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Mapping and validating quantitative trait loci (QTL) for anthocyanin-related genes, coupled with marker analysis for pericarp pigmentation and yield traits in a black and white rice cross

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This study explores the genetic mechanisms underpinning pigmentation traits in rice, with emphasis on the *Kala4* gene responsible for black pigmentation which offers several health benefits. The *Kala4* trait was incorporated into the Improved White Ponni variety to develop a black rice introgression line. Comprehensive genomic analysis identified specific chromosomal regions related to black pigmentation and yield traits. The study evaluated 200 F_2 plants derived from a cross between Manipur Black rice and Improved White Ponni, using 81 distinct polymorphism markers. The results confirmed the crucial role of the *Kala4* loci in black pigmentation and identified key genes regulating anthocyanin synthesis and the *PURPLE PERICARP* trait. This enhanced our understanding of rice pigmentation and its genetic intricacies. The study also evaluated yield-associated traits in the F_2 population, identifying eleven QTLs related to yield, most notably on chromosome 4. These QTLs contribute significantly to phenotypic variance, suggesting their potential for marker-assisted selection in breeding programs. Continued exploration of the *Kala4* gene on chromosome 4 can provide further insights into QTLs for hybrid rice, expediting future rice improvement strategies and allowing for targeted selection in breeding programs, thereby enhancing future rice crop development strategies.

Keywords: Manipur black rice (*Chakhao poireiton*), *Kala4*, QTL mapping, Yield traits

The pigmentation in rice grains, influenced by specific phytochemicals, holds significant importance. While white grains are predominant in rice (*Oryza sativa*), there exist varieties with brown, red, or black grains. Given rice's global importance as a staple food, incorporating coloured rice could have substantial implications for global health. Notably, both proanthocyanidins and anthocyanins, which contribute to grain colour, possess antioxidant properties. Research has extensively explored the health benefits of various foods including rice, soybeans, potatoes, and sweet potatoes[1,2]. In rice, the pericarp of the seed contains two primary anthocyanin pigments are cyanidin-3-o-glucoside (C3G) and peonidin-3-glucoside (P3G) which offer various medicinal benefits, including antioxidant, anti-cancer, and anti-inflammatory properties. Research is carried out in a greater pace for antioxidant properties of several crop sources and their implications[3-6]. However, such benefit in rice offers reach to poor families since it a staple food crop. Pigmentation in rice grains, whether black or red, is attributed to the deposition of anthocyanins and proanthocyanidins, respectively[7]. These pigments serve vital roles in biological processes, such as pigmentation patterns, radio protection[8], plant-microbe interactions, and plant defense mechanisms[9]. The genes *PURPLE PERICARP B (Pb)* and *PURPLE PERICARP A (Pp)*, which are located on chromosomes 4 and 1 respectively, have been identified by conventional genetic research as being critical for black pigmentation[7]. Several molecular markers associated to trait of interest have been identified by statistical approaches[10,11]. Introducing three loci (*Kala1, Kala3* and *Kala4*) from the black rice cultivar "*Hong Xie Nuo*" into the temperate japonica cultivar *Koshihikari* revealed specific genomic regions associated with black pigmentation on chromosomes 1, 3, and 4. The biosynthetic pathway involved in this process also includes structural and functional genes[7]. Additionally, the *C* gene on chromosome 6 plays a pivotal role in anthocyanin synthesis, influencing the presence of red and purple pigments[12].Overall, over 30 genes contribute to the complex regulation of rice pericarp coloration. Key elements include the cloned

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variants like the gene *Pl* responsible for purple leaf, the genes *Rc* and *Rd* for red pericarp, and the *Kala4* gene associated with black rice. Structural alterations in the*Kala4* promoter trigger the anthocyanin biosynthetic pathway, resulting in the emergence of a novel black grain trait. This study sheds light on Manipur black (*Chakhao poireiton*) rice exploration and cultivar enhancement, providing insights into the genetic mechanisms underlying segregation distortion using SSR markers to construct a genetic linkage map within an F_2 population.

Agronomically significant traits, such as yield and its related characteristics, are influenced by multiple genes with diverse effects. Understanding the nature of gene actions, as well as their interactions with other genes and the environment, poses a significant challenge for conventional approaches. Nonetheless, the availability of markers and established linkage maps in rice serve as invaluable instruments for unravelling the genetic underpinnings of quantitative traits *via* QTL analysis[13]. Rice yield is determined by factors including the time taken to reach 50% flowering, the stature of the plant, the number of productive tillers, the length of the panicle, the number of filled grains per panicle, the weight of a hundred grains, and the yield from a single plant. Rice, being a facultative, short-day plant, typically exhibits early flowering under conditions of short days (SD) and postpones flowering under longer daylight (LD) conditions. The characteristic known as "Heading Date" (HD) is of critical importance in determining the adaptability of rice to various cultivation regions and cropping seasons, as it impacts maturation time and grain yield. The development of early- or late-blooming varieties is contingent on environmental factors. Noteworthy QTLs linked to late heading, such as *Ghd7, Hd1, DTH8/Ghd8,* and *DTH7/Ghd7.1*[14], have a strong association with an enhancement in grain yield. This implies that the application of these HD QTLs can markedly influence the productivity of rice and its capacity to adapt to agricultural environments.

The stature of rice plants is an intricate agricultural characteristic guided by a combination of environmental elements and genetic QTL. QTL mapping has emerged as a highly effective method for pinpointing genes that impact rice plant height (PH). A considerable number of PH QTLs have been charted across all 12 rice chromosomes, with a prominent cluster on chromosomes 1, 3, and 4 (www.gramene.org/, accessed on 13 January 2022). Yet, only a handful of principal loci affecting plant height have been isolated, which include the "Green Revolution" genes *sd1/OsGA20ox2*[15], *OsGA20ox1*, and *IPA1*[16]. While *OsGA20ox2* and *OsGA20ox1* contribute to gibberellin synthesis, *IPA1* modifies the structure of the rice plant and boosts grain yield by controlling OsmiR156. On the other hand, multi-effect genes that regulate grain count, plant height, and heading date have been successfully cloned, such as *Ghd7, Ghd7.1, Ghd8*, and *Ghd2*. In recent studies, *qSBM1* was found to augment plant height and aboveground biomass, thereby increasing the number of grains per panicle, grain yield per plant, and nitrogen efficiency[17]. The well-recognized Green Revolution gene, *sd1*, was successfully cloned and defined by three distinct research teams in the year 2002[18].

The pivotal gene *MOC1*, which regulates tiller number, was successfully cloned by Li *et al.*[19].Several genes influencing tiller number have been identified through mutants, including *MOC3*,*FON1* and *DLT10*. A significant number of QTLs impacting Panicle Length (PL) have been meticulously mapped on each of the 12 chromosomes in rice[20]. A minimum of 253 QTLs impacting panicle length have been identified and spread across these chromosomes[21]. Several genes and QTLs that regulate PL have been cloned, most of these influence the growth of primary or secondary branches and spikelets by affecting meristematic activity, including *Ghd7, Ghd7.1,Short Panicle 1, Dense* and *Erect Panicle 1,Dense and Erect Panicle 2,* and *OsRAMOSA2*[22]. Moreover, specific genes like *Dense and Erect Panicle3, OsCD1*, and *OsARG* govern PL by managing cell wall components and crucial growth nutrients. Others, such as *LP, OsPIN5b*, and *OsGRF4,* control PL by affecting hormone metabolism. Additionally*, LONG PANICLE1 (LP1)* a gene that encodes a protein with an unknown function containing a Remorin_C domain has been discovered. Two single nucleotide polymorphisms (SNPs) within *LP1* that lead to amino acid changes have been identified, impacting PL.

The F_2 population has played a crucial role in uncovering QTLs with both additive and dominance effects across the entire rice genome, providing insights into the genetic components of rice yield and its constituents. Notable genes, including *EP3, APO1, DEP/EP2*, *DEP3, SRS3* and *GIF1*, have been identified through QTL mapping. Recent reports emphasize the successful replication of 20 influential grain yield QTLs and associated elements with NIL-F_2 generation, as well as the confirmation of an additional 14 grain yield QTLs in NILs[23]. In this study, an F_2 population derived from the parental lines Improve White Ponni and Manipur Black *(Chakhao poireiton)* revealed the presence of 12 QTLs. Furthermore, the use of SSR markers has aided in the development of a linkage map for all characteristics associated with yield. Insight into molecular markers that are intimately connected to yield-regulating QTLs carries substantial promise for hastening future rice breeding efforts. This understanding enables the swift creation of enhanced strains with specific trait improvements in any high-quality genetic context.

Materials and Methods

Population development

The experimental setup comprised 200 segregating F_2 populations resulting from the cross of two parental lines *viz,* Improved white ponni (characterized by white grains) served as the female parent, while Manipur black (*Chakhao poireiton*) exhibiting black grains was used as the male parent. The distinct grain colors are visually depicted in (Fig. 1). During the 2020 *kharif* season, cultivation encompassed both the F_1 plants and their respective parents, with subsequent identification of successfully crossed plants earmarked for further selection. Following this, the two parental lines, namely Improved white ponni and Manipur black (*Chakhao poireiton*), along with the F_1 plants and the ensuing F_2 population, were evaluated at ADAC & RI, Trichy, during the *Kharif* season of 2022. For this purpose, the nursery was transplanted into a plot measuring 18 x 5 m (length x width), with dense planting at a spacing of 20 cm both within and between rows. This arrangement facilitated the tagging and employment of a total of 200 individuals for genotypic and phenotypic analysis.

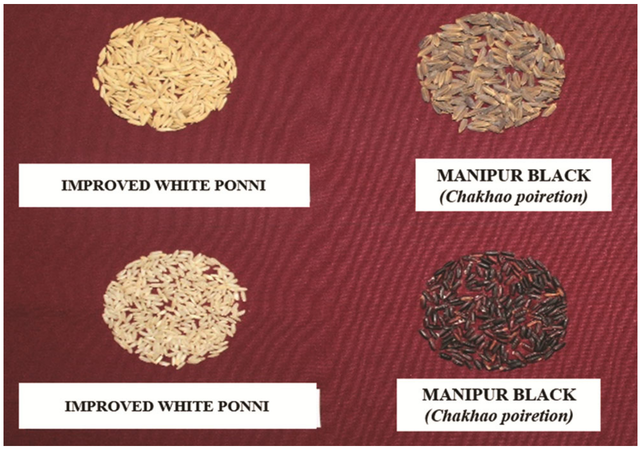

Fig. 1 — Paddy grains and brown rice of parents (Improved White Ponni, Manipur black *(Chakhao poireiton)*

Evaluation of phenotypes

In the current study, a comprehensive set of phenotypic data was meticulously recorded for each F_2 plant, adhering to the DUS guidelines, encompassing a variety of characteristics. Within each row, individual plants were meticulously tagged within the plots. The colour of the grain was visually sorted into three unique categories, namely black, brown, and white. Among these, brown, partial brown, and light brown delineate the finer divisions, though, for the purpose of genetic investigations, the focus remained on the three primary groups. While observations for the parents and F_1 generation were documented on a row-by-row basis, the F_2 population's observations were meticulously recorded on a plant-by-plant basis. To evaluate compatibility with various segregation ratios, recommendation was followed, employing the χ^2 test for individual attribute analysis. In the current investigation, two phenotyping techniques were implemented for evaluating pericarp coloration. Initially, pericarp pigmentation of every accession by noting the presence (1) or absence (0) of pigmentation, a technique referred to as the PA method. To effectively encompass the spectrum of pericarp colour variations within the diverse rice panel, developed a visual scoring card with values from 1 (symbolizing white) to 7 (denoting dark brown), each number signifying a degree of pericarp coloration from light to dark (Fig. 2). Following this, examined the Degree of Pericarp Coloration (DPC) by comparing it to the visual card, and assigned a score to each accession.

