		58	27.88
	Tall	≥142		29	69.0		112	57.1		150	72.12
Heading date	Early	≤ 118	121-143 days	0	0.0	112 -144 days	82	41.8	104 -149 days	26	12.50
	Medium	119 - 132		37	88.1		69	35.2		177	85.10
	Late	≥ 133		5	11.9		45	23.0		5	2.40
Number of panicles	Few	≤ 8	2-24 panicles	19	45.2	2 -33 panicles	60	30.6	1-23 panicles	73	35.10
	Medium	9 - 16		18	42.9		95	48.5		113	54.33
	Many	≥ 17		5	11.9		41	20.9		22	10.58

Table 2. Dissimilarity matrix based on Squared-Euclidean distance in the different lines and control

Lines	C	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12	G13	G14	G15	G16	G17	G18	G19
C		23.86	14.30	26.15	27.58	33.36	16.51	32.71	11.40	28.02	14.66	23.21	27.74	42.21	38.45	69.01	66.10	29.08	19.71	14.82
G1			16.03	18.83	28.60	30.85	31.33	39.04	19.71	34.40	18.09	16.62	29.14	23.45	37.18	48.21	53.08	33.31	36.51	19.78
G2				3.44	9.47	15.84	6.46	14.48	3.17	7.62	7.19	11.72	5.93	25.70	19.28	43.04	37.74	6.95	19.75	3.33
G3					16.36	17.17	10.04	17.05	6.88	7.56	6.82	6.93	4.15	20.52	17.69	37.69	36.48	5.71	19.85	4.30
G4						9.00	15.21	8.60	10.82	8.27	17.58	28.08	11.69	31.00	16.51	36.86	17.93	8.99	37.24	10.33
G5							12.73	2.38	9.67	6.53	10.67	18.40	8.74	13.12	8.33	21.79	10.08	8.11	22.35	9.38
G6								10.28	3.33	6.82	8.16	12.47	7.54	26.14	17.68	46.30	39.84	8.01	7.91	6.29
G7									8.53	3.75	11.88	22.95	5.86	19.83	8.75	25.74	13.90	5.75	21.99	8.99
G8										4.88	2.66	9.77	4.71	16.77	10.95	32.26	30.71	6.24	10.15	1.62
G9											7.43	15.90	1.40	19.52	6.75	26.62	18.47	0.74	18.91	3.60
G10												4.53	5.72	11.34	11.29	29.00	30.60	7.73	8.77	1.61
G11													12.20	14.47	21.66	41.02	43.87	14.50	9.99	7.65
G12														17.94	9.71	28.19	23.67	1.14	17.58	2.90
G13															9.10	9.81	22.16	22.12	22.23	16.66
G14																8.46	11.68	10.50	23.72	10.92
G15																	13.57	31.60	49.21	31.41
G16																		20.86	55.45	27.00
G17																			20.98	3.35
G18																				13.65
G19																				

