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Discrimination of haploids and doubled haploids/diploids in *indica* rice: correlation of morphological indicators with molecular markers

S.K. SINGH[1,2,3], K.P. JEUGHALE[1], B. DASH[1], S.S. BHUYAN[1], M. CHANDRAVANI[1], C. PARAMESWARAN[1], DEVANNA B.N.[1], R.L. VERMA[1], J.L. KATARA[1], and S. SAMANTARAY[1,*]

[1] *Crop Improvement Division, ICAR-National Rice Research Institute, Cuttack, Odisha-753006, India*
[2] *Department of Plant Breeding and Genetics, Odisha University of Agriculture and Technology, Bhubaneswar, Odisha-751003, India*
[3] *Department of Genetics and Plant Breeding, Faculty of Agricultural Sciences, Siksha 'O' Anusandhan, Bhubaneswar, Odisha-751030, India*

[*]Corresponding author: E-mail: smitraray@gmail.com

Abstract

To obtain immediate homozygosity by androgenesis, the doubled haploid method is often used. As a result, a mapping population was created utilizing rice (*Oryza sativa* L.) cvs. Mahulata and IR 20 as parents in order to find QTLs/genes for drought tolerance at the vegetative stage. The effectiveness of the doubled haploids (DHs) approach, on the other hand, is largely dependent on the ability to distinguish haploids from diploids among the green regenerants. Although flow cytometry and cytological screening for pollen sterility can be used to identify haploids, these methods are expensive, time-consuming, and need a sophisticated laboratory with highly trained workers. Plant height and other spikelet features have also been used to differentiate haploids from doubled haploids. However, no systematic analysis of several morphological features for distinguishing haploids in doubled haploids has been published to date. As a result, a cost-effective approach for distinguishing haploids from true DHs obtained from anther culture is required. The goal of this work was to identify haploids using morphological features and simple microscopic examinations without the use of chemicals or complex laboratory facilities. The cross between the IR20 and Mahulata yielded a total of 198 anther culture (AC) derived plants. A group of 41 plantlets was chosen as putative haploids based on their shorter height and Cq values using qPCR-based genotyping and finally validated that, in addition to plant height, other morphological traits such as total number of leaves/plant, total number of tillers/plant, and floral characters can be used to successfully identify haploids. We report a variety of morphological signs as indicators of haploid plants, including smaller plants, higher tiller density, narrower and shorter leaf length, and partial exertion of panicle from the flag leaf sheath. Other morphological markers for identifying haploids from DHs include smaller florets and anthers, and small desiccated microspores.

Keywords: androgenesis, Cq value, doubled haploids, haploids, *indica* rice, Pearson's correlation, principal component analysis, qPCR.

Introduction

Doubled haploids (DHs) are a valuable resource for breeding and genetic research. Doubled haploid breeding shortens the breeding cycle and permits homozygous lines to be produced from a segregating population. Because of their homozygosity and uniformity, DH lines are good materials for genetic investigations, particularly on quantitative

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Abbreviations: AC - anther culture; DH - doubled haploids; gDNA - genomic DNA; PCA - principal component analysis; qPCR - quantitative polymerase chain reaction.
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features. They are also regarded as a permanent mapping population since they may be replicated and reproduced without undergoing genetic change over time (Semagn *et al.* 2006). These characteristics allow for precise quantitative trait measurement through repeated trials, as well as a reduction in the environmental component of overall phenotypic variance. It is important to assess the extent and occurrence of such variation in DH lines since this has important implications for the suitability of using the materials in breeding and genetic studies.

Rice (*Oryza sativa* L; 2n = 2x = 24) is a member of the *Gramineae* family of the genus *Oryza*, which contains 24 species, 22 of which are primitive and the remaining two, *O. sativa* and *O. glaberrima* Steud., are cultivated (Khush 1997). All rice cultivars grown in Asia, Europe, and North America are *O. sativa*, but many cultivars grown in West Africa are *O. glaberrima*. In addition, there are two ecotypes of *O. sativa*: *indica* and *japonica*. Rice is the world's most important crop, with Asia producing and consuming more than 90% of the world's rice. Apart from that, India and China produce more than half of Asian rice. However, while the yield plateau appears to have been reached, more rice production is required to meet the demands of an ever-increasing population.

