Indian J. Genet. Plant Breed., (2024); 84(4): 635-643
https://doi.org/10.31742/ISGPB.84.4.14
ISSN: 0975-6906

www.isgpb.org

RESEARCH ARTICLE

AMP-PCR-based assay for detection and quantification of genome wide natural methylation in rice

Manikala Chandrasekhar[1†], Chandrappa Anilkumar[5†*], Rameswar Prasad Sah[†], Muhammed T.P. Azharudheen[$], Annamalai Anandan[@], Sasmita Behera, Soumya Priyadarsinee Mohanty, Bishnu Charan Marndi, Sanghamitra Samantaray and Geera Roopa Lavanya[1]

Abstract

Natural and artificial selection efforts combined several favorable alleles of economically important traits in crop plants. However, the progress made is insufficient to meet the future food requirements. Hence, exploring new genetic resources and breeding strategies is important for sustainable improvement in production. The epigenetic variation that alters the phenotype expression without altering the gene sequence has played a crucial role in the process of evolution of modern-day crop plants. The methylation-based epigenetic variations are known to inherit more consistently than other types of epigenetic variation. However, detection and quantification of methylation in the plant genome is costly, hence limiting its utility in crop improvement. In the present investigation, we demonstrated the low-cost but effective approach for detecting and quantification of natural DNA methylation variation in the rice genome by employing custom-designed markers called amplified methylation polymorphism polymerase chain reaction markers (AMP-PCR markers). The methylation detected was scored in an effective method which was further used for quantification. The natural methylation in the diverse population of rice was used to showcase the methylation diversity.

Furthermore, the methylation in germplasm accessions, breeding lines, and released varieties indicated the significant influence of artificial selection efforts on methylation in the rice genome. The genotypes cultivated in different ecologies exhibited different types of methylations. The results ensure the utility of the AMP-PCR assay approach in the detection and utilization of methylation variation at lower costs in crop improvement programs for complex economic traits.

Keywords: AMP-PCR assay, Epigenetics, Genetic gain, Internal and External-methylation, Methylation diversity.

Introduction

The process of evolution changed the morphology of many crop species through the pressurized process of natural and artificial selection over thousands of years (Reznick and Ghalambor 2001). Rice, a staple food crop of more than half of the world's population also known to evolve from grasses to today's form. Although the current snapshot of rice production, consumption, and trade indicates a general surplus, population growth in the coming decades may have an impact (Kastner et al. 2014). Considering the cumulative effects of global climate change, reduced farmland, and the increased needs of a growing global population, modern agriculture is in desperate need of solutions that can ensure global food security and sustainable development. The variation created or emerged through the process

ICAR-National Rice Research Institute, Cuttack 753001, Odisha, India

[1]Sam Higginbottom University of Agriculture, Technology and Sciences, Prayagraj 211 007, Uttar Pradesh, India

[$]Present address: ICAR-Indian Institute of Spice Research, Calicut 673 012, Kerala, India.

[@]Present address: ICAR-Indian Institute of Seed Science, Regional Station, Bengaluru 560 001, India.

[5] Present address: Department of Agronomy and Plant Genetics, University of Minnesota, MN, USA.

[†]These authors contributed equally to this work

***Corresponding Author:** Chandrappa Anilkumar, ICAR-National Rice Research Institute, Cuttack 753001, Odisha, India, E-Mail: anilcgpb@gmail.com

How to cite this article: Chandrasekhar M., Anilkumar C., Sah R.P., Azharudheen M.T.P., Anandan A., Behera S., Mohanty S.P., Marndi B., Samantaray S. and Lavanya G.R. 2024. AMP-PCR-based assay for detection and quantification of genome wide natural methylation in rice. Indian J. Genet. Plant Breed., **84**(4): 635-643.

Source of support: Nil

Conflict of interest: None.

Received: April 2024 **Revised:** Sept. 2024 **Accepted:** Oct. 2024

© The Author(s). 2024 Open Access This article is Published by the Indian Society of Genetics & Plant Breeding, NASC Complex, IARI P.O., Pusa Campus, New Delhi 110 012; Online management by www.isgpb.org

of evolution is extensively utilized in many rice breeding programs. Classical crop breeding is still a powerful method to obtain crops with improved economic traits, but its potential is gradually declining owing to exhausted genetic variation (Chepurnov et al. 2011). Furthermore, the variation that exists naturally for a trait may not be completely due to its inherent property or due to genetic sequences. Research advances in the recent past opened avenues to account for non-genetic components of variation in crop improvement programs (Shen et al. 2018; Xu et al. 2019).

Among the non-genetic components, the environment plays an important role in modifying the expression of the character. However, the environment can affect the pattern of expression of genetic variation which is more commonly known as genotype by environment interactions (Gagneur et al. 2013). On the other hand, epigenetic modifications DNA methylations, are known to have a significant impact on the evolutionary journey of many plant species, including rice (Li et al. 2008). DNA methylation-induced epigenetic modifications are known for their stable inheritance (Ganguly et al. 2018). Utilizing the heritable epigenetic variation in breeding programs could be promising (Dalakouras et al. 2021). Understanding the causes as well as the stability of newly incorporated epigenetic variants over generations will be crucial for fully realizing the potential of epigenetic variation to improve crops (Crisp et al. 2022). Some reports documented the phenotypic variation caused by epialleles for agronomic traits without variation in gene sequences. For example, variations in fruit ripening in tomatoes (Manning et al. 2006), fruit yield in oil palm (Ong-Abdullah et al. 2015), and floral morphology in toadflax (Cubas et al. 1999) are reported to be associated with methylation in the DNA sequence. The epigenetic modifications have a range of effects on phenotype, from limited influence on genetic factors to complete control of the trait (Richards 2006; Meng et al. 2021).