DNA marker analysis

Genetic variation was studied using molecular markers technology[24]. Genomic DNA was isolated from the fresh leaves of parental plants and F_2 progeny resulting from the cross between Improved White Ponni and Manipur Black (*Chakhao poireiton*) varieties. A total of 278 SSR markers were strategically employed to achieve comprehensive coverage of all chromosomes throughout the genome. These markers were instrumental in genotyping 200 F_2 lines. Out of the complete marker set, 81 SSR markers exhibiting high polymorphism were selected

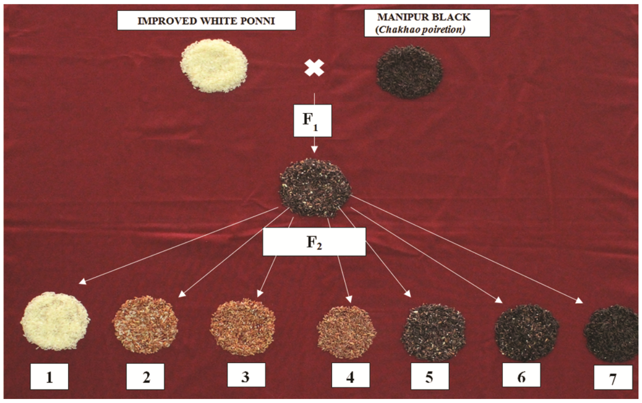

Fig. 2 — Analysis of Pericarp Color Segregation in Black Rice. White pericarp rice Improved white ponni was crossed with a dark purple pericarp Manipur black *(Chakhao poireiton)*

for PCR amplification of the genomic DNA retrieved from the F_2 population, using the CTAB method and PCR was performed as per Gautam *et al.*[25] with slight modifications. The PCR reaction mixture included 10-100 ng of genomic DNA, 0.2 µM primers, 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 0.1 µL of Taq DNA polymerase, which was then amplified. The thermal cycling conditions encompassed an initial denaturation phase at 95°C for 2 minutes, followed by 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and an extension at 72°C for 1 minute, culminating in a final extension phase at 72°C for 3 minutes. Post-amplification, the resulting products were run on 3% agarose gels to identify polymorphic fragments.

In this study, a rigorous investigation into QTLs was conducted utilizing a cohort of 200 F_2 plants and an array of 81 polymorphic SSR markers using specifically designed primers[26] across the landscape of 12 chromosomes. Employing IciMapping, a sophisticated computational tool, Comprehensive Interval Mapping (CIM) was undertaken. The linkage map was used to detect possible QTLs. The examination of QTLs made use of two statistical techniques, interval mapping (IM-ADD) and inclusive composite interval mapping (ICIM-ADD), as elucidated by Li *et al.*[27]. This approach synergistically integrated genotypic and phenotypic data, alongside yield and yield-related parameters, resulting in a comprehensive representation within the final linkage map and putative QTLs. For the assembly of the SSR-based genetic linkage QTL map, we used the Inclusive Composite Interval Mapping method (ICIM) *via* QTL IciMapping software version 4.2[28]. The naming convention for QTLs followed the guidelines set by Mc Couch *et al.*[29]. During the QTL mapping procedure, the Kosambi map function was implemented, with 1,000 permutations used to establish significance levels (at $p = 0.05$), and the LOD threshold was set at 2.5. At significant LOD peaks (that is, equal to or exceeding 2.5), QTL effects were calculated, including the log-likelihood ratio (LOD), additive effect of the found loci, and the phenotypic variation explained (PVE). The LOD test statistic employed was $-2\ln(L0/L1)$, where L0/L1 is the likelihood ratio under the null hypothesis (signifying no QTL) and the alternative hypothesis (indicating the existence of QTL). QTL mapping was carried out for each documented trait along with its corresponding phenotypic means. To ascertain the most appropriate linkage groups, the identified SSR markers were tactically used as anchor identifiers. This decision could involve an explanation of the rationale behind the selection of specific software tools, an elucidation of the significance of using anchor tags in linkage group determination, or a discussion of the broader implications of the genetic linkage map in the context of plant breeding and genetic studies. By incorporating these aspects, the research paper can offer a more comprehensive portrayal of the methodologies and insights derived from the genetic linkage analysis.

QTL significance through LOD scores and linkage analysis

Within the experimental framework, a meticulous strategy was adopted, entailing the utilization of a minimum threshold for the LOD score set at 2.5. The employment of these thresholds facilitated the precise identification of potential QTLs. The establishment of QTL presence hinged upon a rigorous comparison with experiment specific LOD scores, obtained through 1000 permutations at a significance level of $p \leq 0.05$. To ascertain the significance thresholds for QTLs, a meticulous statistical procedure was executed. Through the execution of 1,000 permutations, the Log-likelihood (LOD) threshold for significance ($P < 0.05$) was precisely established. Additionally, a LOD threshold for non-significance ($P > 0.05$) was set at 3 for investigations pertaining to the anthocyanin trait. Specifically, QTLs demonstrating LOD scores below the determined threshold were deemed informative, while those surpassing the threshold were deemed definitive[30]. Leveraging the noteworthy LR peak points from the constructed linkage map, putative QTL locations were effectively pinpointed. For every trait, the additive effect and the efficiency of phenotypic variance (PVE%) were evaluated, thus enriching the understanding of the effect of QTLs. In line with naming conventions, the identified QTLs were judiciously christened. The subsequent identification of QTLs on the genetic linkage map was orchestrated using specific parameters, including a walking speed of 2 cm/s and a significance level of 0.05. The outcome of this process was a meticulous placement of values onto the corresponding genetic linkage map.

Categorizing QTLs, identifying locations and assessing effects

The classification of QTLs was a critical step, distinguishing between those with LOD scores above the designated threshold (definitive) and those falling below (alternative). The putative locations of QTLs were deduced by closely scrutinizing the LR peak points on the linkage map. Furthermore, this analysis encompassed the quantification of additive effects and phenotypic variation efficiency (PVE%) for each trait under examination. This includes an elucidation of the rationale driving the selection of LOD score thresholds, a comprehensive exploration of the significance of composite interval mapping, and an illumination of the broader implications of the QTLs identified within the context of anthocyanin biosynthesis and strategies for crop improvement.

Results

Segregation analysis of F_2 population

A meticulously crafted F_2 population (200 plants) emerged from the crossbreeding of black rice and white rice. This strategic method was designed to analyse the unique roles that the three chromosomal segments have in producing the black pigmentation found in the pericarp. Within this population, a clear division into a coloured group, purple (112 plants) & brown (37 plants) and a white group (51 plants) underscored the segregation pattern, aligning seamlessly with the anticipated 9:3:4 ratio that typifies a gene carrying one dominant and one recessive allele (Table 1). The study capitalized on the potential of the F_2 population for a multifaceted exploration of grain colour. This strategy involved a thorough phenotypic assessment, the creation of a linkage map, and the detection of chromosomal positions of QTLs, all of which were made possible using SSR markers.

Purple pericarp characteristics in this study

Purple pericarp rice is discovered to have a dominating inheritance pattern over white rice. Two genes, *Pp* and *Pb*, which are found on chromosomes 4 and 1, respectively, work in tandem to give rice pericarp its purple color[31]. For the development of purple pericarp in rice, the *Pp* gene works in recessive epistasis with the *Pb* gene[32]. Nevertheless, Lee, 2010[33] recently depicted the *Prp* trait as a synergistic interaction between the *Pb* and *Pp* genes. Leucoanthocyanidin is converted to anthocyanidin during the anthocyanin biosynthesis pathway by the anthocyanin synthase, which is regulated by the *Pp* gene. Specifically, the genotypes *Pb-Pb-* result in a purple pericarp, *Pb-pppp* lead to a brown pericarp, and *pbpbPp* or *pbpbpppp* lead to a white pericarp phenotype. Interestingly, the expression of the *Pp* gene allele can be masked by the presence of the homozygous recessive allele of the *pb* gene. The segregation pattern of the *Pb* and *Pp* genes, pivotal in determining rice pericarp coloration, follows a ratio of 9 purple, 3 brown, and 4 white. In the context of black rice, the pericarp exhibits a range of hues from dark purple to medium purple. Notably, in the F_1 and F_2 generations, medium purple seeds are produced, featuring purple coloration on a brown background. This ratio suggests that the dominance of the *Pp* allele is not sufficient to completely suppress the recessive *pp* allele of the *Pp* gene. Furthermore, the content of cyanidin-3-o-glucoside, a pigmentation compound, in the pericarp of black rice is influenced by the number of *Pp* alleles. Importantly, the dominant *Pp* allele's effect is only partially dominant over the recessive *pp* allele in the context of rice pericarp coloration (Fig. 3).

Biometrical traits

A striking diversity in phenotypic traits was evident within the F_2 population originating from the Improved White Ponni x Manipur Black (*Chakhao poireiton*) cross (Table 2). Among the 200 plants studied, significant variations emerged in attributes

Table 1 — Chi-square analysis of F_2 population IWP x Manipur black (*Chakhao poireiton*) cross

Cross combination	Generation	Plant tested	Purple	Brown	White	Segregation ratio tested	Chi-square (χ^2 test)	Probability (P)
IWP X Manipur black	F_2	200	112	37	51	9:3:4	0.37	0.54

Table 2 — Mean performance parents and F_2 population for biometrical traits

S. no	Variety	Days to 50% flowering	Plant Height (cm)	Number of productive tillers per plants	Panicle length (cm)	Number of filled grains per panicle	Hundred grain weight (g)	Single plant yield (g)
1.	IWP	99	126.19	15.68	26.12	113.85	2.42	34.88
2.	Manipur black	68	101.07	12.09	22.82	96.31	2.36	26.93
3.	F_2 cross	79.02	99.46	15.40	27.92	119.66	2.49	29.91

Cross white (*pbpbpppp*) x Dark purple black (*PbPbPpPp*)

F_1 *PbPppbpp* (Medium purple)

F_2

Gametes	***PbPp***	***pbPp***	***Pbpp***	***pbpp***
PbPp	***PbPbPpPp*** **Purple**	***PbpbPpPp*** **Purple**	***PbPbPppp*** **Purple**	***PbpbPppp*** **Purple**
pbPp	***PbpbPpPp*** **Purple**	***pbpbPpPp*** **White**	***PbpbPppp*** **Purple**	***pbpbPppp*** **White**
Pbpp	***PbPbPppp*** **Purple**	***PbpbPppp*** **Purple**	***PbPbpppp*** **Brown**	***Pbpbpppp*** **Brown**
pbpp	***PbpbPppp*** **Purple**	***pbpbPppp*** **White**	***Pbpbpppp*** **Brown**	***Pbpbpppp*** **White**

Fig. 3 — Genetic variations in the cross between black and white rice varieties

Fig. 4 — Segregation in F_2 generation (Improved White Ponni x *Manipur black (Chakhao poiretion)* and parents

such as Days to 50% flowering (79.02 days), plant height (99.46 cm), number of productive tiller count (15.40), panicle length (27.92), number of filled grains per panicle (119.66), hundred grain weight (2.49), and single plant yield (29.91) (Fig. 4).

Marker banding profiles

The F_2 population's polymorphic marker data unveiled discernible segregation patterns. The banding patterns of markers RM3092 and RM2441 within the F_2 population were visually presented (Figs 5 & 6). Alleles in both parental strains, marked by diverse amplicon lengths, were recognized as polymorphic. This polymorphism resulted in a proportion of 28.08%, evident in the distinctive banding pattern exhibited by select markers when compared to parental profiles (Table 3). Among the total of 200 segregants, 27.22% exhibited banding of parent-1 (Improved White Ponni), 27.53% parent-2 Manipur Black (*Chakhao poireiton*)), and 45.25% showcased heterozygosity (Table 4). For a more comprehensive discourse, potential enhancements could encompass an exploration of the genetic implications underlying these segregation patterns, as well as a broader exploration of the significance of these findings within the context of rice breeding and genetic research.

Construction of genetic linkage map

The linkage map was established using 81 polymorphic SSR markers following the method outlined in Meng *et al.*[28] and implemented through IciMapping v4.2 (Fig. 7). The Kosambi function was employed in constructing the map, resulting in an average marker interval of 156.17 cM. Out of the 278 markers utilized, 81 exhibited varying degrees of segregation distortion across all chromosomes, predominantly favouring the parent Manipur black (*Chakhao poireiton*) (Table 5). The Kosambi mapping function, known for its precision in estimating genetic distances and aiding in the identification of QTLs, was applied adeptly. In this process, a curated selection of 12 polymorphic microsatellite markers was thoughtfully chosen, representing 25% of the total markers The resulting linkage map was methodically organized into eight distinct linkage groups, each thoughtfully aligned with the relevant rice chromosomes. This meticulous mapping approach enabled the accurate allocation of markers to their respective genomic locations.