Note: C: Control, G1: PB-A.5.1.13, G2: PB-A.5.3.36, G3: PB-A.5.3.45, G4: PB-A.6.1.9, G5: PB-A.6.1.12 G6: PB.A.6.1.13, G7: PB-A.6.1.15, G8: PB-A.8.1.5, G9: PB-A.7.1.9, G10: PB-A.7.1.30, G11: PB-A.7.1.41, G12: PB-A.12.2.4, G13: PB-A.12.2.11, G14: PB-A.12.2.12, G15: PB-A.12.2.34, G16: PB-A.14.2.14, G17: PB-A.14.3.1, G18: PB-A.14.4.3, G19: PB-C.20.1.49
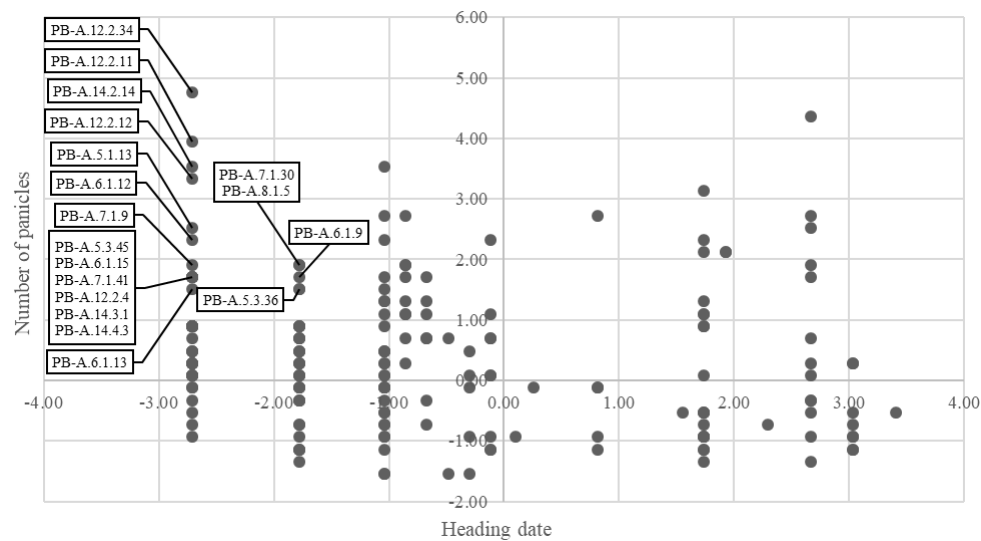
Figure 2. Mapping the selection of heading date and the number of panicles in M_2 plants irradiated with Argon-ion in local Toraja aromatic rice "Pare Bau", Indonesia. Eighteen M_3 lines were nominated
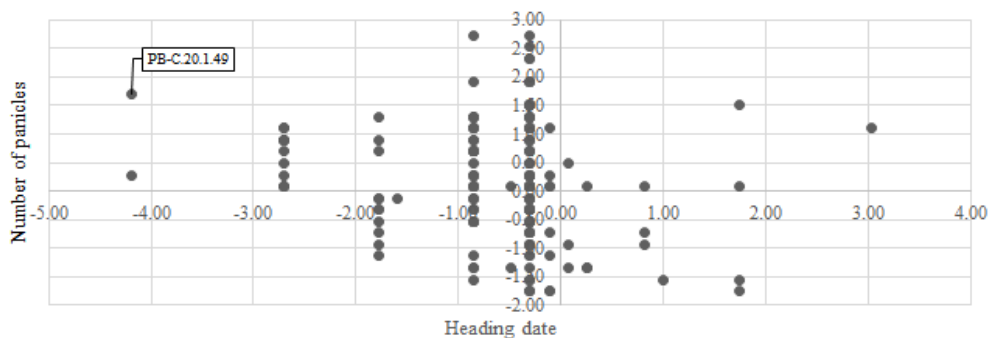
Figure 3. Mapping the selection of days to heading and the number of panicles in M_2 plants irradiated with Carbon-ion in local Toraja aromatic rice "Pare Bau", Indonesia. One M_3 line was nominated

Cluster analysis of 19 selected lines and control based on nine quantitative phenotypic traits showed seven clusters at level 5 (Figure 4). The maximum number of lines was included in cluster III and VII (6 lines), followed by cluster VI (3 lines), cluster IV (2 lines), cluster I, II, and V (1 line). Several studies have previously used this method (Oladosu et al. 2014, Liang et al. 2015, Tahjib-Ul-Arif et al. 2018). The dendrogram shows that varieties in one cluster are primarily identical, have less diversity, and most similar objects are linked by gradually diminished similarity criteria (Sava and Popa, 2011). The aromatic rice lines show significant genetic variations in morphological traits as observed in this study. It was important to note that in the control and two lines formed an independent cluster, which showed that considerable genetic variation has performed quantitative phenotypic traits in some mutant lines compared to control due to mutagenic treatments.

The effectiveness of quantitative phenotypic traits in grouping rice lines has been demonstrated. It has been shown that morphologically based genetic divergence

analysis among rice lines can be used to identify and distinguish different lines in a population (Franco et al. 2001). The phenotypic characterization of mutant populations in their parents is an essential part of the mutation breeding program, and the efficient population of various interrelated traits, especially quantitative traits, is the key to successful selection in the subsequent generations. For the future breeding program, lines are selected from diverged clusters with better phenotypic performance, or each cluster, one parent was selected and then the selected lines are subjected to crossing.
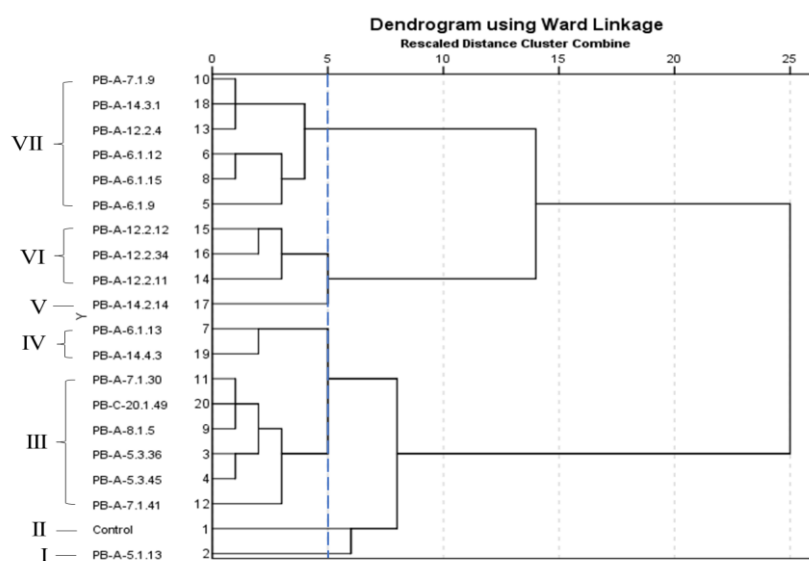
Correlation coefficient analysis