Sources of epigenetic variation have been reported from many crops, from small genome Arabidopsis (Meng. et al. 2021) to orphan legumes like dolichos bean (Ajaykumar et al. 2022) and to major crops like maize (Xu et al. 2020). Yet, limited literature is available on the understanding of epigenetic profiling in major crops. For better utilization of epigenetic variation in crop improvement, exploring the genomic diversity that exists in the crop species and verifying its association with an agronomic trait is crucial because the knowledge on epigenetic variations and regulation of complex traits in crop genomes can help in designing a breeding approach for climate resilience. It further ensures that regular DNA-based markers do not already capture identified epi-alleles. Since DNA methylation epi-alleles are highly stable and heritable, they play a vital role in crop improvement programs along with genetic variants for the trait. Rice being the most extensively researched crop, understanding of the epigenetic variation is limited. Genetic determinants for many agronomic traits including plant characteristics (Qian et al. 2001; Muhammed Azharudheen et al. 2022), yield and nutrition-related characteristics (Jing et al., 2010; Chattopadhyay et al., 2023), panicle characters (Yamagishi et al. 2002; Sah et al. 2023) and grain related characters (Nayak et al. 2022; Anilkumar et al. 2022a) are deeply studied. The genetic variation identified is also being utilized for genomic selection programs for rapid genetic improvement of rice (Anilkumar et al. 2022b; Anilkumar et al. 2023). However, efforts to uncover the epigenetic variation in the rice genome are limited to a few genotypes owing to the cost involved in the epigenome-wide sequence (Kurdyukov and Bullock 2016). Hence, it is important to adopt cost-effective yet efficient approaches that uncover maximum epigenetic variation in rice.

In this vein, the simplest approach of amplified methylation polymorphism polymerase chain reaction (AMP-PCR) assay was utilized by custom designing methylation-specific random amplified polymorphic DNA markers to scan the entire rice genome and identify methylation regions. The objective of the investigation was to capture the maximum methylation variation that exists in the rice genome across a diverse set of genotypes. The results of the investigation have significant scope in the strategic planning of breeding programs to account for epigenetic variation in rice.

Materials and methods

Experimental genetic material

A diverse set of 144 genotypes, which included germplasm lines, released varieties, and advanced breeding lines, was used as genetic material in the investigation (Supplementary Table S1). The released varieties used in the study were developed for cultivation in different cultivation ecologies in various eastern Indian states. These varieties were developed in a time period of over 50 years through different breeding efforts. The various category of genetic material was considered in the present investigation to see the pattern of natural epigenetic modifications in quantitative terms over years of development in comparison to germplasm accessions and advanced breeding lines developed with new breeding approaches.

Methylation-sensitive markers

To detect and quantify the genome-wide methylation in experimental genotypes, an amplified methylation polymorphism-polymerase chain reaction (AMP-PCR) assay was used. AMP-PCR assay utilizes custom-designed random amplified polymorphic DNA (RAPD) primers, which carry a recognition site for restriction enzymes. The restriction enzymes used were isoschizomers, *MspI* and *HpaII*, which recognize and cleave the same recognition site, 5′-CCGG.

Table 1. The information on AMP-PCR-RAPD markers was designed and used in the study

S. No.	Marker name	Marker sequence	Best amplifiable annealing temperature (℃)	Amplicon size (bp) range
1	AMP-PCR-RAPD-1	TGGACCGGTG	39.1	150–600
2	AMP-PCR-RAPD-2	ACCCGGTCAC	39.1	260–610
3	AMP-PCR-RAPD-3	AACCCGGGAA	36.6	180–510
4	AMP-PCR-RAPD-4	TTCCCGGGTT	36.6	230–700
5	AMP-PCR-RAPD-5	TTTGCCCGGT	38.6	255–1000
6	AMP-PCR-RAPD-6	CCCGGCATAA	34.5	140–1200
7	AMP-PCR-RAPD-7	CACCCGGATG	36.8	190–1400
8	AMP-PCR-RAPD-8	TCAGTCCGGG	38.0	160–1200
9	AMP-PCR-RAPD-9	TGCCGGCTTG	41.7	140–700
10	AMP-PCR-RAPD-10	CCCCGGTAAC	36.0	150–1200
11	AMP-PCR-RAPD-11	CAGTGCCGGT	40.4	150–700
12	AMP-PCR-RAPD-12	ACCGGCTTGT	38.1	210–1000
13	AMP-PCR-RAPD-13	GTCCGGAGTG	36.2	200–700
14	AMP-PCR-RAPD-14	ACACCGGAAC	34.9	240–1200
15	AMP-PCR-RAPD-15	CCCGGATGGT	38.5	150–1000
16	AMP-PCR-RAPD-16	AAGACCGGGA	35.5	210–610
17	AMP-PCR-RAPD-17	TCCCGGTGAG	38.0	250–700
18	AMP-PCR-RAPD-18	GAATCCGGCA	35.2	240–700
19	AMP-PCR-RAPD-19	ACCCGGAAAC	34.8	195–1000
20	AMP-PCR-RAPD-20	TGCCGGTTCA	38.1	255–900
21	AMP-PCR-RAPD-21	AGCCGGGTAA	36.2	180–1000
22	AMP-PCR-RAPD-22	CCCGGAAGAG	35.5	160–700
23	AMP-PCR-RAPD-23	CTACCGGCAC	36.9	200–1200
24	AMP-PCR-RAPD-24	ACCTCCGGTC	38.0	310–1250
25	AMP-PCR-RAPD-25	CTCCGGATCA	32.7	160–500
26	AMP-PCR-RAPD-26	TTTCCGGGAG	33.6	140–700
27	AMP-PCR-RAPD-27	AGGCCGGTCA	41.8	130–700
28	AMP-PCR-RAPD-28	CAACCGGTCT	34.3	230–600
29	AMP-PCR-RAPD-29	CCGCCGGTAA	40.1	180–450
30	AMP-PCR-RAPD-30	TCCGGGACTC	37.4	260–600

However, they differ in their sensitivity to the methylation at the recognition site (Salmon et al. 2008). A set of 30 AMP-PCR-RAPD markers were designed by including methylation-sensitive restriction enzyme sites, which enhance the specificity and efficiency of detecting methylation in the rice genome. Detailed information on markers designed and used in the study is provided in Table 1.

