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Detection of genomic loci associated with days to heading in tropical japonica rice through QTL-seq

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Abstract: This study investigated the genetic basis of days to heading (DTH) in tropical japonica rice using F_2 populations derived from late-maturing Rojolele and early-maturing Rojolele Srinuk varieties. Phenotypic analysis of DTH showed continuous distribution and positive skewness. Whole genome sequencing (WGS) derived single nucleotide polymorphism (SNP) from early and late-heading bulks were used to identify three candidate regions with strong association to DTH: *qDTH3.1* and *qDTH3.2* on chromosome 3, and *qDTH7.1* on chromosome 7, with the latter linked to the *Oryza sativa Pseudo-Response Regulator 37* (*OsPRR37*) gene. InDel markers validated *qDTH7.1*'s significant linkage to DTH, particularly marker ID14, which is effective for marker-assisted selection of early DTH in Rojolele background.

Keywords: bulked segregant analysis; flowering time; genetic regulation; *Oryza sativa*; whole genome sequencing

Rojolele, a prized tropical japonica rice from Klaten, Central Java, Indonesia, is known for its superior taste and fragrance but has a long maturation period and low productivity per year when compared to modern rice cultivars. A variety with Rojolele's exceptional taste and aroma but a shorter maturation cycle would be ideal for commercial cultivation. In Indonesia, Indica rice varieties are preferred for their quick

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maturation and high yield (Muhamad et al. 2017), though they lack the flavour and fragrance of Rojolele and other japonica varieties. Introducing temperate japonica to Indonesia is difficult because they flower prematurely in tropical conditions, thereby reducing their yield significantly.

Cao et al. (2021) classified 79 rice genes involved in flowering into seven groups: light receptors, temperature sensors, circadian clock genes, light-regulated output genes, temperature-regulated output genes, flowering pathway integrators, and floral meristem identity genes. Environmental cues like day length and temperature, which set the plant's circadian clock, determine flowering timing. While light sensors such as phytochromes are well-known, temperature sensors are less understood, although photoreceptors like *PHYB* may also respond to temperature. Flowering is initiated when circadian clock genes activate integrators (florigens) like *Hd3a* or *RFT1*, which move to the shoot apex to trigger the transition to floral organs (Sun et al. 2014). Different rice cultivars use different regulatory genes and pathways, and some genes like *Hd1* and *OsPRR37* can either repress or promote flowering based on environmental signals and the allelic compositions of other regulatory genes (Sun et al. 2022). Thus, the genetic regulation of days to heading (DTH) is complex and environment-specific, requiring separate studies for different environments.

Identification of candidate genes for DTH in rice can be effectively achieved through a combination of next-generation sequencing techniques and bulked segregant analysis, which is also known as quantitative trait loci by sequencing analysis (QTL-seq). It has been used to successfully map important genes related to various agronomic traits in rice (Takagi et al. 2013; Wang et al. 2022). This method is also valuable in gene cloning and functional research, making the process of identifying candidate genes more efficient (Tiwari et al. 2016). Using QTL-seq, we aimed to identify the genetic determinants of DTH regulation in Rojolele and its progeny. The insights from this investigation will be useful for enhancing the agronomic performance of Rojolele and facilitate the integration of superior japonica varieties into tropical cultivation, which will contribute to food security and agricultural sustainability in Indonesia and other tropical regions.

MATERIAL AND METHODS

Plant material. A mapping population consisting of 209 F_2 progenies was developed by crossing Rojolele Srinuk and Rojolele varieties in the dry season of 2020. The F_1 seeds were grown and selfed during the wet season of 2020/2021. Rojolele is a local rice variety with a long growth duration (155 days from sowing to harvesting), high susceptibility to lodging due to its tall culm, and low grain yield (Chairunisa et al. 2020). Rojolele Srinuk is a new variety with early maturity, semi-dwarf stature, resistance to lodging, and high grain yield. DTH, defined as the number of days from transplanting to panicle exertion, was measured in the dry season (March to August 2021). Following Takagi et al. (2013), we selected 20 plants with the earliest and 20 plants with the latest headings from the F_2 population to establish two bulks: early and late heading. These bulks were used to identify loci associated with DTH through QTL-seq. The remaining F_2 plants were set aside for marker validation. Additionally, five plants each with early, late, and intermediate headings from the F_2 generation were self-pollinated to develop F_3 populations for marker validation. All planting experiments were conducted single location and season without replication in a screen house National Research and Innovation Agency at South Jakarta, Jakarta, Indonesia (6°17'41''S; 106°46'27''E; 25 m a.s.l.)