The ten linkage groups were assigned to their corresponding chromosomes in accordance with the rice chromosome map proposed by Rani *et al.*[34]. The linkage groups were further delineated into specific chromosomes, yielding insights into their

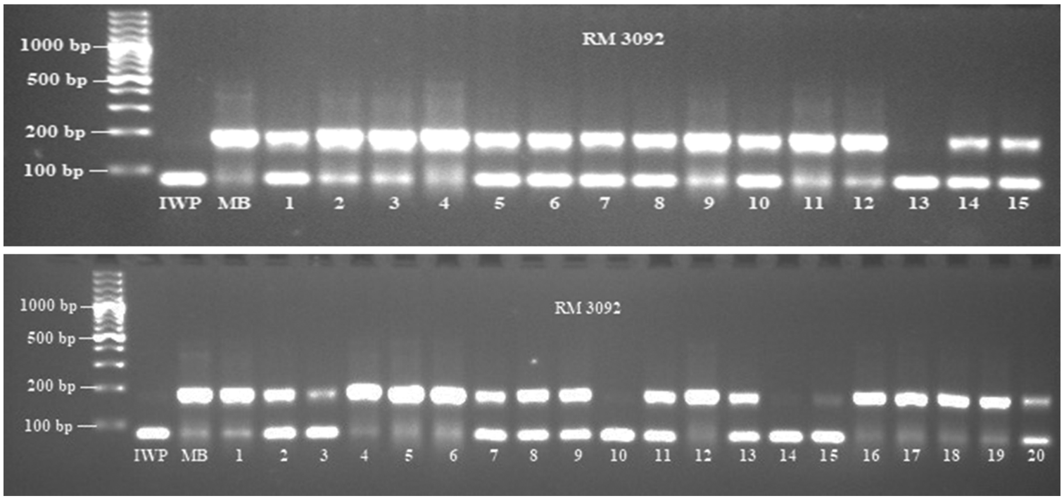

Fig. 5 — SSR-banding pattern analysis for marker RM 3092 in F_2 population using a 100-3000 bp ladder

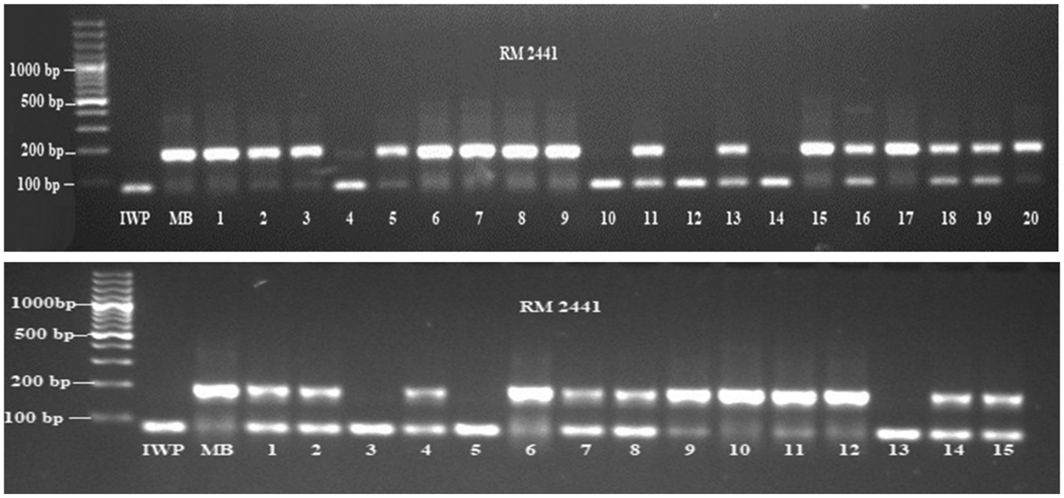

Fig. 6 — Depicting SSR-banding pattern for RM 2441 marker in F_2 population using a 100-3000 bp ladder

Table 3 — Details of polymorphism as characterized by SSR (Simple Sequence Repeat) Markers

Number of SSR primers used	289
Number of polymorphic primers	81
Percentage polymorphism (%)	28.08

Table 4 — Distribution pattern of polymorphic markers in the F_2 generation

Total number of segregants	Segregation percentage
Parent-1 type (A)	27.22
Heterozygote (H)	45.25
Parent -2 type (B)	27.53

genetic landscapes. On chromosome 1, a noteworthy assembly of eight markers collectively spanned a comprehensive map distance of 229.35 cM. Meanwhile, chromosome 2 accommodated a more extensive span, featuring a collection of six markers that encapsulated a genetic region spanning 114.69 cM. The analysis of chromosome 3 revealed a coverage of five markers across a genetic span of 47.80 cM. Similarly, chromosome 4 featured eleven markers within a genetic region encompassing 161.1 cM. Chromosomes 5 and 6 exhibited a slightly reduced marker count, with seven and eight each. These markers corresponded to genetic stretches of 80.80 cM and 90.40 cM, respectively. The distinctive chromosome 7 exhibited a distinct arrangement, hosting seven markers that collectively spanned a genetic distance of 93.60 cM. Transitioning to chromosome 8, a compendium of six markers coalesced to form a genetic span of 57.4 cM. The unique chromosome 9 housed a more modest collection of six markers, collectively contributing to a genetic region spanning 75.50 cM. Furthermore,

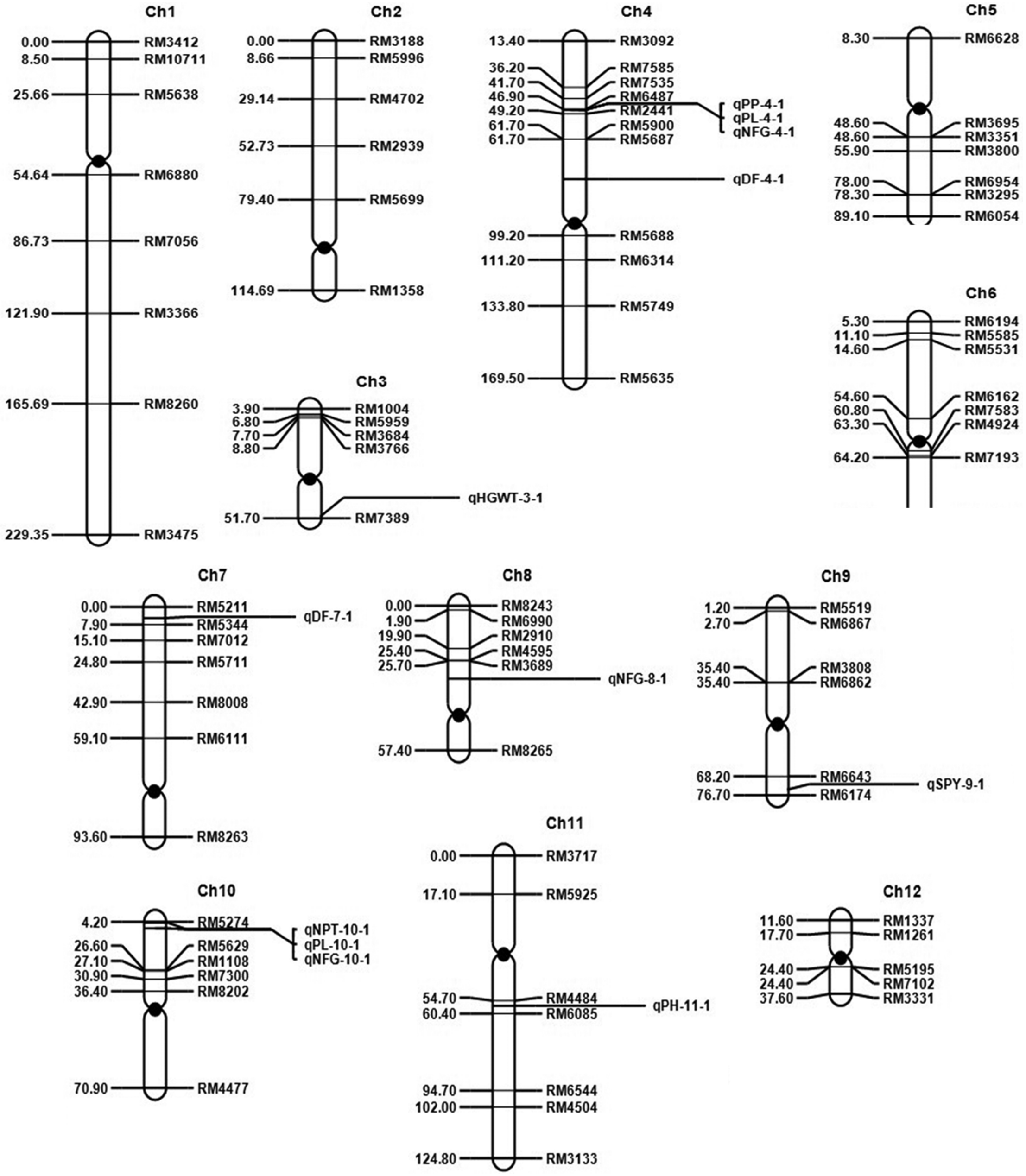

Fig. 7 — Identified QTLs for IWP x Manipur black (*Chakhao poireton*) for anthocyanin (*Kala4*), yield and yield traits related QTLs in F_2 population using IciMapping v4.2

chromosome 10 accommodated six markers, thus contributing to a genetic distance of 66.70 cM. Chromosomes 11 and 12 were also meticulously assessed, with seven and five markers, respectively. These markers conveyed valuable genetic information, contributing to chromosomal spans of 124.80 cM and 26.00 cM. The construction of this genetic linkage map, followed by the QTL analysis, represented a significant progress in deciphering the genetic structure that underlies various traits of interest. The delineation of marker distribution across chromosomes facilitated insights into potential genetic regions associated with trait variations. The utilization of the Kosambi mapping function ensured accurate estimation of genetic distances, thus enhancing the precision of QTL identification.

Detection of QTL for anthocyanin Trait

The standout QTL *qPP-4-1*, situated at 4.67-27.86 Mb on chromosome 4, has emerged as a pivotal regulator of the anthocyanin trait. With a notable

Table 5 — Utilization of Polymorphic SSR Markers for Genotyping the F_2 Generation