When compared to traditional methods, which take at least 6 - 7 generations, producing doubled haploids by anther culture is a fast way to homozygosity that reduces the time to generate new rice cultivars. Hu and Zeng (1983) compared three rice breeding strategies for the development of novel rice cultivars: anther culture, bulk method, and pedigree method. Anther culture was the best approach for producing homozygous lines in the least amount of time, *i.e.*, one to two years. Furthermore, anther culture-produced doubled haploids (DH) improve selection efficiency, especially when dominance variation is high. Despite all of these benefits, the creation of a mixed population with ploidy varying from 1x to 5x is a major disadvantage of anther culture-derived DH lines. Although most are haploids (30 - 35%) or diploids (50 - 65%) (Chen and Wu 1983, Mercy and Zapata 1986, Rout and Sarma 1990) prior identification of haploids will save time and cost involved in doubled haploid production. Besides, molecular marker analysis cannot be used to differentiate haploids and doubled haploids. To date, the reports are meager on differentiating haploids and DHs in elite *indica* rice hybrids (Mishra *et al.* 2015). The present study aims to investigate the haploids in detail and to generate some combinations of agro-morphological indicators that can be used to distinguish the haploid plants from the putative DHs in *indica* rice without any cost-effective and time-consuming measures.

Materials and methods

Experimental material: We used 198 dihaploids generated from the F_1 generation of rice drought susceptible cv. IR20 and drought tolerant cv. Mahulata in the experiment conducted in a greenhouse at the ICAR (Indian Council of Agriculture Research)-National Rice Research Institute (NRRI) in Cuttack. The anther culture-derived DHs were developed as per the standard protocol described by Naik *et al.* (2017) with slight modifications. A set of 136 and 62 anther cultured derived plantlets were planted in pots in the greenhouse during the autumn 2019 and spring 2020. The earthen pots (height 45 cm and diameter 30 cm) were filled with homogenized NRRI farm soil and sealed at the bottom. Further, three holes were drilled at the bottom and plugged with stoppers to allow suitable drainage. The lower portion (80%) of the pots was filled with a bulk density equivalent to 1.15 g mL^{-1} of dry soil by compacting the soil even after every 5 cm during the filling process (Bernier *et al.* 2009). The pots were saturated with water for a few days before transplanting the seedlings. The upper (20%) portion was filled with lowland irrigated soil. The soil level was set aside 5 cm below the edge of the pots. Phosphorus and potassium were applied at an amount corresponding to 40 kg ha^{-1} along with nitrogen at 30 kg ha^{-1} as a basal dose. The rest amount of N (60 kg ha^{-1}) was applied in two split doses. Seeding was performed by hand and prophylactic measures were taken whenever it was required, to maintain the disease and pest-free conditions.

Data collection: The characteristics like total number of leaves per plant, total number of tillers per plant, plant height, leaf length, leaf breadth, and chlorophyll content were recorded at the vegetative stage. Also, the nature of panicle exertion, floret size, anther position inside the spikelets, and anther length were recorded and visualized under the microscope at the reproductive stage. All the traits under consideration were taken from five randomly selected tillers from all the haploid rice plantlets derived from the embryogenic callus and selected according to their height and they were compared with the diploid rice plants (Zapata-Arias 2003, Mishra *et al.* 2015). The microscopic study of individual florets was done in a stereo microscope (*OLYMPUS SZX16*, Tokyo, Japan). The microspore density was determined by simply dusting individual florets on a slide and counting them under the microscope.

Extraction of genomic DNA: The leaf samples of 159 rice lines (putative DH/putative haploids) and their diploid parents were collected and kept at -80°C in the refrigerator. A modified cetyltrimethylammonium bromide (CTAB) method of Doyle and Doyle (1987) was used for the genomic DNA (gDNA) extraction. Then, 0.8% (m/v) agarose gel electrophoresis was used to determine DNA quality, and quantity was measured using a spectrophotometer. Further, high-quality gDNA was diluted to 50 ng L^{-1} and used for genotyping.

Standardization of qPCR: A set of putative haploids/ doubled haploids, parental (diploids), and tetraploid rice lines gDNA was taken in six different (0, 10, 20, 30, 40, and 50 ng L^{-1}) concentrations for the standardization of gDNA concentration for qPCR (*BIO-RAD CFX96* real-time system, Hercules, CA, USA) analysis. From this analysis, 30 ng L^{-1} DNA concentration was found to be

appropriate for qPCR quantification. Therefore, DNA concentration in all the samples was normalized to 30 ng and subjected to qPCR.

Design of the qPCR genotyping system: The putative haploids/DHs, parental (diploids), and tetraploids (based on their gigantic growth habits) gDNA were subjected to qPCR genotyping. The *25s* housekeeping gene was used for the screening of the anther culture-derived rice plantlets with different ploidy levels. The qPCR system included gDNA (1.0 µL), primers (1.0 µL), ddH_2O (3.0 µL), and 2× *Brilliant III SYBR® qPCR Super Mix Plus* (*Agilent*, Santa Clara, CA, USA) (5 µL). The reaction program was 95°C for 4 min, followed by 40 cycles (95°C for 15 s, 60°C for 30 s), and then melting curve analysis at 60 - 95°C.