Whole genome sequencing (WGS) of bulked samples. The genomic DNA from 100 mg fresh leaves of parents and the F_2 bulks for DTH were extracted using Plant Genomic DNA Mini Kit (GP100, Geneaid, Taiwan). A total of four libraries (early and late heading bulks, Rojolele and Rojolele Srinuk) were prepared and sequenced. We used the Illumina Novaseq 6000 platform (Modi et al. 2021) to obtain ~50X of genome coverage for both F_2 bulks and Rojolele Srinuk, while Rojolele was sequenced with ~15X coverage.

Read mapping and variant calling. Cleaned raw reads from each bulk were mapped to MSU 7 rice reference genome (http://rice.uga.edu/annotation_pseudo_current.shtml) using bwa mem command with default settings in bwa aligner software (Li & Durbin 2009). After the alignments were sorted using samtools (Li et al. 2009), single nucleotide variants were identified among the bulks using mpileup and call commands in bcftools (Danecek et al. 2021), by turning on the FORMAT/AD option to obtain allelic depth data from each variant. The identified variants were then filtered using SNPsift (Cingolani et al. 2012) to remove variants that are multiallelic, heterozygous in either parent, monomorphic among the bulks and have quality score lower than 30.

Identification of contrasting of allele distribution. Candidate QTL regions were identified by cal-

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culating the Δ(SNP-index) as outlined by Takagi et al. (2013). SNP indexes were calculated separately in each bulk, by dividing the number reads that have the Rojolele Srinuk's allele in a locus by the total read depth in that locus. The Δ(SNP-index) values were then obtained by subtracting SNP indexes from the early heading bulk with SNP indexes from the late heading bulk. The resulting values were then plotted as scatter plots across the whole genome using the ggplot2 library in R software (Ver. R 4.4.1).

Marker design and validation. Six insertion/deletion (InDel) markers, which were polymorphic between the two parental lines and located within regions with the highest SNP indexes on chromosomes 3 and 7, were developed (Table 1). Primers for those markers were designed using NCBI Primer Blast (Ye et al. 2012). These markers were then used to genotype 172 F_3 plants selected from eight $F_{2:3}$ populations comprising 24 plants per population and a random sample of 105 F_2 plants to validate their efficacy in identifying plants with early and late heading dates.

Total genomic DNA was extracted from leaves using a modified potassium acetate method (Dellaporta et al. 1983). PCR was performed in 15 µL reaction mix containing 1.5 mM $MgCl_2$, 200 µM of each dNTP, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.2 µM of each primer, 0.75 units of *Taq* polymerase, and approximately 16 ng of template DNA. The PCR program was initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. PCR products were analysed on 3% agarose gels using a 1 kbp DNA ladder in 0.5× TBE buffer at 125 V for 50 min. Gels were stained with 1% Safe Red and visualized under UV light.

Marker effects were visualized by plotting the DTH of each F_3 individual using boxplot geometry in R software, grouping them according to the parental genotype they carried. The marker effect was estimated by subtracting the mean DTH in each allelic group, and the significance of the mean differences was calculated using Wilcoxon signed-rank test in R. Additionally, genotype and phenotype data from F_2 and F_3 populations were analysed in Windows QTL Cartographer (Ver. 2.5), using composite interval mapping in default settings (Wang et al. 2012).

RESULTS

Phenotype of F_2 population. In South Jakarta, the DTH values for the F_2 population ranged from 60 to 97 days (mean 70.58, standard deviation 7.31), while the DTH values for Rojolele Srinuk and Rojolele were 62 and 94 days, respectively (Figure 1A). This indicates that the significant difference in DTH observed in the parent plants was inherited and segregated in the F_2 progeny. The frequency distribution of DTH in the F_2 population has a slight positive skewness (Figure 1B). From this population, 20 F_2 plants with the earliest heading (61–63 days) and 20 with the latest heading (78–97 days) were selected to create early and late bulks, which were then sequenced to examine the distribution of parental alleles in each bulk.