S. No	Markers	Chr. No	Mb	Repeat Motif	Tm	Product size	Forward sequence	Reverse sequence
1	RM3412	1	11.58	(CT)17	55	211	AAAGCAGGTTTTCCTCCTCC	CCCATGTGCAATGTGTCTTC
2	RM10711	1	11.16	(GAG)9	55	172	GCTTCGATCGATGAGAAAGTAGAGG	GAATCTCCCATCCTTCCCTTCC
3	RM5638	1	21.87	(AAG)13	55	203	GGCTTCCTCATCGCCATC	CTGAGCAGCATTCCAGTCTG
4	RM7056	1	23.11	(AATT)6	50	149	GAAACGTGTAGCAGTACGCC	ACCAAGCTCTTCATCAACGG
5	RM3366	1	24.26	(CT)16	55	137	TGTTTTGCGTATTTATAGGATG	CAAGAAGTACATGGGACCTG
6	RM8260	1	25.31	(TAT)5	55	192	AATCTAACGTTTGACTATCCATC	TCTACCAGTACTCCCTTCACC
7	RM3475	1	18.72	(CT)22	55	150	GTCGGTTTGCCTAGTTGAGC	TTCCTCGGTGTATGGGTCTC
8	RM3188	2	3.45	(CT)12	50	114	TCACGAGTCGTTCGTTCTTG	CTTGCTGCTCAAGTGGTGAG
9	RM5996	2	2.21	(CCG)8	55	158	CGATTCGCTTCGTTTCCTAC	AAACCAACAGCGACACGC
10	RM4702	2	6.21	(TA)25	55	113	AAATCATGCATGTGAAAG	GAGAAGCAACTCTAATCAAAC
11	RM2939	2	7.42	(AT)39	55	202	CAAGAACTAGACCGGTGTC	CATGGGACCAGCTATTACT
12	RM5699	2	8.98	(AAT)13	55	167	ATCGTTTCGCATATGTTT	ATCGGTAAAAGATGAGCC
13	RM1358	2	10.88	(AG)24	55	180	GATCGATGCAGCAGCATATG	ACGTGTGGCTGCTTTTGC
14	RM1004	3	31.93	(AC)12	55	137	ACGACCCCTCCTGGTTCTG	CTCGTGGTTCTGGTCACAAC
15	RM5959	3	33.51	(CAG)8	55	101	CAACACCACTACCACCTCCC	TTGCTCTCACTCTCCTGCAC
16	RM3684	3	34.61	(GA)15	55	156	TATTTCACCTTCCTGCCACG	GAATGAGGTGGAGGATCGAC
17	RM3766	3	6.93	(GA)18	55	152	TTATAGAGCCAACACAACGG	ATCGATCTCTCTCCTGGAAA
18	RM7389	3	36.51	GATA)7	55	111	AGCGACGGATGCATGATC	TTGAGCCGGAGGTAGTCTTG
19	RM3092	4	27.5	(AT)49	55	181	GTTAAGGTGAAATTCATTGG	ACGACCAGACTCCTACTACA
20	RM7585	4	0.22	(TCTT)6	50	157	CCTCCTCCCTCGACTACCTC	GGTGTGTCGGTGTGATATGC
21	RM7535	4	1.16	(TATG)8	50	125	GACGAAAACCGGTCGAATTC	TCCAACAAGAGTGAGCATGC
22	RM6487	4	4.67	(GCG)8	61	129	AGAAGCTGTAGACGATGGCC	CTAGACCTCATCCCCTTCCC
23	RM2441	4	27.86	(AT)27	55	140	CCATGTGAGTTTAAATTCAC	ATTAACAGATGATGCAAATC
24	RM5900	4	13.76	(ATT)16	55	191	TTCTACGTTTGACCGTCA	TCTAGGAGCGTTTGTAGGAG
25	RM5687	4	15.74	(AAT)17	50	158	GATCGCTGGCGATTGATC	GACTTGTGGGGTGGTTTTTG
28	RM5688	4	1.71	(AAT)17	55	150	GCAGTGTCCAACCATCTGTG	ATCTGGTCACCCTTTGCTTG
29	RM6314	4	18.44	(CTT)11	50	169	GATTCGTGTCGGTTGTCAAG	GGTTCAGGGACGAATTTCAG
30	RM5749	4	19.95	(ACT)8	55	162	GTGACCACATCTATATCGCTCG	ATGGCAAGGTTGGATCAGTC
31	RM5635	4	20.70	(AAG)8	55	167	AGCTGAACACTGCGTTTTAC	GCTAGCTTAGCTTGCTCTCC
32	RM6628	5	17.70	(GGT)8	55	201	GCAGATCTCGTCGGTAGGAG	GCATGGGGTTGGAGATGTAC
33	RM3695	5	19.95	(GA)15	50	203	TCAGTCCACTGCTCACCCC	CCAGAGCGGTTTTGTCCTAC
34	RM3351	5	20.69	(CT)15	55	174	ATGGAAGGAATGGAGGTGAG	TACCCCTACGTCGATCGATC
35	RM3800	5	21.39	(GA)19	55	193	CCTGGAATGATGATGGAAGG	GTTTTGCTTCCTGGAAGTGC
36	RM6954	5	22.01	(TTC)8	67	151	CACAGATGCGAAATGCAGAG	GCGCTGCTGCTAAATTAAGC
37	RM3295	5	22.26	(CT)14	55	92	TCGTGTCATGCGATCGAC	GCTTCGACTCGACCAAGATC
38	RM6054	5	22.77	(CCG)12	55	128	CCCTCCGTACGGATACACAC	CTCTTCGGCTTCATCTCCTC
39	RM6194	6	6.05	(CGG)8	61	167	AGGACCAAGCGTGACAAGAC	ATTGGCTTCTCGTCGTCATC
40	RM5585	6	7.60	(TG)27	55	159	TCAGAGGTGGCAGCTTATTTTATACC	ATGTAAATGGTCACACACACACACAC
41	RM5531	6	7.17	(TG)13	55	158	TTTGTGTTGGTAAGTTGCTTC	TTAAGGAGAGTGTTTTCTTTTCTC
42	RM6162	6	9.19	(CGC)10	55	224	CCCACTCCCACACACTTTTC	ACCGCCGGAGCTAGTGAG
43	RM7583	6	12.44	(TCTT)6	55	160	AGCAGTCAGTCATTCAGCCC	CTGACAAGCCCTTTTCATCC
44	RM4924	6	18.47	(TA)30	55	173	GAAGAGAAGTTTTCGTCTGT	GGATACATTAATCGTTTTCA
45	RM7193	6	20.25	RM7193	50	139	ATGTGGGAATTTCTAGCCCC	CCCTAGTTTTCCAAATGGCC
46	RM3827	6	22.29	(GA)21	55	160	GGACGGATTGTAGGTAGGAC	CCTTTCTTCAATCTGCATTC
47	RM5211	7	0.05	(TA)40	55	197	ATGATGAGATGCCTCAAATC	GAATTGTGCTAGGTAGGCTG
48	RM5344	7	19.04	(TC)13	55	116	ACGAACGGGAGCAAGGTC	CTCTCAACCAAGACGCCTTC
49	RM7012	7	25.24	(AAAG)6	50	89	ATGATTTCTGCTCAGAGGGG	GGCCCAAACAAAAGTCACTC
50	RM5711	7	3.14	(AAT)24	55	145	GTCCATGCATCCATCTCTAG	ACGGAAGGAATACGTCTGTA
51	RM8008	7	4.19	(AT)26	55	144	ACCAAATCTTTAATTCAATG	CCTTCTTCCTTCCTAGTCTA
52	RM6111	7	4.09	(CGC)8	50	94	GAGTCGTCGTCTTCGTCTCC	TCTAGGGCTAGCTCTTCCCC
53	RM8263	7	4.65	(TC)13	55	196	TTTGCTGTCCCTTTGTTC	TGCAATTCAAAGTCTTAGGG
54	RM8243	8	8.93	(CA)11	55	207	CTCGTGCAACCATTATATTC	ACCTTAGCTGTCCTGAATTG
55	RM6990	8	14.36	(TTG)8	50	130	GGTGTGATCCTTTCTGATGC	ACGGGTGTGATCCCAGATAC
56	RM2910	8	16.94	(AT)39	55	182	CAGCTGCTCATATTCATATA	ATAAGGTACTTCATCCGTTA

(*Contd.*)

Table 5 — Utilization of Polymorphic SSR Markers for Genotyping the F_2 Generation (*Contd.*)

S. No	Markers	Chr. No	Mb	Repeat Motif	Tm	Product size	Forward sequence	Reverse sequence
57	RM4595	8	7.03	(TA)23	55	163	AATAGTTGTTGTTTTGGACA	AAATTTAAGTGATTTTGTGC
58	RM3689	8	19.33	(GA)15	55	138	GAAGTTGAGGAACGCGTCAC	GCTGCTCTGTTTTCCTCTGC
59	RM8265	8	20.48	(TC)12	55	198	TCAAAATCACGTGTATGTAAGC	TTTACAAAGGACAGAGGGC
60	RM5519	9	18.92	(TG)12	55	139	GGCCTTTGGTTACCCCTAAC	TGTACAGCAAAAGCAACCCC
61	RM6867	9	19.64	(TGG)8	55	114	AGAGAGCACAATCGGAGTCG	GCAGCAGCAACAAGATGTTC
62	RM3808	9	20.54	(GA)20	55	119	CGTTAGCGAAACGAACAGTG	CAGTGGCTCGGTAATCGC
63	RM6862	9	8.94	(TGC)9	50	113	GGCAAGATCGTTGGAAGAAC	TTACCTGTCGTTTCCCTTCG
64	RM6643	9	21.70	(GTC)8	50	143	TGGTGTTATTCCGAGGCTTC	GAGAGAGAGAGGAGATTTTGGG
65	RM6174	9	22.26	(CGG)8	61	108	TCGAGGTGGAGAAGCAGC	TAGTCTTCGTGTCACGCAGC
66	RM5274	10	17.84	(TA)46	55	179	AATGTTTATCCAACTCATGT	ATTTAGAAACGAGTGTTGTG
67	RM5629	10	18.67	(AAG)11	55	117	AGCTCAACTCGACAACTCCC	CCATCTCCTCTTTCACCTCG
68	RM1108	10	19.16	(AG)12	55	124	GCTCGCGAATCAATCCAC	CTGGATCCTGGACAGACGAG
69	RM7300	10	19.93	(ATTA)6	50	102	TCCGTATCCTAGTCGCGATC	CGCCGTCATGACTCATACTC
70	RM8202	10	11.67	RM8202	55	188	TTGGTCAATAAGAGTGGCCC	TTCTCTCTACCGGAGGATGG
71	RM4477	10	21.10	(TA)20	55	114	AGTAAACATGTCTTCGGGAT	CAGTGCATATTCCACTGGTA
72	RM3717	11	1.17	(GA)16	61	123	AGCTCTACCTTTGCTGTCGG	AACTCCCTAGACCCACCTGC
73	RM5925	11	6.80	(ATT)29	55	231	GTGGAACTCACGGCTAGCC	AGTGTGGCATGACACGAGAG
74	RM4484	11	2.38	(TA)20	55	153	CACTTTATCAAATCGCAATG	CAGTTCGTCCCAAAATAAAT
75	RM6085	11	3.04	(CCT)9	55	200	GGTGAGAGATGGCTAAAGCG	CATCGCCTCTAGCACCTCC
76	RM6544	11	3.85	(GCT)9	50	174	ACCACTATGCACCCTTCGTC	GAATGCTCTGCTTCGTTTCC
77	RM4504	11	5.47	(TA)20	55	127	TAATTGATGAGCTTGATGTA	AGAGAGATTTTATGAAACCA
78	RM3133	11	6.18	(CA)14	55	98	TCAATAGACACACGGGCATG	CGATTTTGCTCACTGCACAG
79	RM1337	12	11.93	(AG)21	55	210	GTGCAATGCTGAGGAGTATC	CTGAGAATCTGGAGTGCTTG
80	RM1261	12	17.53	(AG)16	50	167	GTCCATGCCCAAGACACAAC	GTTACATCATGGGTGACCCC
81	RM5195	12	19.15	(TA)39	55	184	TCTCTGTTCTTGGGTTTAAC	CCGACCAATTTTATTAAGAT
82	RM7102	12	13.21	(AGAT)8	55	169	TTGAGAGCGTTTTTAGGATG	TCGGTTTACTTGGTTACTCG
83	RM3331	12	23.46	(CT)15	50	129	CCTCCTCCATGAGCTAATGC	AGGAGGAGCGGATTTCTCTC

LOD value of 4.09, this QTL underscores its genetic influence in determining anthocyanin content. The substantial phenotypic variance explained (10.29%) suggests that *qPP-4-1* is a key factor contributing to the observed variations in anthocyanin levels. Further investigations into the genes encompassed by the marker intervals RM6487 and RM2441 will illuminate the underlying molecular mechanisms that *qPP-4-1* governs (Fig. 8).

Identifying QTLs for different yield traits

In the present study, used Composite Interval Mapping (CIM), combining genotypic data along with phenotypic and genetic linkage maps, which allowed us to identify a total of eleven QTLs linked with seven yield and yield-contributing traits. Each of these QTLs was defined by specific parameters, such as the chromosome number, marker-interval of peak LOD, additive and dominance effects, peak LOD value, and the proportion of phenotypic variation explained (R^2). These details are presented in (Table 6).

Days to 50% flowering

Two QTLs were identified for days to 50% flowering, located at distances of 15.74-17.71 Mb on chromosome 4 and 0.05-19.04 Mb on chromosome 7, respectively. These QTLs, designated as *qDF4.1* and *qDF7.1*, were delimited by the flanking markers RM5687 (left) and RM534 (right), with corresponding LOD values of 3.32 and 2.64. The additive effects were -12.05 and -5.72, while the dominance effects were -15.48 and 2.68 for *qDF4.1* and *qDF7.1,* respectively. The phenotypic variability explained was 10.09% for *qDF4.1* and 1.84% for *qDF7.1.* The peak is illustrated in the (Fig. 9).