Genomic DNA isolation and restriction digestion

The genomic DNA of experimental genetic material was extracted from 15 days old seedlings germinated at a laboratory in the optimal conditions without imposing any stress. The genomic DNA was isolated using the cetyl-trimthyl-ammonium-bromide (CTAB) approach (Doyle and Doyle 1987). The quantity and quality of DNA were tested using a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). The working stocks were prepared by diluting the DNA to 20 ng/µL. The working samples of each DNA sample were replicated in three sets, where two sets were subjected to restriction digestion with two restriction enzymes and one set was maintained as a control set.

Two restriction enzymes used, MspI and HpaII, which are isoschizomers known to have a high frequency of restriction recognition sites in plants (at least one in 256 bp) (Fulnecek

and Kovarik 2014). Considering the small genome size of rice, we hypothesized that restriction recognition sites for these enzymes are highly frequent in the rice genome. The enzymes MspI and HapII have sensitivity to methylation at restriction sites. *MspI* is highly sensitive to methylation at external cytosine, while *HpaII* is sensitive to methylation at internal methylation. However, methylation at both internal and external cytosine in the recognition site is not digested by both enzymes. The digestion of DNA with restriction enzymes was done as per the guidelines of the manufacturer of enzymes (New England Biolabs).

AMP-PCR amplification

Following an AMP-PCR assay, a cleaved restriction fragment of DNA was used for amplification. The digested genomic DNA was amplified using a set of 30 customized AMP-PCR-RAPD markers, each of which contained a CCGG recognition sequence for the *Msp I* and *Hpa II* enzymes. Amplification was done in three sets, one control and one each for MspI and HpaII digested samples. The PCR reaction mixture was prepared with 1-µL of 20 ng/µL template DNA, 1-µL AMP-PCR-RAPD primer, 5 µL standard PCR premix, and 3 µL of nuclease-free water to make up the volume to 10 µL. The PCR program started with an initial denaturation step at 94°C for 2 minutes, followed by an array of annealing temperatures starting from 42°C for 1-minute, 39°C for 1-minute, 36°C for 1-minute, 33°C for 1-minute, and 30°C for 1-minute along with two minutes of elongation was provided for 40 cycles. Additionally, 8 minutes final elongation at 72°C was provided at the end of amplification. The PCR amplified products were separated on a 3.5% agarose gel in an electrophoresis unit and amplicons were visualized in a gel documentation unit.

Methylation amplicon scoring

The AMP-PCR products were scored from a minimum of 4 amplicons per marker to 8 amplicons per marker based on the separation of amplicons for the control DNA sample in the agarose gel. Methylation amplicon scoring was made by comparing the presence of amplicon in the control sample and the presence/absence of amplicon in the *MspI/HpaII* digested samples. The scoring pattern followed for different types of methylation is detailed in Table 2. This scoring was followed for all 30 markers across 144 genotypes.

Quantification of genome-wide DNA methylation

The presence of different types of methylations for a marker allele across genotypes was considered methylation polymorphism. For example, the presence of internal methylation in one genotype and external/full methylation in another genotype for the first amplicon of marker one was counted as methylation polymorphism at that locus. The DNA methylation polymorphism across genotypes was quantified as frequency (%) of genotypes having different methylation patterns or methylation types. The frequencies of different types of methylations were calculated using the following formulae:

Non-methylation (%):

$$\left[\frac{\text{Number of genotypes with Type I amplicons}}{\text{Total number of genotypes}}\right] \times 100$$

Internal methylation (%):

$$\left[\frac{\text{Number of genotypes with Type II amplicons}}{\text{Total number of genotypes}}\right] \times 100$$

External methylation (%):

$$\left[\frac{\text{Number of genotypes with Type III amplicons}}{\text{Total number of genotypes}}\right] \times 100$$

Full methylation (%):

$$\left[\frac{\text{Number of genotypes with Type IV amplicons}}{\text{Total number of genotypes}}\right] \times 100$$

Total methylation (%) = Internal methylation (%) + External methylation (%) + Full methylation (%)

Genome-wide methylation diversity assessment

Genome-wide methylation diversity of experimental genotypes was assessed by converting methylation scores into a binary format considering internal, external, and full methylations as the presence of methylation (1) and no methylation as the absence of methylation (0). The binary pattern of methylation scores was used for the estimation of epigenetic diversity in the experimental genotypes. Methylation score information was used in neighbor-end joining tree construction based on allelic distances in DARwin software, and the distances were subjected to phylogenetic tree construction using iTOL online tool (Letunic and Bork 2007). The scoring information was subjected to principle component analysis using the 'factoextra' package in R software (Kassambara and Mundt 2017) to reconfirm the possible clusters based on methylation patterns. The methylation-based clustering of genotypes was subjected to analysis of molecular variance (AMOVA) to explore the variation within and among methylation groups using 'GenAlex' software version 6.5 (Peakall and Smouse 2012). Furthermore, the different types of methylations were compared among different types of genetic material and genetic material developed for different ecologies used in the study.