Statistical analysis: The data generated were analyzed using *XLSTAT 2020 v. 22.5.1050* and *RStudio v. 4.2.2*.

Results

Initially based on the morphological assessment at the seedling stage, 41 plants (20.70%) were identified as putative haploids and 118 as putative doubled haploids or diploids out of 159 regenerants. Further, morphological analysis between putative haploids and doubled haploids/diploids showed apart from plant height, three traits namely leaf number per plant, tiller number per plant, and leaf length also showed significant differences between the putative haploids and doubled haploids/diploids. In support of our observation, Mishra *et al.* (2015) also observed that haploid plants were smaller and had small glumes and sterile spikelets. Additionally, our analysis identified that tiller number, leaf number, and leaf length can also be taken as phenotypic markers for haploid plant identification.

The Cq values of the *25s* reference gene (Mbéguié-A-Mbéguié *et al*. 2007, Thomas-Hall *et al.* 2007, van den Berg *et al.* 2007, Elitzur *et al*. 2010, Wang *et al.* 2010, Shekhawat *et al.* 2011) showed a linear relationship with ploidy level. Putative haploids/DHs, diploid parents, and tetraploid rice lines were subjected to qPCR, and it was found that the average Cq value for all the putative haploids was the highest (14.23) whereas the lowest Cq (12.76) was observed for the tetraploids (Fig. 1). The putative DHs and the diploid parents showed a Cq value of 13.4 and 13.0, respectively, that fall in between the tetraploids and haploids.

Total leaf number per plant showed a very strong positive correlation (Table 1) with total tiller number per plant (0.924) and a weak positive correlation with chlorophyll content index (0.302), whereas a moderate negative correlation was observed with leaf length (-0.587), plant height (-0.562), and leaf breadth (-0.426). The total tiller number per plant was observed to have a weak positive association with the chlorophyll content index (0.353). Like total leaf number/plant, total tiller number per plant was moderately negatively correlated with leaf length (-0.663), plant height (-0.657), and leaf breadth (-0.457). Unlike total leaf number per plant, leaf length was negatively correlated with total leaf number per plant, total tiller number, and chlorophyll content index (-0.434). However, it showed a strong positive association with plant height (0.931) and it positively correlated with leaf breadth (0.398). The chlorophyll content index had a negative correlation with plant height (-0.418). Plant height had a positive correlation with leaf breadth (0.406).

The amount of variance shared by plant height and leaf length was highest followed by tiller number per plant, leaf number per plant, and leaf length, tiller number (Table 2). In contrast, the lowest variance was shared by leaf breadth and chlorophyll content index followed by chlorophyll content index and leaf number per plant, and chlorophyll content index and total tiller number per plant. These findings indicate that, in the case of chlorophyll content index and leaf breadth, a very large amount, *i.e.*, 98% of variance was explained by other factors that were

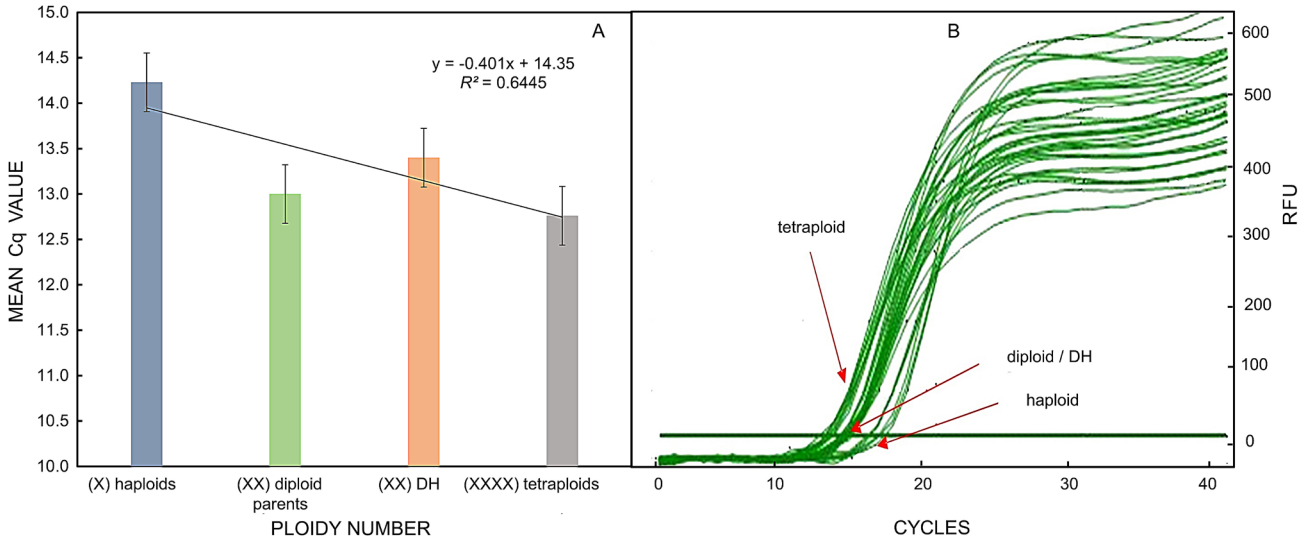

Fig. 1. The Cq values of the reference sequence (*A*) and the quantification graph of the qPCR genotyping system (*B*) in anther cultured derived rice lines of different ploidies.