Plant Height

On chromosome 11, a significant QTL, *qPH11,* was discovered spanning from 2.38 to 3.04 Mb. It was flanked by the markers RM4484 on the left and RM6085 on the right. The LOD value for this QTL was 3.09, with an additive effect of -2.39, complemented by a dominance effect of 5.81. Additionally, *qPH11* accounted for 8.15% of the phenotypic variability. The peak is depicted in (Fig. 10).

Number of productive tillers

The only QTL detected for panicle weight was *qNPT1,* situated in the 17.84-18.67 Mb region of chromosome 10, flanked by the markers RM5274 on

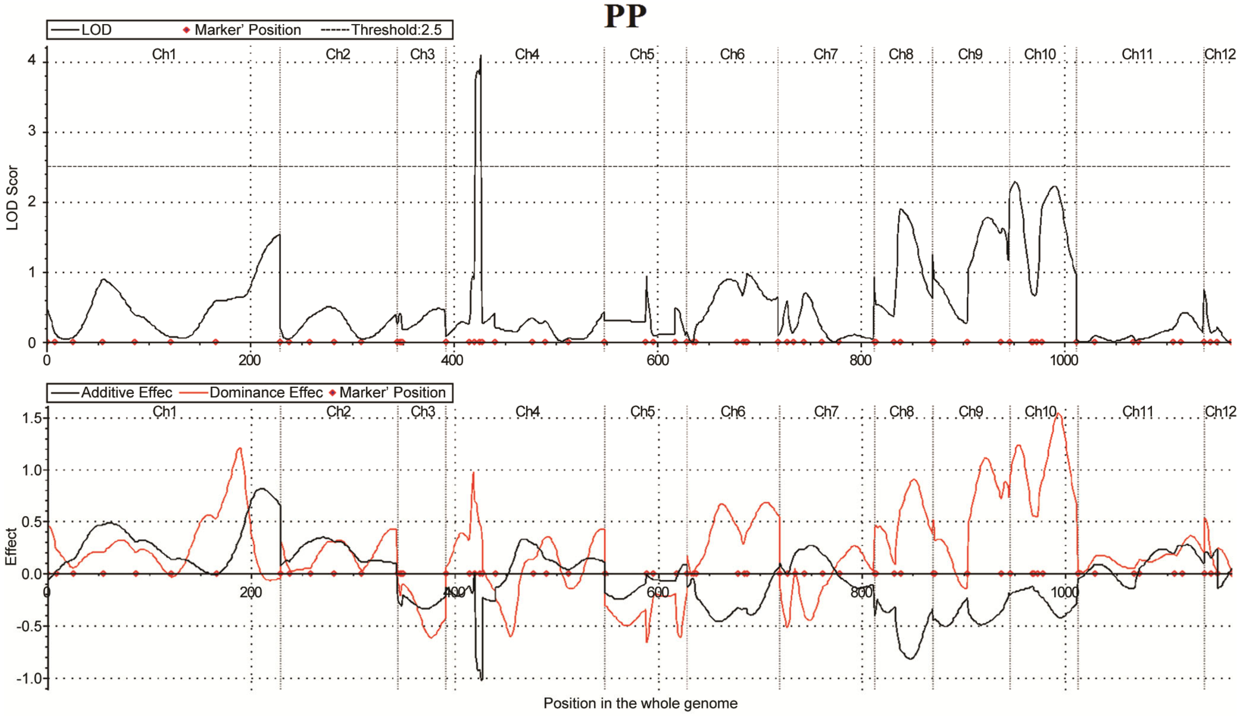

Fig. 8 — QTLs likelihood plots indicating LOD scores, Additive, Dominance effect for anthocyanin pericarp pigment using CIM using IciMapping v4.2

Table 6 — QTL identified for anthocyanin from F$_2$ population

Trait	Chr. no^a	No. of QTLs	Major QTLs identified in present study	Left Marker	Right Marker	Position (Mb)^b	LOD value^c	Effect^d		PVE % (R2)^e	QTLs/genes in this region	References
								Additive effect	Dominance effect			
Anthocyanin	4	1	qPP1	RM6487	RM2441	4.67-27.86	4.09	-1.03	0.44	10.29	(*Kala4*)	Maeda *et al.*[1]
Days to 50 % flowering	4	1	qDF4.1	RM5687	RM5688	15.74-17.71	3.32	-12.05	-15.48	10.09	(qDTF4.34a, qDTF4.34b)	Rajat *et al.*[57]
	7	1	qDF7.1	RM5211	RM5344	0.05-19.04	2.64	-5.72	2.68	1.84	(qDFF7-1)	Marathi *et al.*[21]
Plant height	11	1	qPH11	RM4484	RM6085	2.38-3.04	3.09	-2.39	5.81	8.15	(qPH11.1)	Yuxiang *et al.*[46]
Number of productive tillers	10	1	qNPT1	RM5274	RM5629	17.84-18.67	3.63	-0.36	1.58	11.90	(qTN90-10)	Nagabhushana *et al.*[49]
Panicle length	4	1	qPL4.1	RM6487	RM2441	4.67-27.86	6.67	-1.68	0.26	12.20	(pl4.1)	Zhengzheng *et al.*[58]
	10	1	qPL10.1	RM5274	RM5629	17.84-18.67	2.76	-0.24	1.51	5.17	(LP1)	Erbao *et al.*[59]
Number of filled grains per panicle	4	1	qNFG4.1	RM6487	RM2441	4.67-27.86	5.09	-13.94	4.13	9.19	(qFGP4-1, qFGP4-2)	Marathi *et al.*[21]
	8	1	qNFG8.1	RM3689	RM8265	19.33-20.48	2.61	-11.58	10.36	6.18	(qfgn8.1)	Baoyan *et al.*[52]
	10	1	qNFG10.1	RM5274	RM5629	17.84-18.67	2.77	-3.19	14.29	5.06	(qGP-10)	Wang *et al.*[60]
Hundred grain weight	3	1	qHGWT3.1	RM5548	RM7389	34.89-36.15	3.89	-0.14	0.29	11.36	(wt100-vb3.1)	Untung *et al.*[61]
Single plant yield	9	1	qSPY9.1	RM6643	RM6174	21.70-22.26	3.95	-0.09	1.69	10.81	(qYLD9-1)	Marathi *et al.*[21]

a. Number of Chromosomes, b. Position of QTLs from the initial marker (Mb), c. Individual QTL's Maximum Likelihood LOD score, d. Genotypic effect of the allele, e. Proportion of phenotypic variance attributed to the individual QTL

the left and RM5629 on the right. The LOD value for this QTL was 3.63. It accounted for 11.9% of the phenotypic variation, with an additive effect of -0.36 and a dominance effect of 1.58. Notably, it was contributed by Manipur black (*Chakhao poireiton*) as the donor. The peak is illustrated in (Fig. 11).

Panicle length

Two QTLs were identified for panicle length, located at distances of 4.67-27.8 Mb on chromosome 4 and 17.84-18.67 Mb on chromosome 10, respectively. These QTLs, designated as *qPL4.1* and *qPL10.1,* were delimited by the flanking markers

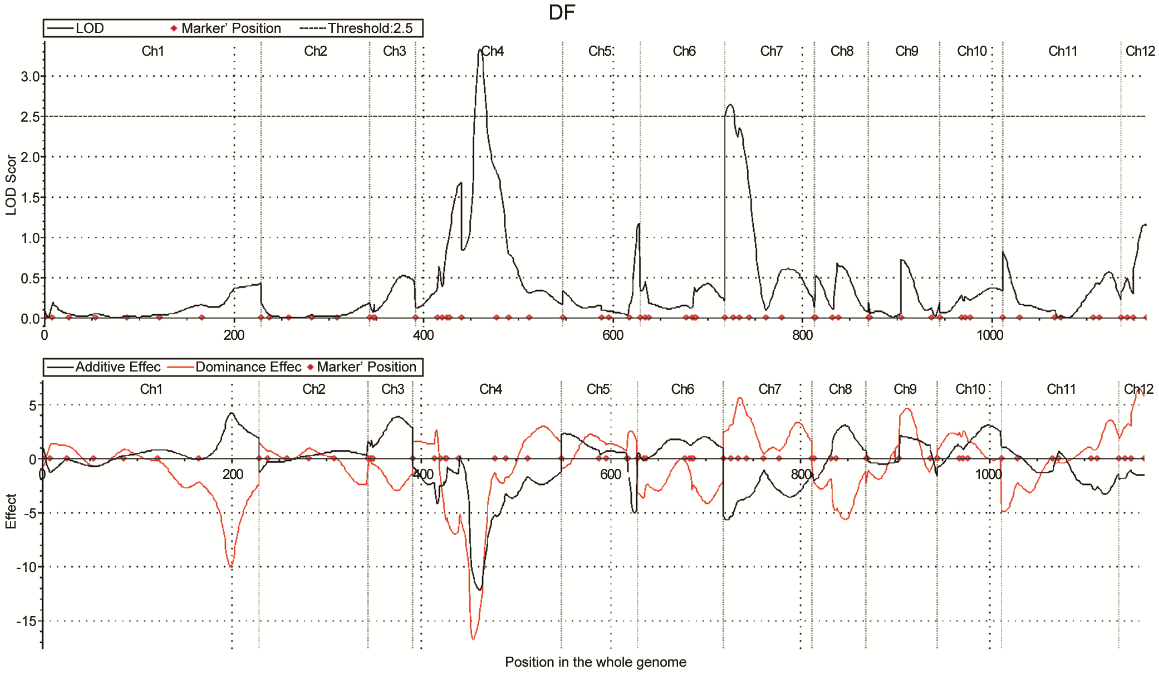

Fig. 9 — QTLs likelihood plots indicating LOD scores, Additive, Dominance effect for Days to 50 % flowering using CIM using IciMapping v4.2

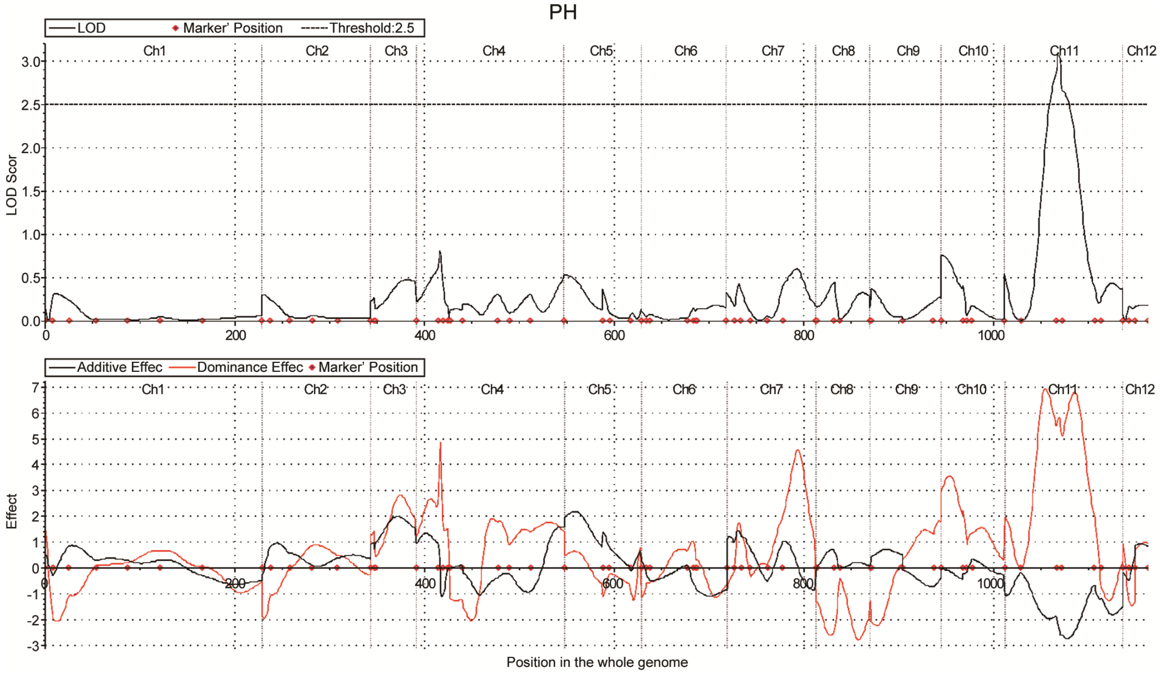

Fig. 10 — QTLs likelihood plots indicating LOD scores, Additive, Dominance effect for Plant Height using CIM using IciMapping v4.2

RM6487 (left) and RM2441 (right), and RM5274 (left) and RM5629 (right), with corresponding LOD values of 6.67 and 2.76. The additive effects were -1.68 and -0.24, while the dominance effects were 0.26 and 1.51 for *qPL4.1* and *qPL10.1* respectively. The phenotypic variability explained was 12.20% for *qPL4.1* and 5.17% for *qPL10.1*. The peak is illustrated in the (Fig. 12).