Results

Methylation polymorphism and quantification

The AMP-PCR assay with 30 custom-designed RAPD markers amplified a large number of loci across genotypes in the experimental material. Among the several amplifications, four to eight clearly differentiating amplicons on agarose gel were considered for scoring. A total of 120 amplicons

Table 2. Details on methylation scoring pattern and type of methylation

Possible methylation at restriction site	Control	MspI digested	HpaII digested	Score		Methylation status	Methylation type
				MspI	HpaII		
5′-CCGG-3′	+	-	-	0	0	No methylation	Type I
5′-CC^{M}GG-3′	+	-	+	0	1	Internal cytosine methylation	Type II
5′-C^{M}CGG-3′	+	+	-	1	0	External cytosine methylation	Type III
5′-C^{M}C^{M}GG-3′	+	+	+	1	1	Full methylation	Type IV

of different sizes over all the markers were scored. These 120 loci were scored for their differential methylation status based on the property of restriction digestion of isoschizomers. The loci were differentiated into different patterns of methylations following the description presented in Table 2. Combining the information of all the markers alleles over all genotypes, methylation frequencies were calculated and expressed in percentage. Among 144 experimental genotypes, Poornabhog recorded the lowest (15.83%) full methylation, and the advanced breeding line 19 recorded the highest (61.66%) full methylation. On an overall basis, the average total methylation recorded was 48.65% of which full methylation accounted for 41.21% and internal cytosine (5.34%) and external cytosine (2.10%) methylation accounted for very low methylation in the experimental genotypes (Fig. 1). Average of 19.25% of the loci recorded no methylation in the corresponding genomic regions. On the other hand, among 120 marker loci generated by 30 custom-designed AMP-PCR-RAPD markers, AMP-PCR-RAPD 1.1 locus produced the highest methylation (95.10%) alleles, and AMP-PCR-RAPD 11.4 locus produced the lowest (1.38%) methylation alleles in the population.

Methylation variation in different genetic material

The frequency of different types of methylations recorded over all the marker loci across genetic material used in the experimentation was compared. The genetic material considered included germplasm accessions, released varieties, and advanced breeding lines. Comparatively equal frequencies of full methylation were observed for advanced breeding lines and released varieties. However, the full methylation pattern was observed lowest in germplasm accessions (Fig. 2). Internal cytosine methylation and external cytosine methylation also followed a similar trend. Correspondingly, germplasm accessions recorded the significantly highest no methylation frequency, while released varieties and advanced breeding lines recorded relatively low no methylation frequencies.

Methylation variation in genotypes cultivated in different ecologies

The methylation variation in genotypes cultivated in different ecologies was assessed. Irrigated, lowland and upland rice cultivation conditions were considered as different growing ecologies. The experimental material was classified according to the ecology of cultivation. The frequency of different types of methylation was compared with the genotypes cultivated in different ecologies. Different types of methylations showed varied levels in genotypes cultivated in different ecologies. However, the variations were not significant from each other (Fig. 3). However, there was no variation was observed in methylation alleles in genotypes of different ecologies.

Methylation-based epi-genetic diversity in rice

The complete methylation variation explained by different types of methylation in the experimental material was converted into binary score considering methylation and no methylation as only two categories. The binary information on methylation and no methylation was then used for assessing the genome-wide diversity in the rice population. The methylation variation was used to classify the experimental genotypes following the neighbor-end joining algorithm. The phylogenetic tree constructed using iTOL online tool classified the genotypes into three subgroups (Fig. 4a). Among the three subgroups, subgroup 1 had the highest genotypes (91) followed by subgroup 2 with 49 genotypes and subgroup 3 with 4 genotypes. The methylation alleles between the subgroups differentiated subgroups based on the methylation-based molecular variance assessed using AMOVA. The methylation variation between genotypes within subgroups was very less (14%) and the methylation variation among genotypes in different subgroups was high (86%) (Fig. 4b). The methylation alleles detected using AMP-PCR assay were successful in capturing methylation variation between genotypes.

Discussion

In the direction of exploring the epigenetic variation in crop improvement, detection and quantification of sources of epigenetic modifications remains crucial. Among different epigenetic modifications, DNA methylations are most useful owing to their highly stable Mendelian inheritance (Li et al. 2014). Except for the basic studies in the model crops like Arabidopsis (Dubin et al. 2015), there is a research gap that exists in the area of detection and utilization of DNA methylation in genetic improvement programs of higher crops. One of the main reasons for the slow advancement of

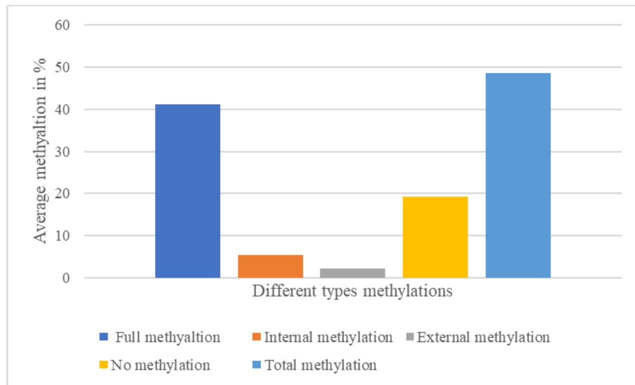


Fig. 1. Quantification of type of methylation in the population. AMP-PCR assay differentiated the type of methylation based on the cytosine molecule methylated. The percentage of methylated alleles of different methylation groups from among the total methylation alleles was used to estimate the percent methylation in each category

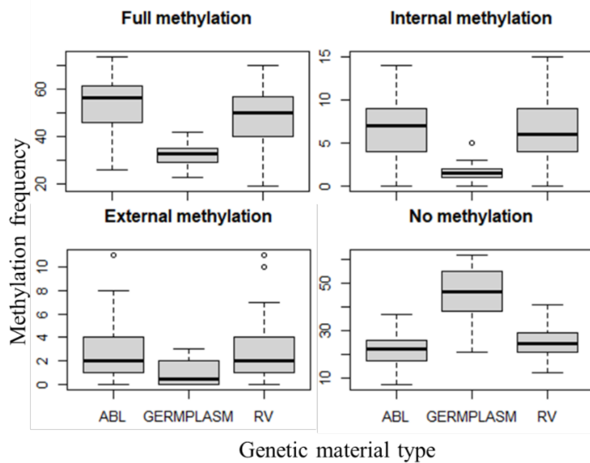


Fig. 2. Differential methylation in different genetic materials. The quantum of methylation in each methylation type was compared among different genetic materials used in the study. ABL: advanced breeding lines, RV: released varieties