QTL-seq analysis. The results of WGS for the early and late DTH bulks indicated that a sufficiently high sequencing depth was achieved. A total of 50.10 Gb

Table 1. List of InDel markers for validation of candidate loci for days to heading (DTH)

Markers ID	QTL	Chr	InDel position	Product size	InDel size	Primer sequence (5'-3')
ID08	*qDTH3.1*	3	10 239 138	164	14	F: CACGAGTGTTTTTCATCTTCCAA R: GTTCTCGCTGGTAAAGCAAGT
ID09	*qDTH3.1*	3	10 938 773	175	19	F: TTTTCGGTACACCGATCACC R: CATGGAGTTGACGGCTTTTG
ID11	*qDTH3.2*	3	26 541 257	274	31	F: CACATCAAACGTTTAAACACTCAC R: CGAGCCCAGTTATTCCATCC
ID12	*qDTH3.2*	3	30 436 041	167	20	F: ATATCGTGGGCGACATGATTG R: TCCTGCTCCATCTTTTCTAACC
ID13	*qDTH7.1*	7	28 778 152	175	23	F: GCAGTAGAGGAAACTTCTCGC R: CAATGCCTTACTAGCATTCCAA
ID14	*qDTH7.1*	7	29 514 173	205	17	F: GGCATGAAGTGGTCCATGTTA R: CAAGAAGCTTCGGAATGAGGAA

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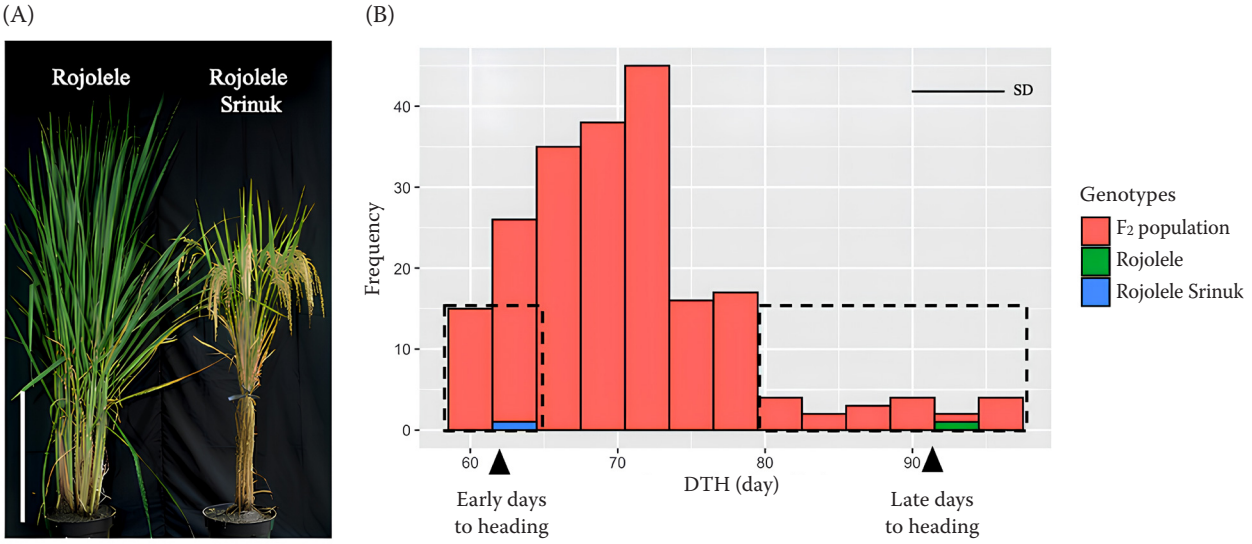

Figure 1. Morphological features of varieties Rojolele and Rojolele Srinuk (bar = 50 cm) (A), frequency distribution histogram of days to heading (DTH) trait of 209 F_2 plants (B)

The dashed boxes indicate F_2 plants selected to build the early and late DTH bulks; SD – standard deviation

of raw read data was obtained, with 26.2 Gb from the early bulks (Q30 ≥ 91.08%) and 23.9 Gb from the late bulks (Q30 ≥ 91.57%). For the parents, 24.5 Gb was obtained from Rojolele Srinuk (Q30 ≥ 91.45%) and 6.2 Gb from Rojolele (Q30 ≥ 90.92%) (Table S1 in Electronic Supplementary Material (ESM)).