Table 1. Pearson's correlation matrix. Values in bold are different from 0 with a significance level of alpha = 0.05.

Variables	Total leaf number/ plant	Total tiller number/ plant	Leaf length [cm]	Chlorophyll content index	Plant height [cm]	Leaf breadth [cm]
Total leaf number/plant	**1**	**0.924**	**-0.587**	**0.302**	**-0.562**	**-0.426**
Total tiller number/plant		**1**	**-0.663**	**0.353**	**-0.657**	**-0.456**
Leaf length [cm]			**1**	**-0.434**	**0.931**	**0.398**
Chlorophyll content index				**1**	**-0.418**	-0.141
Plant height [cm]					**1**	**0.406**
Leaf breadth [cm]						**1**

Table 2. Coefficient of determination (R^2) [%] of variables. Values in bold are the three largest contributors, and values in bold italics are the three smallest contributors.

Variables	Total leaf number/ plant	Total tiller number/ plant	Leaf length [cm]	Chlorophyll content index	Plant height [cm]	Leaf breadth [cm]
Total leaf number/plant	100	**85.42908**	34.43422	***9.10581***	31.53113	18.16455
Total tiller number/plant		100	**44.01451**	***12.49535***	43.14559	20.78462
Leaf length [cm]			100	18.83695	**86.62441**	15.80169
Chlorophyll content index				100	17.44343	***1.99117***
Plant height [cm]					100	16.44786
Leaf breadth [cm]						100

Table 3. Eigenvalue of different principal components (PCs) with variability [%].

	PC1	PC2	PC3	PC4	PC5	PC6
Eigenvalue	3.669	0.911	0.667	0.617	0.077	0.060
Variability [%]	61.150	15.177	11.110	10.279	1.284	1.000
Cumulative [%]	61.150	76.327	87.437	97.716	99.000	100.000

not taken in the experiment whereas for plant height and length and tiller number and leaf number per plant a very small amount of variance, *i.e.*, 13 and 14% contributed by other factors, respectively.

Principal component analysis (PCA) is the most widely used multivariate analysis technique as it reduces data dimensionality and generates component scores that capture variance in the multivariate study (Siebert 2001, Leardi 2002). Based on an eigenvalue greater than 1, PCA was performed, yielding a single main principal component PC1. Furthermore, PC1 covered 61.15% of overall variability, whereas PC2 covered 15.17%, for a total variation of 76.32% across the two PCs (Table 3, Fig. 2). Furthermore, the contribution of each variable under study to each PC's variability was noticed (Table 4). For PC1, all variables except chlorophyll content index and leaf breadth showed a very high contribution, whereas these two variables had a major contribution for PC2 and PC3. The biplot of the PCA (Fig. 3) explains the traits that can be used for the identification of haploids. Also, the box plot (Fig. 4) illustrates that the observed variations among the studied traits were statistically significant between the putative DH/diploid groups and haploid groups.

The clustering of genotypes was also performed to confirm the results obtained in PCA. The two major clusters were formed using the Ward's dissimilarity that divides the entire population under the experiment into two clusters (Fig. 5). Under cluster-I, 41 individuals were grouped and all were haploids whereas cluster-II contained 120 individuals that were diploids/DHs. Thus, both principal component and clustering analyses using morphological markers clearly distinguished haploids and doubled haploids.

Pollen structure and fertility were studied in all 41 haploid lines. All these haploid lines had infertile pollen (Mishra *et al.* 2015). All lines identified as haploids had smaller leaves (Fig. 6*A*), partial panicle exertion from flag leaf sheath (Fig. 6*B*), smaller floret, stamen, and carpels which were positioned towards the tip of the floret (Fig. 7*A*), and the very dry stigmatic surface as compared to the diploids (Fig. 7*B*). Besides these characters, haploids had smaller and desiccated anthers (Fig. 8*A*) and had very few desiccated microspores per unit area (Fig. 8*B*) when compared to individual diploids.