Correlation coefficient analysis was carried out to determine how selecting one character will cause a simultaneous change in other characters and determine the relationship between character and fitness. Breeders used correlation coefficient analysis to show a positive relationship between yield and other traits that enhance yield in cereals, such as rice (Ogunbayo et al. 2014),

sorghum (Mutava et al. 2011), wheat (Lopes et al. 2012), and corn (Zabed et al. 2016).

All calculated correlation coefficients are listed in Table 3. The number of tillers showed positive correlation with plant height (0.59). The number of panicles exhibited significantly positive correlation with plant height (0.54) and the number of tillers (0.94). Grain weight per panicle was found to be positively correlated with plant height (0.49). The number of fertile grains had positive association with plant height (0.45) and grain weight per panicle (0.98). The trait of 100-grain weight was found to be positively correlated with the heading date (0.62). Significant positive correlations with plant yield were recorded for the traits of plant height (0.71), the number of tillers (0.81), the number of panicles (0.87), and grain weight per panicle (0.46). Close relationships between traits can play a positive or negative role in the transmission of traits through genetic introgression since strong selection for the desired trait can support the presence of other traits.

In this study, plant height was closely related to yield. Dwarf mutant lines may be restricted in the rice breeding program due to negative effects on reduction in the number of panicles, grain weight per panicle, number of fertile grains, and plant yield. According to Peng et al. (1994), short plant height causes leaf crowding, poor ventilation, and reduced crowding conditions, which result in the decreased photosynthetic efficiency. The mutant plants with the dwarf phenotype were not suitable for direct commercial cultivation. They can be used in hybridization to pass some of their beneficial traits to other high-yielding rice varieties. The number of panicles was correlated with the number of tillers, and the number of tillers that are too high or low will affect the yield of rice. This finding is consistent with previous research that showed a positive association between the number of panicles and rice yield (Ranawake and Amarasinghe 2014). The grain weight per panicle also correlated with plant yield, and the results conformed to Akinwale et al. (2011). Therefore, it can be suggested that lines should possess more plant height, number of tillers, number of panicles, and grain weight per panicle for increasing rice yield. These traits are important yield contributing traits, and selection based on these traits would be most effective.

Figure 4. Dendrogram of 19 selected lines and one control based on nine quantitative traits

Table 3. The phenotypic correlation coefficient of different traits obtained from 19 rice lines

Traits	Plant height	Heading date	No. of tillers	No. of panicles	Grain weight per panicle	No. of fertile grains	No. of sterile grains	100-grain weight	Plant yield
Plant height	1.00	-0.38^{ns}	0.59^{**}	0.54^{*}	0.49^{*}	0.45^{*}	-0.13^{ns}	0.16^{ns}	0.71^{**}
Heading date		1.00	-0.30^{ns}	-0.33^{ns}	-0.30^{ns}	-0.28^{ns}	0.02^{ns}	0.62^{**}	-0.32^{ns}
Number of tillers			1.00	0.94^{**}	0.20^{ns}	0.12^{ns}	0.14^{ns}	0.22^{ns}	0.81^{**}
Number of panicles				1.00	0.13^{ns}	0.07^{ns}	0.19^{ns}	0.26^{ns}	0.87^{**}
Grain weight per panicle					1.00	0.98^{**}	-0.21^{ns}	0.17^{ns}	0.46^{*}
Number of fertile grains						1.00	-0.21^{ns}	0.09^{ns}	0.38^{ns}
Number of sterile grains							1.00	-0.33^{ns}	0.01^{ns}
100-grain weight								1.00	0.32^{ns}
Plant yield									1.00

Note: ns: indicates non-significance; *: indicates significance at $p \leq 0.05$; **: indicates significance at $p \leq 0.01$

The evaluation of these mutants in subsequent breeding cycles can be useful in the development of selection indices for important traits in rice. Nineteen selected lines (18 PB-A and 1 PB-C) for early heading and high yield compared to control, were suitable for aromatic rice breeding programs from this population. Consequently, it is expected that there will be significant advancements in the use of ion beam breeding. We recommended that future studies should explore molecular markers to validate the findings of this study.

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