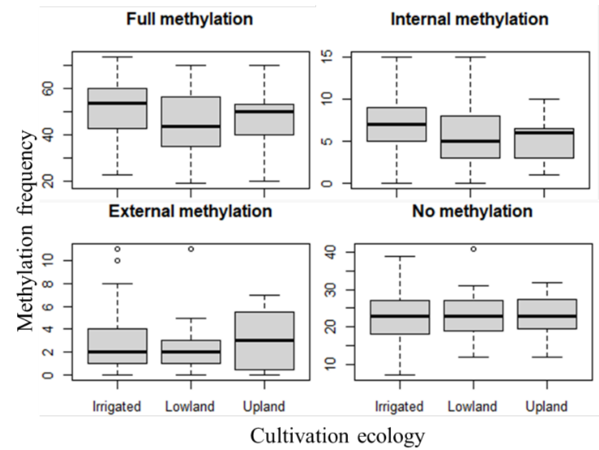


Fig. 3. Differential methylation in genetic materials of different cultivation ecologies. The quantum of methylation in each methylation type was compared among genetic materials cultivated under different growing ecologies

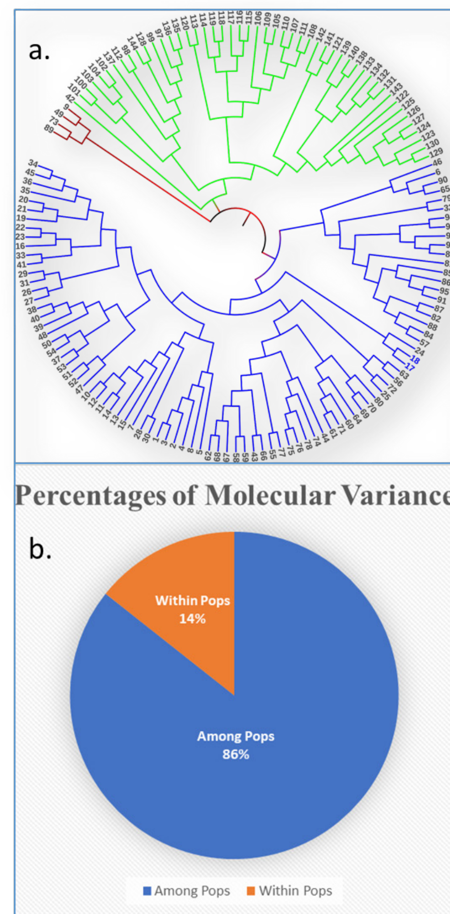


Fig. 4. Methylation diversity in the rice population. (a) phylogenetic tree constructed based on methylation score information depicting the three subpopulations formed from the experimental rice population based on the methylation information and, (b) the subpopulations showed a higher significant variation among subpopulations than within subpopulations

epigenetic improvement in higher crops is the cost involved in base-pair resolution sequencing approaches, including whole genome bisulfite sequencing (WGBS) (Crisp et al. 2022). However, it is important to adopt an approach that can scan for methylation in the entire genome and detect significantly higher coverage of methylation in the genome at a low cost is more useful for implementing regular crop improvement programs.

The AMP-PCR assay adopted in this investigation was found highly useful in capturing genome-wide methylation based on the restriction amplification principle. AMP-PCR markers were successful in differentiating methylated and non-methylated DNA sequences, showing greater polymorphism between DNA-methylated and non-methylated loci in a diverse set of rice genotypes. Since the AMP-PCR markers were custom-designed or modified

versions of RAPD markers, using them in smaller laboratories is quite easy, and the cost involved is also very marginal compared to the sequence-based approach (Phutikanit et al. 2010). Further, this approach is very useful to detect various methylation patterns like internal cytosine methylation, external cytosine methylation, and full/complete methylation of DNA sequences. The methylation alleles were scored in binary form and used for the quantification of methylation in every genotype. The methylation variation in various genetic materials, including advanced breeding lines, released varieties, and germplasm accessions, was captured and quantified. The higher methylation content in the genomes of systematically bred genetic material than germplasm accession suggested artificial selection pressure exerted during breeding varieties inducing the methylation in the rice varieties. Contrasting results of low methylation in breeding lines than germplasm was reported in dolichos bean (Ajaykumar et al. 2021). The induced methylation during the breeding process may produce phenotypic expressions suitable to achieve breeding goals in favorable directions. However, there were no significant differences in methylation variation between genotypes of different growing ecologies because the adoptive traits for each ecology are different. Hence, methylation variation between genotypes of different ecologies is not comparable.

Similar to the genetic sequence-based diversity among genotypes, there exists a diversity in methylation content in genotypes. In experimental rice genotypes, methylation profiles of genotypes detected by AMP-PCR assay explained the methylation divergence among the experimental genotypes. Methylation epi-alleles divided the experimental population into three subgroups based on the epi-allele-based distance between genotypes. Further, the methylation-based molecular variance between the subgroups was more significant than within subgroup variance, suggesting the efficiency of epi-alleles in assessing the divergence of experimental genotypes. Similar results were discussed in maize, where whole genome bisulfite sequencing was used to assess the methylation (Xu et al. 2019). These results may be further associated with any quantitative economic trait and the genotypes from distant groups may be utilized in methylation-based trait improvement.

The results of the experiment have scope in the understanding association of methylation variation with some quantitative economic traits in rice. The methylation allele in the population may be used in the binary format considering methylation and no methylation for further exploitation in trait prediction experiments (Hu et al. 2015). Further, methylation allele information can be utilized to improve the prediction accuracy of genomic selection programs to improve the genetic gain in rice breeding. The AMP-PCR assay approach cannot replace the WGBS approach completely. However, AMP-PCR assay may be utilized for the initial quantification of methylation variation in the plant genome. The results of this investigation provide a new avenue for detecting and exploiting methylation variation in rice improvement programs at a minimal cost.