Number of filled grains per panicle

The number of filled grains per panicle, three QTLs were identified. QTL *qNFG4.1* was situated on

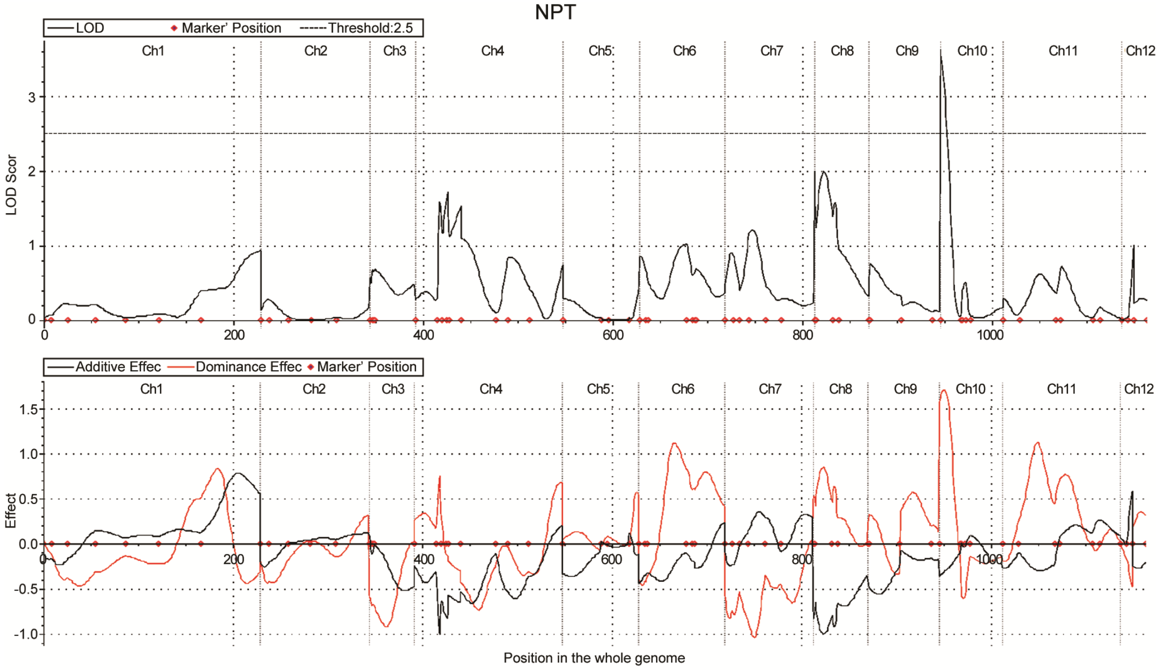

Fig. 11 — QTLs likelihood plots indicating LOD scores, Additive, Dominance effect for Number of productive tillers using CIM using IciMapping v4.2

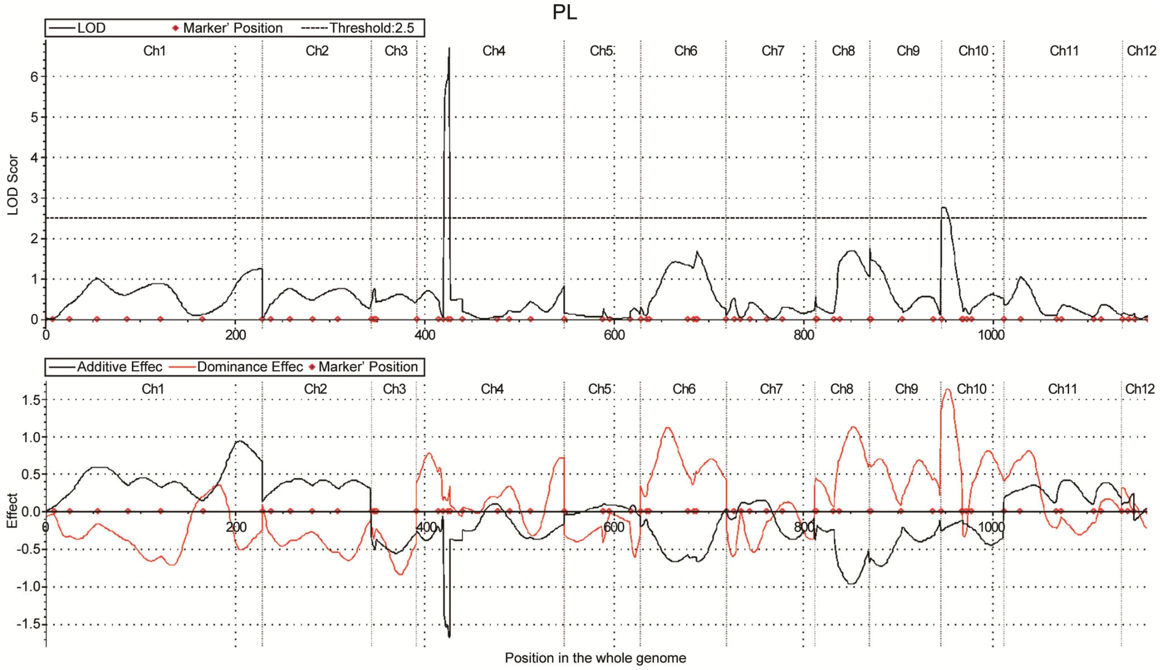

Fig. 12 — QTLs likelihood plots indicating LOD scores, Additive, Dominance effect for Panicle length using CIM using IciMapping v4.2

chromosome 4, flanked by RM6487 on the left and RM2441 on the right, with 4.67-27.86 Mb on the linkage map. QTL *qNFG8.1* was located on chromosome 8, flanked by RM3689 on the left and RM8265 on the right, spanning 19.33-20.48 Mb. Finally, QTL *qNFG10.1* was positioned on chromosome 10, between the flanking markers RM5274 (left) and RM5629 (right), covering 17.84-18.67 Mb. All three QTLs, *qNFG4.1, qNFG8.1,* and *qNFG10.1,* exhibited LOD values of 5.09, 2.61, and 2.77, respectively. The additive effects were -13.94, -11.58, and -3.19, while the dominance effects were 4.13, 10.36, and 14.29.

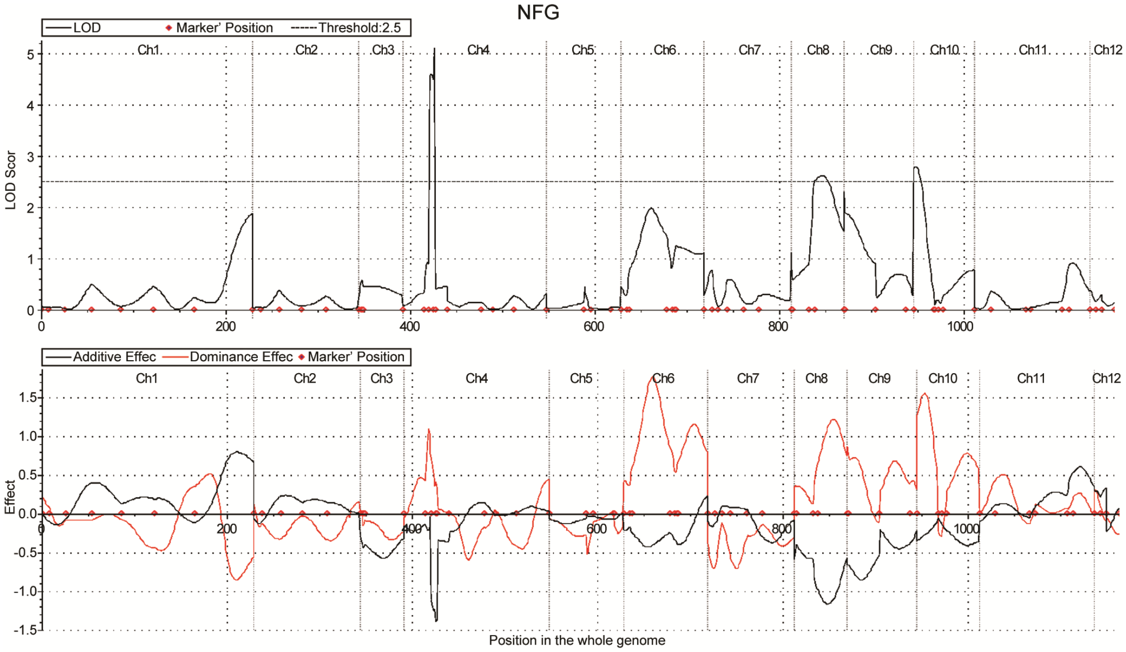

Fig. 13 — QTLs likelihood plots indicating LOD scores, Additive, Dominance effect for Number of filled grains per panicle using CIM using IciMapping v4.2

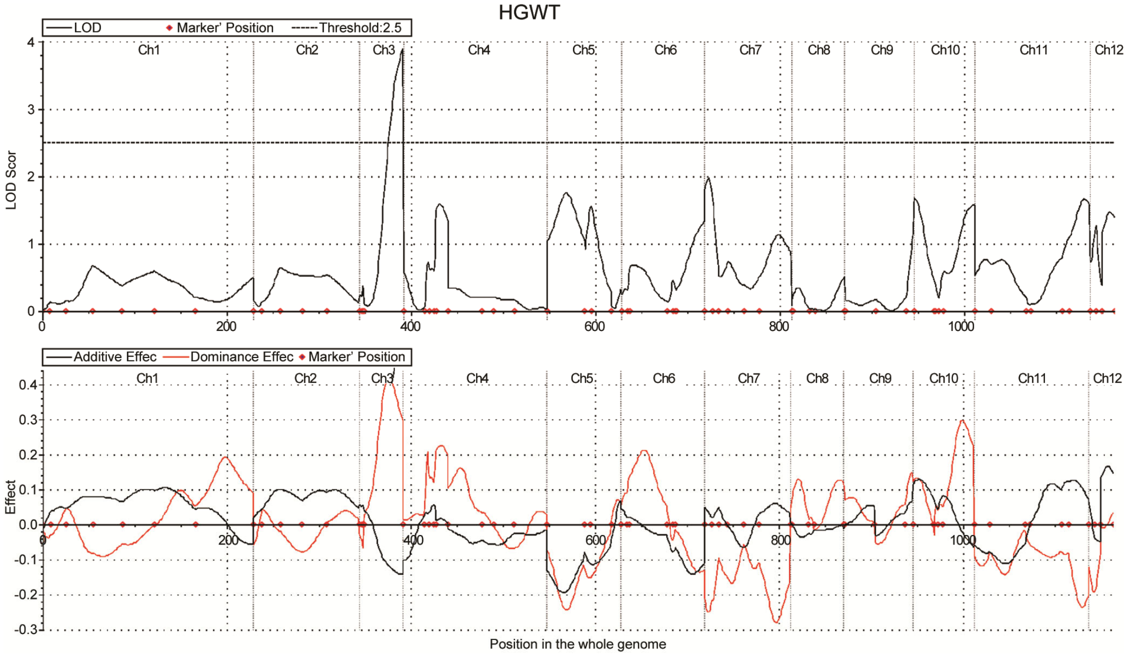

Fig. 14 — QTLs likelihood plots indicating LOD scores, Additive, Dominance effect for Hundred grain weight using CIM using IciMapping v4.2

Additionally, the phenotypic variability explained was 9.19%, 6.18%, and 5.06%, respectively. The peak is depicted in (Fig. 13).

Hundred grain weight

On chromosome 3, a significant QTL, *qHGW3.1*, was discovered spanning from 34.89 to 36.15 Mb. It was flanked by the markers RM5548 on the left and RM7389 on the right. The LOD value for this QTL was 3.89, featuring an additive effect of -0.14 and a dominance effect of 0.29. Additionally, *qHGW3.1* explained 11.36% of the phenotypic variability. The peak is depicted in (Fig. 14).