Discussion

Over traditional breeding, doubled haploid breeding has several advantages, including a shorter breeding

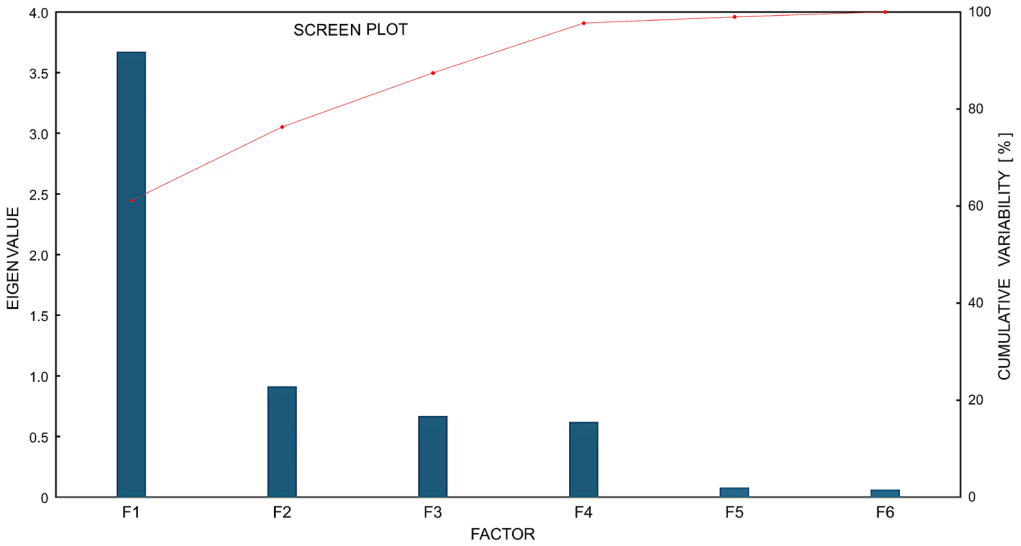

Fig. 2. Screen plot analysis of principal components with their cumulative variability [%] and eigenvalue. F1 - principal component 1, F2 - principal component 2, F3 - principal component 3, F4 - principal component 4, F5 - principal component 5, F6 - principal component 6.

Table 4. Contribution of the variables [%] towards different principal components.

Variables	PC1	PC2	PC3	PC4	PC5
Leaf number/plant	19.260	7.488	27.847	1.580	20.386
Tiller number	21.836	4.310	17.734	0.528	27.925
Leaf length	21.321	3.984	5.143	18.012	30.255
Chlorophyll content index	7.516	52.439	0.065	39.932	0.001
Plant height	20.907	3.621	6.855	19.656	21.398
Leaf breadth	9.159	28.158	42.356	20.293	0.034

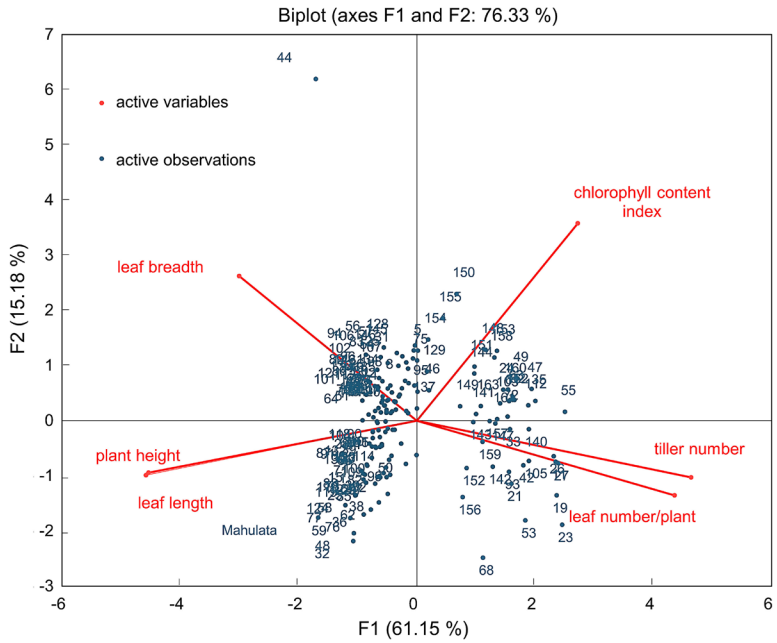

Fig. 3. Biplot representing the degree and direction of association between the different morphological traits in putative diploids and haploids. F1 - principal component 1, F2 - principal component 2, values in parentheses are the cumulative variability of the respective principal component.