Supplementary material

Supplementary Figure 1 and Supplementary Table S1 are provided and can be accessed at www.isgpb.org

Authors' contribution

Conceptualization of research (CA, MC); Designing of the experiments (CA, AC, MC); Contribution of experimental materials (RPS, MTPA, BCM); Execution of field/lab experiments and data collection (MC, SB, SP, AA); Analysis of data and interpretation (MC, AC, CA); Preparation of manuscript (MC, AC, CA, SS, GRL).

Acknowledgments

The authors acknowledge the support of ICAR-National Rice Research Institute, Cuttack during the period of this investigation.

References

Ajaykumar H., Ramesh S., Sunitha N.C. and Anilkumar C. 2021. Assessment of natural DNA methylation variation and its association with economically important traits in dolichos bean (*Lablab purpureus* L. Var. Lignosus) using AMP-PCR assay. J. Appl. Genet., **62**: 571-583. https://doi.org/10.1007/s13353-021-00648-x

Anilkumar C., Muhammed Azharudheen T.P., Sah R.P., Sunitha N.C., Devanna B.N., Marndi B.C. and Patra B.C. 2023. Gene based markers improve precision of genome-wide association studies and accuracy of genomic predictions in rice breeding. Heredity, **130**: 335-345. https://doi.org/10.1038/s41437-023-00599-5

Anilkumar C., Sah R.P., Muhammed Azharudheen T.P., Behera S., Singh N., Prakash N.R., Sunitha N.C., Devanna B.N., Marndi B.C., Patra B.C. and Nair S.K. 2022a. Understanding complex genetic architecture of rice grain weight through QTL-meta analysis and candidate gene identification. Sci. Rep., **12**: 1-13. https://doi.org/10.1038/s41598-022-17402-w

Anilkumar C., Sunitha N.C., Harikrishna,Devate N.B. and Ramesh S. 2022b. Advances in integrated genomic selection for rapid genetic gain in crop improvement: a review. Planta, **256**: 87. https://doi.org/10.1007/s00425-022-03996-y

Chattopadhyay K., Bagchi T.B., Sanghamitra P., Sarkar S., Anilkumar C., Marndi B.C., Kumar A., Moharana N., Mohapatra S.S. and Sahoo S.K. 2023. Mapping genetic determinants for grain physicochemical and nutritional traits in brown and pigmented rice using genome-wide association analysis. Euphytica, **219**: 57. https://doi.org/10.1007/s10681-023-03184-3

Chepurnov V.A., Chaerle P., Roef L., Van Meirhaeghe A. and Vanhoutte K. 2011. Classical breeding in diatoms: scientific background and practical perspectives. In: Seckbach J, Kociolek J P. (Eds.), The diatom world, Springer, Dordrecht,

pp. 167-194. https://doi.org/10.1007/978-94-007-1327-7_7

Crisp P.A., Bhatnagar-Mathur P., Hundleby P., Godwin I.D., Waterhouse P.M. and Hickey L.T. 2022. Beyond the gene: epigenetic and cis-regulatory targets offer new breeding potential for the future. Curr. Opin. Biotechnol., **73**: 88-94. https://doi.org/10.1016/j.copbio.2021.07.008

Cubas P., Vincent C. and Coen E. 1999. An epigenetic mutation responsible for natural variation in floral symmetry. Nat., **401**: 157-161. https://doi.org/10.1038/43657

Dalakouras A. and Vlachostergios D. 2021. Epigenetic approaches to crop breeding: current status and perspectives. J. Extl. Bot., **72**: 5356-5371. https://doi.org/10.1093/jxb/erab227

Doyle J.J. and Doyle J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull., **19**: 11–15.

Dubin M.J., Zhang P., Meng D., Remigereau M.S., Osborne E.J., Paolo Casale F., Drewe P., Kahles A., Jean G., Vilhjálmsson B. andJagoda J. 2015. DNA methylation in Arabidopsis has a genetic basis and shows evidence of local adaptation. elife, **4**: e05255. https://doi.org/10.7554/eLife.05255

Fulneček J. and Kovařík A. 2014. How to interpret methylation sensitive amplified polymorphism (MSAP) profiles?. BMC Genet., **15**: 1-9. https://doi.org/10.1186/1471-2156-15-2

Gagneur J., Stegle O., Zhu C., Jakob P., Tekkedil M.M., Aiyar R.S., Schuon A.K., Pe'er D. and Steinmetz L.M. 2013. Genotype-environment interactions reveal causal pathways that mediate genetic effects on phenotype. PLoS Genet., **9**: e1003803. https://doi.org/10.1371/journal.pgen.1003803

Ganguly D.R., Crisp P.A., Eichten S.R. and Pogson B.J. 2018. Maintenance of pre-existing DNA methylation states through recurring excess-light stress. Plant Cell Environ. ,**41**: 1657-1672. https://doi.org/10.1111/pce.13324

Hu Y., Morota G., Rosa G.J. and Gianola D. 2015. Prediction of plant height in Arabidopsis thaliana using DNA methylation data. Genet., **201**: 779-793. https://doi.org/10.1534/genetics.115.177204

Jing Z., Qu Y., Yu C., Pan D., Fan Z., Chen J. and Li C.2010. QTL analysis of yield-related traits using an advanced backcross population derived from common wild rice (*Oryzaru fipogon* L). Mol. Plant Breed., **1**: 1-10. https://doi.org/10.5376/mpb.2010.01.0001

Kassambara A. and Mundt F. 2017. Factoextra: extract and visualize the results of multivariate data analyses. R Package Version 1, 337–354

Kastner T., Erb K.H. and Haberl H. 2014. Rapid growth in agricultural trade: effects on global area efficiency and the role of management. Environ. Res. Lett., **9**: 034015. https://doi.org/10.1088/1748-9326/9/3/034015

Kurdyukov S. and Bullock M. 2016. DNA methylation analysis: choosing the right method. Biol.,**5**: 1-21. https://doi.org/10.3390/biology5010003