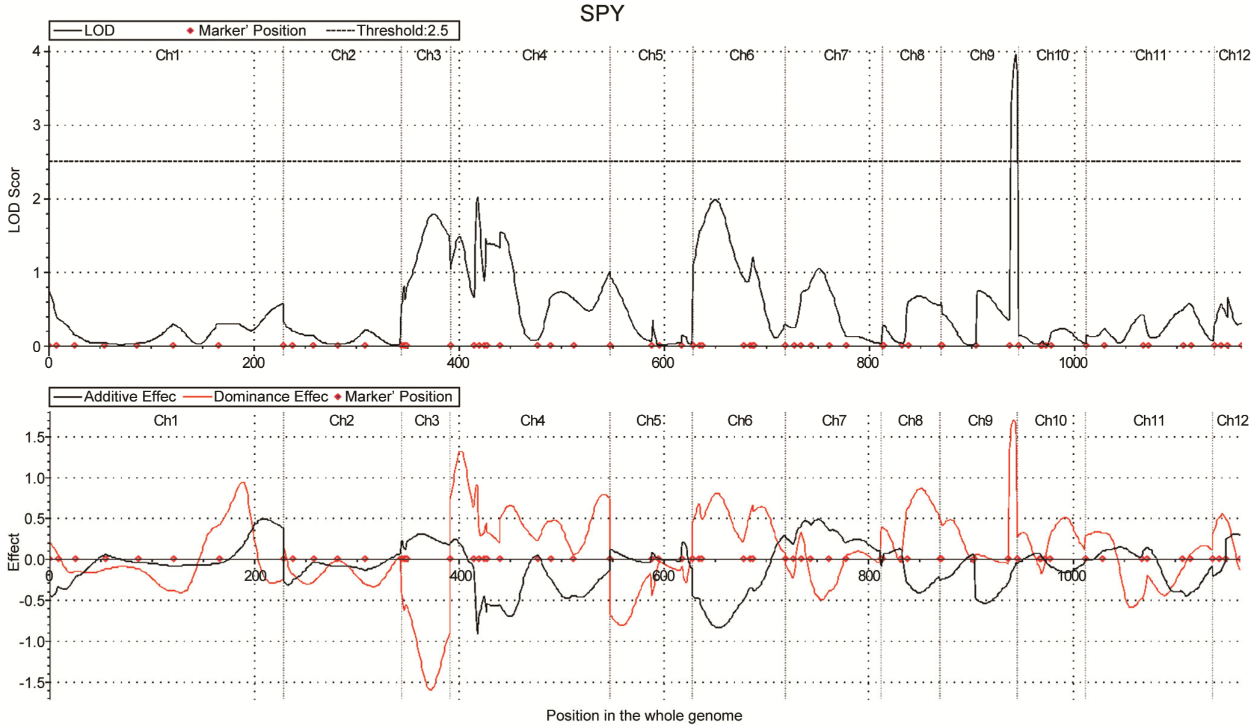

Fig. 15 — QTLs likelihood plots indicating LOD scores, Additive, Dominance effect for Single plant yield using CIM using IciMapping v4.2

Single plant yield

One QTL was identified in association with single plant yield, namely *qSPY9.1*, located at 21.70-22.26 Mb on chromosome 9. It was delimited by the flanking markers RM6643 (left) and RM6174 (right). The LOD value for this QTL was 3.95, possessing an additive effect of -0.09 and a dominance effect of 1.69. Additionally, *qSPY9.1* accounted for 10.81% of the phenotypic variability. The peak is depicted in (Fig. 15).

The regular correlation observed underscored the significance of parameters such as days to 50% flowering, plant height, number of productive tillers, panicle length, number of filled grains per panicle, and hundred grain weight, as criteria for efficient yield enhancement. Our findings indicated that the F_2 population is highly effective for identifying QTLs with an additive effect and can also be utilized to determine the dominance degree of the identified QTLs. Incorporating these findings into the broader context of the studied trait's biology will pave the way for targeted molecular investigations and potential applications in crop improvement. By unraveling the genetic basis of trait variations, these QTLs offer valuable tools for precision breeding and the development of improved cultivars with desired traits.

Discussion

Diverserice pigmentation and segregation patterns in F_2 crosses

Among those phenotypic variations observed between wild and cultivated rice, pigmentation stands out as a key differentiator. Reports indicate that the intricate control of anthocyanin pigmentation extends to various parts of the plant, encompassing leaves, leaf sheaths, pericarp, and hull. Notably, this variation is orchestrated by a complex interplay of multiple genes. The segregation trends observed in F_2 generation crosses between white and red kernel colours. Remarkably, the 9:3:4 ratio observed for the distribution of red and white kernel plants within the F_2 population signifies a close adherence to Mendelian inheritance patterns. This classic ratio underscores the genetic mechanisms underpinning kernel colour inheritance. An intriguing revelation surfaced from the observations made regarding hull coloration. Despite the male parent possessing a black hull colour and the female parent displaying a white hull colour, all F_1 plants exhibited a brown hull. This intriguing phenomenon hints at the complex genetic interactions influencing hull coloration. Remarkably, it is evident that the dominance of black hull coloration is subdued in comparison to the white hull colouring.

Table 7 — Regulatory genes involved in anthocyanin biosynthesis between three loci *Kala1*, *Kala3* and *Kala4* in seed pericarp of rice

Locus	Possible allelic locus/ gene locus	References	Cloned genes	References
Kala1	A	Nagao and Takahashi, 1963[36]	-	-
	Rd	Furukawa *et al.*[37]	DFR	Furukawa *et al.*[37]
	Pp	Wang and Shu, 2007[32]	-	-
	Kala1	Maeda *et al.*[1]	-	-
Kala3	P	Nagao and Takahashi, 1963[36]	MYB	Saitoh *et al.*[39]
	Kala3	Maeda *et al.*[1]	-	-
Kala4	C	Nagao and Takahashi, 1963[36]	-	-
	Pl	Sakamoto *et al.*[40]	bHLH, OSB1 and OSB2	Sakamoto *et al.*[40]
	Pb	-	OSB1	Wang and Shu, 2007[32]
	Kala4	Maeda *et al.*[1]	OSB2	Oikawa *et al.*[7]

Hull colourexpression in F_2generation

The intricacies of hull coloration unfolded further in the F_2 generation. The observed ratio of 9:3:4, in which plants bearing black and white hull colours predominated, offers compelling evidence for the presence of one or both primary genes working in tandem with another dominant gene that governs hull colour expression. These findings suggest an intricate genetic network influencing hull coloration, involving multiple interacting genes. These revelations echo the complexity of trait inheritance in rice pigmentation, underscoring the involvement of multiple genetic factors acting in concert. Unravelling these underlying genetic mechanisms will offer deeper insights into the interplay of genes governing pigmentation, with potential implications for breeding strategies and the development of rice varieties with desired pigmentation traits[35].

Regulatory genes involved for anthocyanin biosynthesis of black rice

The regulatory genes underlying anthocyanin biosynthesis in black rice have been extensively studied, with particular focus on three key loci: *Kala1, Kala3*, and *Kala4*[1] (Table 7). These loci play pivotal roles in pericarp pigmentation and anthocyanin accumulation within black rice. Three genes play a vital role in anthocyanin pigmentation, as per Nagao and Takahashi, 1963[36]reported the Chromogen gene (C), the Activator gene (A), and the gene that regulates tissue-specificity (P).It is plausible that *Kala1, Kala3,* and *Kala4* correspond respectively to *C, A,* and *P* genes. Specifically, *Kala1* aligns with the *A* gene, encoding dihydroflavonol-4-reductase (*DFR*) and mapping to the *Rd* locus on rice chromosome 1[37]. *DFR*, a conserved gene, participates in anthocyanin and proanthocyanin synthesis pathways. *Kala3* likely functions as a *myb* family member, acting as a tissue-specific transcription factor regulator, paralleling the role of *P* genes. Expanding this understanding, In Sweeny *et al.*[38] made the discovery that the Rc gene is responsible for the encoding of a *bHLH* protein. In the context of *Kala4*, the *Pb* gene encodefurus a crucial *bHLH* protein essential for color development in black rice. Intriguingly, a DNA duplication event at the 5' end was correlated with *Kala4*[7]. Both *Kala1* and *Kala4* are imperative for anthocyanin synthesis, *Kala1* correspondence to the *A* gene emphasizing its significance. Molecular cloning efforts have further enriched our knowledge of rice genes involved in anthocyanin synthesis, including *Ra1, Rb, C1*[39] and *Pb*[40]. This multifaceted exploration underscores the intricate genetic network governing anthocyanin production in rice.

Utilization of SSRs markers and IciMapping

In this investigation, the exploration of anthocyanin-related QTLs was conducted using a comprehensive set of 278 SSRs. The IciMapping 4.2was employed to delineate linkage groups and marker order. This systematic approach facilitated the categorization of twelve linkage groups, closely associated with chromosomes 3, 4, 7, 8, 9, 10, and 11 as determined by the CIM analysis. Remarkable variability in LOD values and Phenotypic Variance Efficiency (PVE%) was observed across the chromosomes. Notably, for days to 50% flowering, chromosome 7 exhibited the lowest LOD value (2.64) and the lowest PVE% (1.84). Conversely, for the number of filled grains per panicle, chromosome 4 claimed the highest LOD value (5.09), and forpanicle length,chromosome 4 secured the highest PVE% (12.20%). These distinct values denote varying genetic influences and phenotypic contributions across different chromosomes.

The intricate interplay between anthocyanin-related genes and overall yield traits is noteworthy. Phenotypic expression of yield and its composite attributes is intricately affected by the pleiotropic impacts of anthocyanin genes and environmental conditions. QTL analysis aids in linking phenotypes, marker genotypes, and population characteristics, illuminating the multifaceted relationships that define these traits. The significance of individual QTLs was determined based on their contribution to phenotypic variance. The distinction between major and minor QTLs was drawn based on their capacity to account for phenotypic deviation. Major QTLs explain over 10% of phenotypic variation, while minor QTLs contribute less than 10%. This classification provides a foundation for understanding the genetic weight carried by each QTL.Moreover, small and environment-specific QTLs tend to be overlooked in QTL analyses, despite the limited number of stable QTLs identified[41]. The findings were evaluated considering the studies, the QTLs identified were named in accordance with the nomenclature criteria established by McCouch *et al.*[29].

Chromosome 4 (*Kala* 4) major QTLs for anthocyanin

Anthocyanin is predominantly found in the pericarp of black rice varieties. The gene known as *Ra*, discovered in a prospective region on chromosome 4, is responsible for encoding the basic helix-loop-helix (*bHLH*) transcription factor. This transcription factor is significant for regulating anthocyanin synthesis. A comparative analysis of the phenotypes of the BAB-type Near Isogenic Line (NIL), which carries the *Hong Xie Nuo* alleles of *Kala1* and *Kala4*, and the BBA-type NIL, carrying the *Hong Xie Nuo* alleles of *Kala1* and *Kala3*, emphasized the more crucial role of *Kala4* in black rice[7]. The *Hong Xie Nuo* alleles located at *Kala4* are crucial for determining grain color and have been mapped to a region between RM1354 and RM7210 on chromosome 4, which is a QTL[32]. The cloned genes of anthocyanin biosynthesis were found adjacent to or within three genomic candidate regions. The *Rd* gene, which is involved in proanthocyanidin biosynthesis in rice pericarp, was discovered[37]. However, in rice, only a few genes related to anthocyanin biosynthesis have been identified, such as *Rd*[37], *OsCHI* and *Kala4*[7]. The study identified a total of eight anthocyanin-related QTLs. Among these, one is a minor QTL (*qPP1*) designated as a major QTL, accounting for a significant 80% of the total QTLs. Chromosome 4 (*Kala 4*) emerged as a hotspot for most QTLs, suggesting a pivotal role for this chromosome in governing anthocyanin traits. Further investigation into chromosome 4 holds promise for unravelling key factors influencing anthocyanin QTLs. To enhance QTL validation and stability, creating Recombinant Inbred Lines (NILs) through successive selfing of the F_2 population is proposed. These NILs could serve as crucial tools for confirming the QTLs identified within the population.