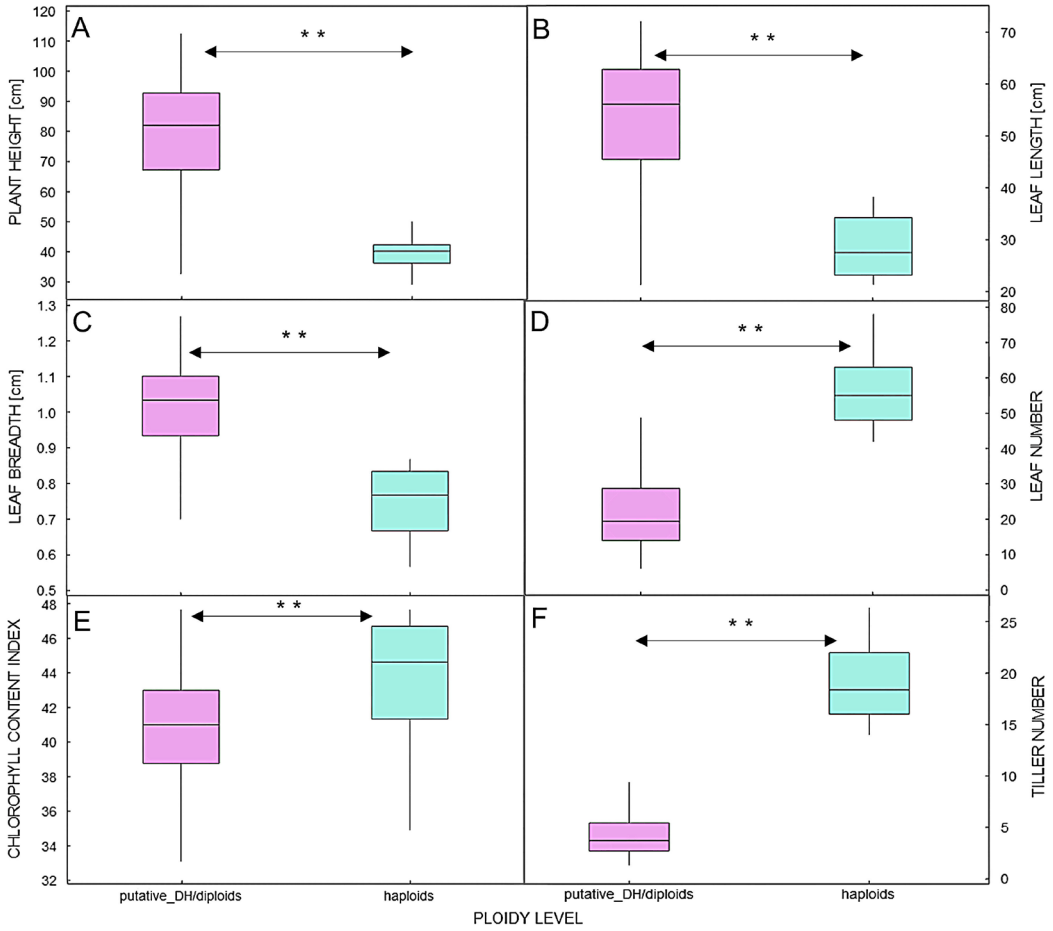

Fig. 4. A series of box plots illustrating the morphological variations observed between the putative DH/diploid and haploid groups. The horizontal line within each box indicates the median value for the respective trait being measured. *A* - depicts the difference in plant height [cm]. *B* - show the leaf length [cm]. *C* - depicts leaf breadth [cm]. *D* - represents the leaf number per plant. *E* - illustrates the chlorophyll content index. *F* - the tiller number per plant among the putative DH/diploids and haploids. ** indicate significant difference at $P < 0.01$ between the putative DH/diploids and haploids.

cycle due to immediate fixation of homozygosity, high selection efficiency, increased genetic variability due to the production of gametoclonal variants, and earlier expression of recessive genes suitable for breeding (Devaux and Pickering 2005). The production of a population of mixed ploidy levels is a major drawback in androgenesis. Further, the identification of haploids using flow cytometry is laborious and requires sufficient expertise in handling samples. Besides, molecular marker analysis cannot differentiate haploids and diploids which require normalization of DNA concentrations and accurate quantification. Though ploidy status of the green plants was determined based on morphological evaluation such as plant height, glume length, pollen grain size, pollen fertility, and spikelet fertility, and confirmed through flow cytometry analysis (Zapata-Arias 2003, Mishra *et al.* 2015, Naik *et al.* 2017) no comprehensive report on discrimination of haploids and DHs are available.

The qPCR genotyping system uses PCR amplification to quantify the amplicons and can be used for the copy number analysis. The Cq value in the PCR cycle indicates the number at which the sample's reaction curve intersects the threshold line. This value tells how many cycles are necessary to detect a real signal from the sample. Cq values are inverse to the amount of target nucleic acid that is in the sample and correlate to the number of target copies in the sample (Bustin *et al.* 2009, Schrader *et al.* 2012). Therefore, the Cq value is inversely proportional to the ploidy level. Also, this phenomenon has already been reported in crops like cotton (Yoo *et al.* 2013), wheat (Powell *et al.* 2017), *Brassica napus* (Wu *et al.* 2018), and very recently in loquat (Wang *et al.* 2021).