Letunic I. and Bork P. 2007. Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. Bioinformatics, **23**: 127-128. https://doi.org/10.1093/bioinformatics/btl529

Li Q., Eichten S.R., Hermanson P.J. and Springer N.M. 2014. Inheritance patterns and stability of DNA methylation variation in maize near-isogenic lines. Genet., **96**: 667-676. https://doi.org/10.1534/genetics.113.158980

Li X., Wang X., He K., Ma Y., Su N., He H., Stolc V., Tongprasit W., Jin W., Jiang J. and Terzaghi W. 2008. High-resolution mapping of epigenetic modifications of the rice genome uncovers interplay between DNA methylation, histone methylation, and gene expression. The Plant Cell, **20**: 259-276. https://doi.org/10.1105/tpc.107.056879

Manning K., Tör M., Poole M., Hong Y., Thompson A.J., King G.J., Giovannoni J.J. and Seymour G.B. 2006. A naturally occurring epigenetic mutation in a gene encoding an SBP-box transcription factor inhibits tomato fruit ripening. Nat. Genet., **38**: 948-952. https://doi.org/10.1038/ng1841

Meng F., Zhao H., Zhu B., Zhang T., Yang M., Li Y., Han Y. and Jiang J. 2021. Genomic editing of intronic enhancers unveils their role in fine-tuning tissue-specific gene expression in *Arabidopsisthaliana*. The Plant Cell, **33**: 1997-2014. http://dx.doi.org/10.1093/plcell/koab093.

Muhammed Azharudheen T.P., Nayak A.K., Behera S., Anilkumar C., Marndi B.C., Moharana D., Singh L.K., Upadhyay S. and Sah R.P. 2022. Genome-wide association analysis for plant type characters and yield using cgSSR markers in rice (*Oryza sativa* L.). Euphytica, **218**: 69. https://doi.org/10.1007/s10681-022-03021-z

Nayak A.K., Anilkumar C., Behera S., Sah R.P., Lavanya G.R., Kumar A., Behera L. and Muhammed Azharudheen T.P. 2022. Genetic dissection of grain size traits through genome-wide association study based on genic markers in rice. Rice Sci., **29**: 462-472. https://doi.org/10.1016/j.rsci.2022.07.006

Ong-Abdullah M., Ordway J.M., Jiang N., Ooi S.E., Kok S.Y., Sarpan N., Azimi N., Hashim A.T., Ishak Z., Rosli S.K. and Malike F.A. 2015. Loss of Karma transposon methylation underlies the mantled somaclonal variant of oil palm. Nat., **525**: 533-537. https://doi.org/10.1038/nature15365

Peakall R.O.D. and Smouse P.E. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol. Ecol. Notes, **6**: 288-295. https://doi.org/10.1111/j.1471-8286.2005.01155.x

Phutikanit N., Suwimonteerabutr J., Harrison D., D'Occhio M., Carroll B. and Techakumphu M. 2010. Different DNA methylation patterns detected by the Amplified Methylation Polymorphism Polymerase Chain Reaction (AMP PCR) technique among various cell types of bulls. Acta Veterinaria Scandinavica, **52**(1): 1-9. https://doi.org/10.1186/1751-0147-52-18

Qian Q., He P., Teng S., Zeng D. and Zhu L. 2001. QTLs analysis of tiller angle in rice (*Oryza sativa* L.). Yi chuanxuebao= Actagenetica Sinica, **28**: 29-32.

Reznick D.N. and Ghalambor C.K. 2001. The population ecology of contemporary adaptations: what empirical studies reveal about the conditions that promote adaptive evolution. In: Hendry A.P., Kinnison M.T. (Eds), Microevolution Rate, Pattern, Process. Contemporary Issues in Genetics and Evolution, Springer, Dordrecht. pp. 183-198. https://doi.org/10.1007/978-94-010-0585-2_12

Richards E.J. 2006. Inherited epigenetic variation—revisiting soft inheritance. Nat. Rev. Genet., **7**: 395-401. https://doi.org/10.1038/nrg1834

Sah R.P., Nayak A.K., Chandrappa A., Behera S., Azharudheen T.P.M. and Lavanya G.R. 2023. cgSSR marker-based genome-wide association study identified genomic regions for panicle characters and yield in rice (*Oryza sativa* L.). J. Sci. Food Agric., **103**: 720-728. https://doi.org/10.1002/jsfa.12183

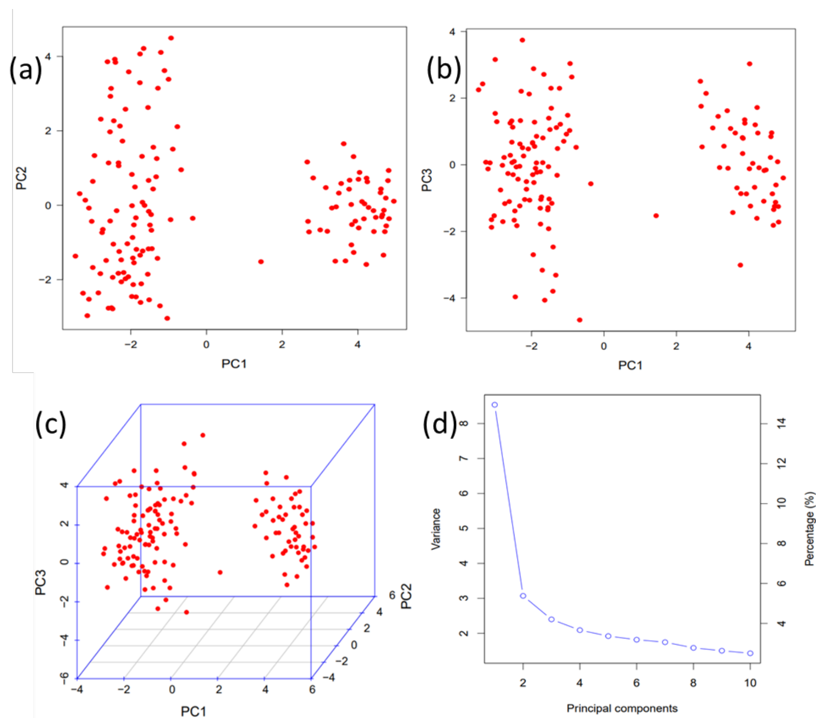
Salmon A., Clotault J., Jenczewski E., Chable V. and Manzanares-Dauleux M.J. 2008. Brassica oleracea displays a high level of DNA methylation polymorphism. Plant Sci., **174**: 61–70. https://doi.org/10.1016/j.plantsci.2007.09.012