Major yield QTLs identified in this study

Eleven putative QTLs governing eight agronomic traits were identified with these loci situated on chromosomes 3, 4, 7, 8, 9, 10, and 11. While these methods yielded largely congruent results, CIM analysis emerged as the most robust in drawing conclusive findings. This is because CIM effectively mitigated background effects stemming from adjacent markers and facilitated a more precise localization of the QTLs.

In the present investigation, one major QTL (*qDF4.1,* 10.09%) and one minor QTL (*qDF7.1*, 1.84%) were identified for days to 50% flowering. Moncada *et al.*[42]conducted a study utilizing the *Oryza rufipogon* genetic background and identified four QTLs that influence heading date. These QTLs were found on chromosomes 2, 3, and 7, accounting for 6-14% of the observed variation. In a separate study, Linh *et al.*[43] working with a population from *O. sativa/O. minuta*, pinpointed two QTLs that regulate heading date or days to heading. These QTLs, located on chromosomes 6 and 9, accounted for a considerable 43.2% of the total variation. Marathi *et al.*[21] discussed the variability in the number and location of detected QTLs, suggesting that the genetic control of the trait can differ based on the mapping populations or genetic backgrounds used in the study. A polymorphism survey was conducted using 15 genes, including *sd1, Ebisud2, Ghd7, Hd1, Hd3, Hd6, PLA1, D10, TB1, HTD1, MOC1, Lax1, GS3, GW2,* and *CKX2*. In a recent study, Amir Sohail *et al.*[44] reported the existence of 14 QTLs associated with heading date, located on various chromosomes: Chr2 (q*HD2a*), Chr4 (*qHD4a* and *qHD4b*), Chr5 (*qHD5a*), Chr6 (*qHD6a* and *qHD6b*), Chr7 (*qHD7b* and *qHD7c*), Chr8 (*qHD8a*), Chr10 (*qHD10a* and *qHD10b*), Chr11 (*qHD11*), and Chr12 (*qHD12a* and *qHD12b*). The findings related to *qDF4.1* and *qDF7.1* will significantly contribute to Marker-Assisted Selection (MAS) and facilitate the development of late-maturing varieties adaptable to a wide range of geographical regions.

In our study, a minor QTL named *qP11.1* was discovered, which influences plant height. This finding is similar to the work of Moncada *et al.*[42], who reported six QTLs responsible for controlling plant height. These QTLs are located on chromosomes 1, 2, 4, and 5, and they account for 6–21% of the variation. On the other hand, You *et al.*[45] reported 17 QTLs distributed across all 12 rice chromosomes that control plant height, explaining variations ranging from 5-23%. In the Lemont′Yangdao4 Recombinant Inbred Line (RIL) population, QTLs in different lines were categorized into eight distinct groups based on the number of height-increasing alleles they carried at 12 loci, namely *qPH1.1, qPH1.2, qPH3.1, qPH3.3, qPH5.2, qPH5.1, qPH8.2, qPH9.1, qPH10.2, PH11.1, qPH12.1,* and *qPH12.3*[46].

In the current research, a major QTL, termed *qNPT1*, was discerned on chromosome 10. It exhibited a Logarithm of the Odds (LOD) value of 3.63 and a significant Phenotypic Variation Explained (PVE) of 11.90%. Furthermore, QTLs *qTN3.1* and *qTN6.1* were identified for the number of tillers, while *qPTN4.1* was recognized for the number of productive tillers[47]. *qTN4* was further narrowed to a 4.08 Mb region on chromosome 4 and divided into two QTLs, *qTN4.1* and *qTN4.2*, utilizing the secondary F_2 population. The respective QTLs explained 34.31% and 32.05% of the phenotypic variance. A similar QTL was reported by Lim *et al.*[48] on chromosome 6, with a LOD value of 3.79. In a study by Nagabhushana *et al.*[49], QTLs on chromosome 10, namely *qTN90-10* and *qTNmat-10*, detected between the markers RM239 and G1084, were specific to tiller number at 90 DAS and maturity.

Panicle length is a key determinant of rice yields and is crucial for optimal plant breeding. As indicated by prior research, panicle length is a complex quantitative trait, regulated by numerous genes, and can be significantly affected by environmental factors[41]. In the present investigation, a major QTL, *qPL4.1*, accounting for 12.20% of phenotypic variance, and a minor QTL, *qPL10.1*, accounting for 5.71% of the variance, were identified on chromosomes 4 and 10, respectively. Research has previously shown variations in the number and impact of QTLs for panicle length, identified through linkage mapping within segregating populations like F_2 populations, Recombinant Inbred Lines (RILs), or backcrossed inbred lines. These variations could explain the identification of roughly 253 QTLs for panicle length across all 12 chromosomes[50]. The gene known as *LARGER PANICLE (LP)* encodes a protein containing Kelch repeats, which is vital for increasing the numbers of both spikelets and branches. This gene also plays a regulatory role in cytokinin concentration within plant tissues. Another QTL was discovered on chromosome 10, accounting for 7.67% of the phenotypic variation. *qPL4.1* was consistently identified early in the season in Nanning in 2014 and 2015, whereas *qPL10.1* was exclusively identified. QTLs for productive tiller number on chromosome 4 were reported by Lim *et al.*[48] in an indica-japonica recombinant inbred line population. Kavitha *et al.*[51] reported that *qPL4.1* was identified with different flanking markers in F_2 and $F_{2:3}$. It explained 8.1% of the phenotypic variance on chromosome 4 with a LOD value above 2.5.

Three minor QTLs, namely *qFGN4.1, qFGN8.1,* and *qFGN10.1*, were identified for the number of filled grains per panicle. The quantity of primary branches per panicle displayed a significant increase in the following QTL-NILs: *PB1121-qGN4.1*, Samba Mahsuri-*qGN4.1*, Swarna-*qGN4.1, IR64-qGN4.1, CSR30-qGN4.1, Ranjit-qGN4.1*, and *PB1-qGN4.1*. Similarly, the count of secondary branches per panicle showed a significant rise in *PB1121-qGN4.1,* Samba Mahsuri-*qGN4.1, IR 64-qGN4.1, MTU1010-qGN4.1*, and Ranjit-*qGN4.1*. The total grain (spikelets) count and the number of well-filled grains per panicle both saw a significant increase in the QTL-NILs across all 12 Recurrent Parent (RP) genetic backgrounds. Three QTLs for full grain number (FGN) were identified on chromosomes 8, 11, and 12, explaining 63.7% of the phenotypic variation. The alleles from Nagdong contributed to a rise in the full grain number at *qfgn11.1* and *qfgn12.1*[52].

A QTL named *qHGW3.1* on chromosome 3, with a LOD value of 3.89, was detected for the trait of hundred grain weight. This QTL accounted for 11.36% of the phenotypic variation, indicating its significance as a major QTL. Moncada *et al.*[42] identified five QTLs linked to hundred grain weight, all originating from *Oryza rufipogon*. These QTLs were located on chromosomes 1, 3, and 11, explaining variations between 5% and 22%. The QTL on chromosome 1 could be in proximity to the one identified in the current study (*wt100-vs1.1* at 66 cM). Septiningsih *et al.*[53] also reported five QTLs governing grain weight, situated on chromosomes 1, 2, 3, and 7, accounting for variations ranging from 4% to 11%. A QTL on chromosome 3 was also detected in the present study. However, due to the use of different markers in various studies, it is difficult to ascertain if it could be the same QTL. Two QTLs, *wt100-vb2.1* and *wt100-vb3.1*, were identified for the weight of a hundred grains. The first one, contributed by IR75862-206-2-8-3-B-B-B, was located at 108 cM on chromosome 2 (between RM5430 and RM526), with an additive effect of 0.14 g, explaining 18.9% of variation. The second one was contributed by IR64, located at 68 cM on chromosome 3 (between RM1324 and RM6931), explaining 19.3% of the variation.

A significant QTL, *qSPY10.1*, was pinpointed on chromosome 9 within the 21.70-22.26 Mb location, explaining 10.81% of the phenotypic variation (PVE). Another QTL, *qSPY4.1*, which accounted for a PVE of 8.5% and had a LOD value of 2.6, was also detected in the current study. This finding corroborates the previous studies by Haritha *et al.*[54], who also reported QTLs linked to grain yield on chromosome 4. Peng *et al.*[55] also reported *qGYPP4* in a Backcross Inbred Line (BIL) population derived from *Oryza longistaminata*. Moreover, a key QTL, termed *qYLD4-1*, was recognized at Aduthurai with a LOD score of 3.28, accounting for 15% of the phenotypic variation. In New Delhi, a location-specific QTL, *qYLD9-1*, was pinpointed within the marker interval RM160-RM201, with a LOD score of 6.88, it contributed to 11% of the phenotypic variation[21].

Rice yield is decreased by accumulation of anthocyanin pigment in the pericarp

In comparison to hybrid and white rice, coloured rice generates reduced yield[55]. The deposition of pigment in coloured rice's spikelets might be responsible for the reduced chlorophyll content, thus contributing to the yield decline. The reduction in hundred seed weight could be attributed to the smaller sink, indicating that purple pericarp rice possesses a smaller sink. Black rice with smaller seeds exhibits slower rates of photosynthetic activity and grain filling, leading to ultimately lower yields.

To enhance mapping precision, high-resolution mapping techniques can be employed to augment the QTL-enriched chromosomal regions with additional markers. The frequent occurrence of potential QTLs in both homozygous and heterozygous states further underscores their genetic significance. With additional validation, the genotypes harboring putative QTLs could serve as valuable resources in marker-assisted breeding initiatives, propelling the development of improved rice cultivars with enhanced anthocyanin-related traits. The elucidation of anthocyanin-related QTLs through a comprehensive exploration of genetic markers and sophisticated software tools contributes significantly to our understanding of complex trait inheritance. Anthocyanins serve as a valuable asset, even utilized as environment friendly biosensors[56]. The intricate interplay of genes, their varying impacts on different chromosomes, and the underlying genetic mechanisms have important implications for both fundamental research and practical breeding applications.

Conclusion

In conclusion, the vibrant purple colour exhibited by rice tissue and organs can be attributed to anthocyanins, a crucial group of flavonoid compounds[62-64]. The growing trend towards developing anthocyanin-rich functional foods underscores their significance. Gaining insights into various aspects such as assimilate distribution, source-sink dynamics, anthocyanin buildup during chlorophyll degradation, grain maturation, and yield in the context of black rice holds significant importance. The observed phenotypic diversity among the F_2 population resulting from the cross between Improved White Ponni and Manipur Black (*Chakhao poireiton*)varieties underscores the complex inheritance patterns underlying rice pigmentation. Incorporating our F_2 lines, characterized by high antioxidant activity and good palatability, into a regular diet can lead to beneficial health effects. The utilization of SSR markers and the IciMapping software facilitated the construction of a comprehensive genetic linkage map, revealing distinct linkage groups on different chromosomes. Notably, chromosome 4 emerged as a hotspot for anthocyanin-related QTLs, with both major and minor QTLs contributing to the observed genetic variation. These findings emphasize the importance of understanding the interactions between multiple genes and their impact on trait expression and yield traits. The identification of QTLs, both major and minor, provides valuable insights into the genetic architecture of anthocyanin and yield traits. Chromosome 4 *(Kala4*) plays a pivotal role in shaping anthocyanin-related variations. Furthermore, we detected a total of 11 QTLs using 200 individuals of the F_2 population of Manipur black (*Chakhao poireiton*) crosses, including the identification of six major QTLs, namely, *qPP1.1,qDF4.1, qNPT1, qPL4.1, qHGW3.1,* and *qSPY9.1* for anthocyanin and yield-related traits. The presence of potential QTLs in both homozygous and heterozygous states underscores their genetic significance.

To enhance mapping precision, we recommend the use of high-resolution mapping techniques and the development of Recombinant Inbred Lines (RILs). These strategies will contribute to a finer understanding of the genetic architecture underlying anthocyanin and yield traits. Importantly, our findings have implications for both fundamental research and practical breeding applications. By leveraging the knowledge gained from this study, it becomes possible to develop rice varieties[65] with enhanced anthocyanin-related traits and good yield through strategic breeding programs. The genetic insights provided here contribute to a deeper understanding of rice pigmentation and provide a foundation for the advancement of rice crop improvement strategies.

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Conflict of Interest

All authors declare no conflict of interest.

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