Besides, confirmation of haploids through principal component analysis using different morphological traits could significantly save resources and manpower in the characterization of regenerants developed through androgenesis. The correlation study gives us an idea of the degree and direction of association of two or more variables, whereas Pearson's correlation is used to measure the strength and direction of this linear association (Schober *et al.* 2018). The positive correlation between leaf number and tiller number further substantiates that both these traits could be taken as phenotypic markers for haploid identification. Similarly, Babar *et al.* (2008) also

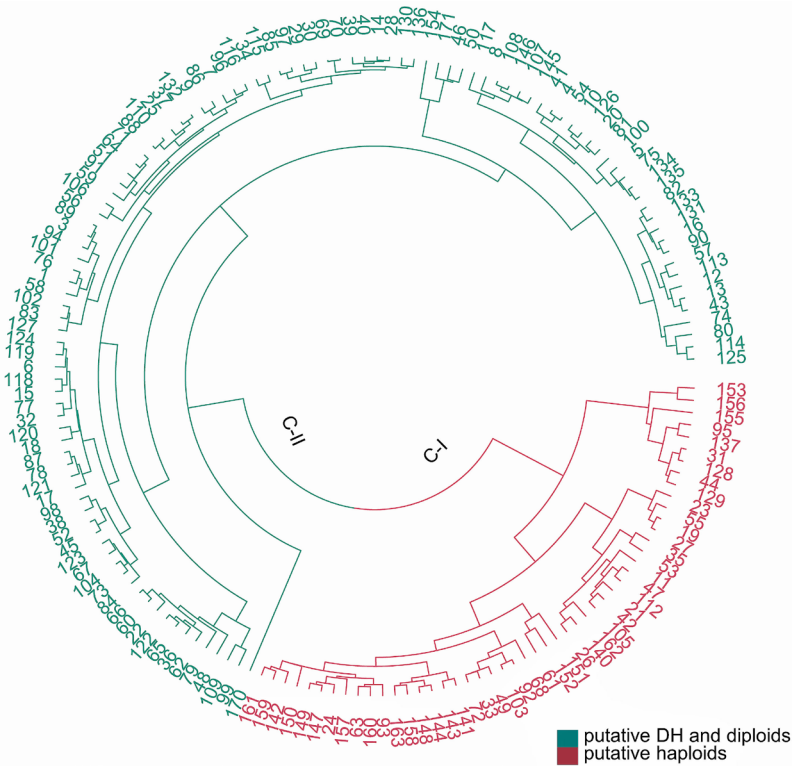

Fig. 5. Circular dendrogram clustering of 159 putative DH/haploids and two parents based on morphological traits. The parents are represented by 170 and 171 as Mahulata and IR20, respectively. Putative DH/diploid plants are numbered in Persian green colour and the magenta colour represents the haploids. C-I and C-II represent the cluster-I and cluster-II, respectively.

Fig. 6. Morphological difference between diploid and haploid rice plants. *A* - plant height difference among diploid and haploid rice plants. *B* - difference in panicle exertion from flag leaf sheath in diploid and haploid rice panicle.

observed a strong correlation between plant height and flag leaf length in the doubled haploid lines in rice.

The coefficient of determination (R^2) points out the amount of variance shared between the two variables. Therefore, to distinguish the haploids from the diploids, one should consider the combination of characters namely plant height, leaf length, total tiller number per plant, and total leaf number per plant as discriminating morphological characters. In agreement with our findings, Ilyushko and Romashova (2019) reported that haploids could be differentiated from double haploids using three to four traits in *japonica* rice lines. Thus, haploids regenerated