Shen Y., Zhang J., Liu Y., Liu S., Liu Z., Duan Z., Wang Z., Zhu B., Guo Y.L. and Tian Z. 2018. DNA methylation footprints during soybean domestication and improvement. Genome Biol., **19**: 1-14.https://doi.org/10.1186/s13059-018-1516-z

Xu G., Lyu J., Li Q., Liu H., Wang D., Zhang M., Springer N.M., Ross-Ibarra J. and Yang J. 2020. Evolutionary and functional genomics of DNA methylation in maize domestication and improvement. Nat. Commun., **11**: 5539. https://doi.org/10.1038/s41467-020-19333-4

Xu J., Chen G., Hermanson P.J., Xu Q., Sun C., Chen W., Kan Q., Li M., Crisp P.A., Yan J. and Li L. 2019. Population-level analysis reveals the widespread occurrence and phenotypic consequence of DNA methylation variation not tagged by genetic variation in maize. Genome Biol., **20**: 243. https://doi.org/10.1186/s13059-019-1859-0

Yamagishi M., Takeuchi Y., Kono I. and Yano M. 2002. QTL analysis for panicle characteristics in temperate japonica rice. Euphytica, **128**: 219-224. https://doi.org/10.1023/A:1020893731249



Supplementary Fig. **1**. Principle component analysis using three components differentiating the population in to two sub-populations based on methylation information. (a) Principle component one and component two differentiating the main population in to subpopulations, (b) Principle component two and component three differentiating the main population in to subpopulations, (c) Three dimensional view of principle components and (d) scree plot showing number of principle components and respective percentage of methylation variation explained

Supplementary Table S1. A list of material used in the present study

S.No.	Genotype	Ecology	S. No.	Genotype	Ecology
1-40	Advance Breeding Lines 1-40	All Irrigated			
Released varieties			*Released varieties*		
41	CR-DHAN-311	Irrigated	83	CR-DHAN-1014	Lowland
42	CR-DHAN-909	Irrigated	84	CR-DHAN-306	Lowland
43	CR-DHAN-800	Irrigated	85	DHARITRI	Irrigated
44	CR-DHAN-508	Irrigated	86	RATNA	Lowland
45	CR-DHAN-506	Lowland	87	SUPRIYA	Irrigated
46	CR-DHAN-209	Upland	88	BINADHAN-10	Upland
47	CR-DHAN-310	Irrigated	89	VANAPRAVA	Irrigated
48	CR-DHAN-301	Lowland	90	TARA	Irrigated
49	CR-DHAN-307	Irrigated	91	UDAYA	Lowland
50	CR-DHAN-203	Irrigated	92	INDIRA	Upland
51	CR-DHAN-101	Irrigated	93	TAPASWINI	Irrigated
52	CR-DHAN407	Irrigated	94	DURGA	Lowland
53	IMPROVED TAPASWINI	Upland	95	CR-DHAN-802	Upland
54	IMP-LALAT	Irrigated	96	KALASHREE	Irrigated
55	SAKTIMAN	Irrigated	97	RADHI	Irrigated
56	JALAMANI	Upland	98	SATTARI	Upland
57	PHALGUNI	Lowland	99	ABHISHEK	Irrigated
58	KETAKIJOHA	Lowland	100	SADABAHARI	Lowland
59	GEETANJALI	Lowland	101	UTKALPRABHA	Irrigated
60	LUNISHREE	Lowland	102	SNEHA	Irrigated
61	CR-SUGANDH DHAN 907	Irrigated	103	Tulsi	Upland
62	CR-DHAN-100	Lowland	104	POORNABHOG	Lowland
63	CR-DHAN-701	Lowland	105	LUNASAKHI	Lowland
64	CR-DHAN-500	Lowland	106	KHIRA	Irrigated
65	SAHABHAGIDHAN	Lowland	107	DHALA HEERA	Lowland
66	ANNADA	Upland	108	JAYA	Irrigated
67	LUNA SUVARNA	Lowland	109	SAKET 4	Lowland
68	KALINGA-1	Lowland	110	SAMALEI	Lowland
69	POOJA	Lowland	111	NEELA	Lowland
70	CR-DHAN-401	Lowland	112	KALANI-2	Lowland
71	SWARNA SUB-1	Upland	113	KHITISH	Lowland
72	CR-DHAN 10	Irrigated	114	TN 1	Irrigated
73	SHATABDI	Irrigated	115	NUA-CHINIKAMINI	Lowland
74	HEERA	Lowland	116	NUAKALJEERA	Irrigated
75	CR-DHAN-507	Lowland	117-136	Advance Breeding Lines, 40-1 to 59	All Irrigated
76	CR-DHAN-510	Upland		Germplasm Lines	
77	Ajay	Upland	137	NIPPONBARE	Irrigated
78	SUMIT(CR-DHAN-404)	Lowland	138	THAVALAKANA	Lowland

79	RAJALAXMI	Upland	139	M-BLACK	Upland
80	CR-DHAN-200	Lowland	140	BINDLI	Lowland
81	CR-BORODHAN-2	Lowland	141	IC 301206	Lowland
82	CR DHAN 309	Upland	142	IC 343465	Lowland
			143	IG -53	Lowland
			144	IG-40	Lowland