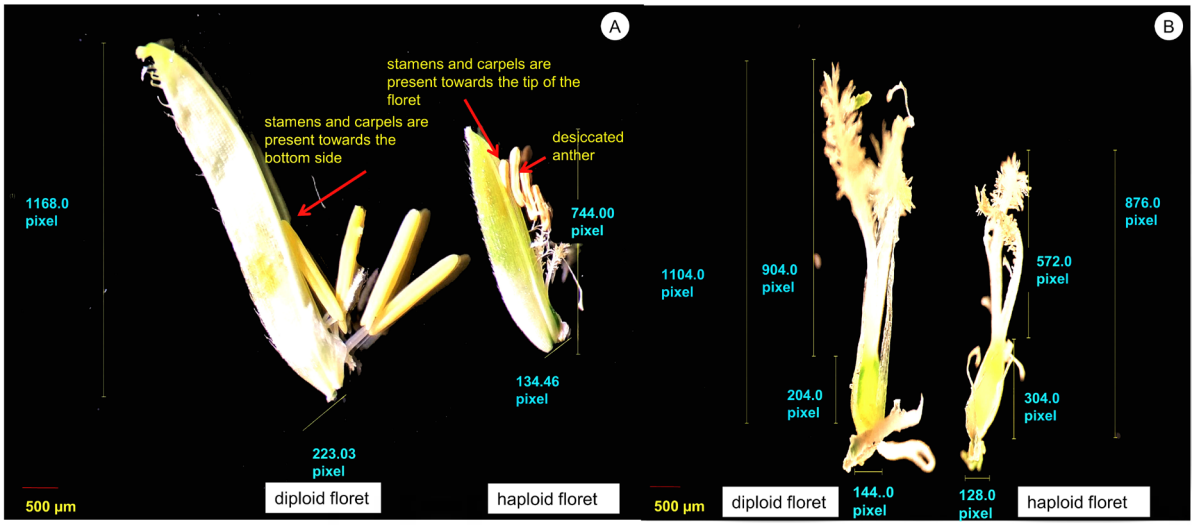

Fig. 7. Morphological discrimination among the floret of diploid and haploid rice plant (*A*), difference in carpel morphology in between diploid and haploid rice flower (*B*).

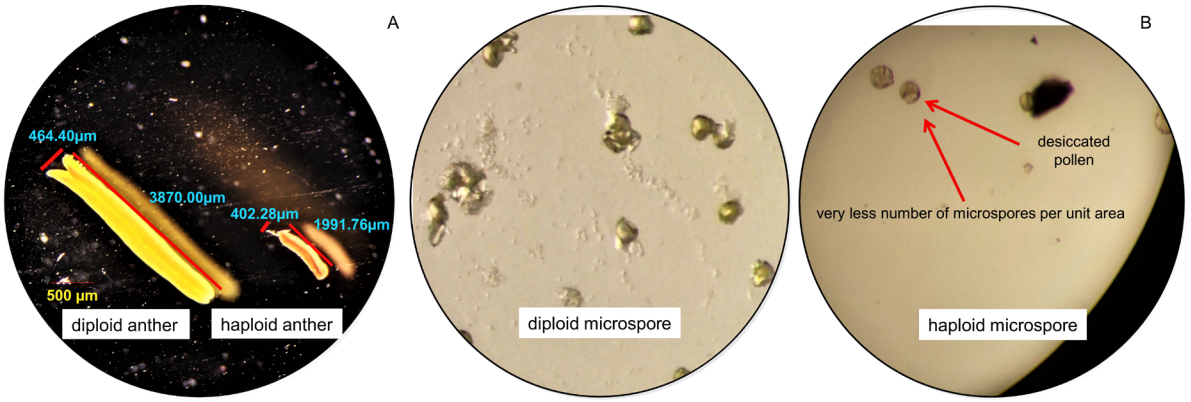

Fig. 8. Variability in anther and microspore among diploid and haploid rice genotypes. *A* - difference in anther dimensions between the pair, *B* - variability in microspore structure and density per unit area in between diploid and haploid rice genotypes.

using *indica* rice as explants could also be differentiated using leaf length, tiller number, and plant height traits.

The traits like tiller number per plant, leaf number per plant, and plant height can be used as morphological characteristics to identify the haploids at the seedling stage. In PCA, the length of the line and its closeness to the circle indicates a good representation of the variable in the plot. Though chlorophyll content index and leaf breadth were poorly represented by the PC1, these two traits cannot be considered for haploid differentiation.

To differentiate the haploids from the putative doubled haploids/diploids, we found that they had smaller leaves, partial panicle exertion from flag leaf sheath (Fig. 5), smaller floret, stamen, and carpels positioned towards the tip of the floret, and very dry stigmatic surface as compared to the diploids (Fig. 6). In addition to the above-discussed traits, all the haploids under study possessed smaller and desiccated anthers and they had very few desiccated microspores per unit area (Figs. 7, 8) when compared to diploids. Thus, improper pollen development and dry stigmatic surface may be the reason for which the pollen was not able to germinate after pollination and produce fertile spikelets in haploids derived from anther culture.

Conclusions

Androgenesis results in the development of plants with different ploidy levels. Hence discriminating the androgenesis-derived plants is one of the major factors in its utilization. The traditional morphological characteristics such as plant height may not always be truthful to the ploidy as environments influence most of them. However, the combination of plant height, tiller number, number of leaves, partial panicle exertion from the flag leaf sheath, anther position, and size along with the floret size was found ploidy dependent. Also, the ploidy level of the experimental material was tested by the qPCR. Thus, this study highlighted the uniqueness of the combination of traits for the identification of haploids in androgenesis in rice. Further, haploid plants identified could be either used for colchicine treatment for doubling chromosomes

or discarded from further analysis due to the sterile nature of haploids.

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