Using homozygous Rojolele Srinuk allele as the baseline, SNP indexes from each bulk were calcu-

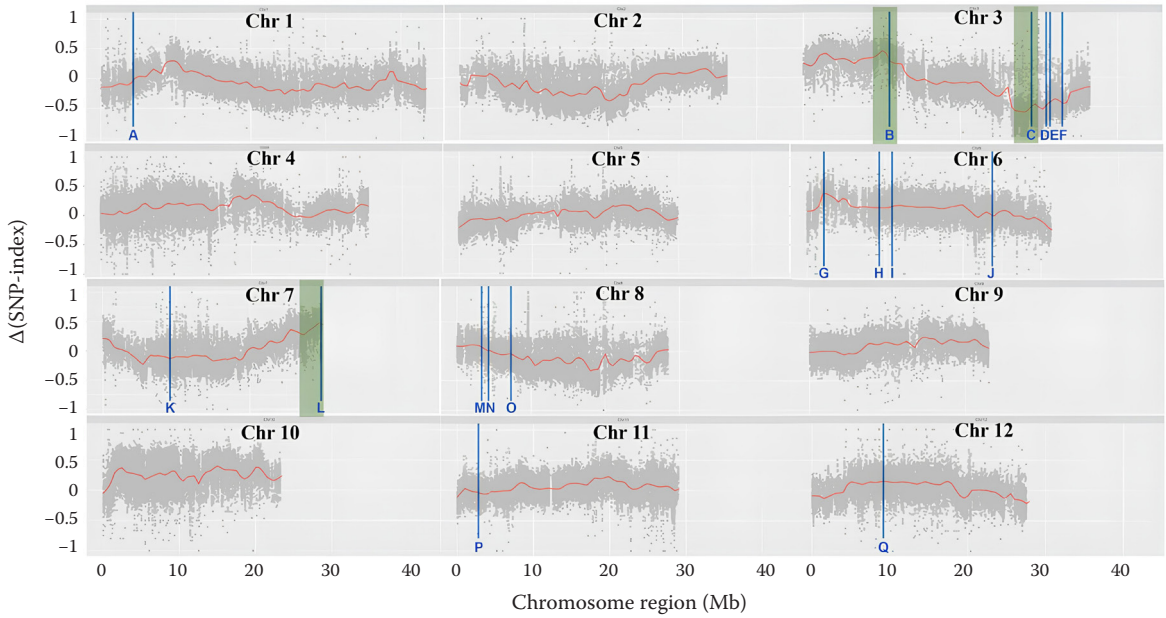

Figure 2. Genome-wide scatterplot of Δ(SNP-index) values

Red lines represent the average Δ(SNP-index) values for each 1 Mbp bins; green-shaded areas are segments with the most extreme average Δ(SNP-index) values, which were used to develop indel markers to test the segment's association to flowering time in F_3 and F_2 populations; blue lines represent the locations of known bifunctional flowering regulation genes, as compiled by Sun et al. (2022): A – *OsGI*; B. *PHYB*; C – *PHYA*; D – *PHYC*; E – *Hd6;* F – *Hd16*; G – *OsELF3*; H – *Hd1*; I – *OsCCT22*; J – *SE5;* K *Ghd7*; L – *OsPRR37*; M – *OsLHY*; N – *DTH8*; O – *OsVIL1*; P – *OsCCT38*; Q – *OsCCt41*

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lated following the method of Takagi et al. (2013) and plotted onto the 12 rice chromosomes. Regions with the top five lowest and highest average Δ(SNP-index) were chosen (Figure 2, Table S2 in ESM), corresponding to three QTL candidates: *qDTH3.1* (10.0 to 10.5 Mb) and *qDTH3.2* (26.5 to 31.0 Mb) on chromosome 3, and *qDTH7.1* (29.0–30.0 Mb) on chromosome 7 (Figure 2).

Validation of marker candidates. To validate the QTLs, six InDel markers flanking *qDTH3.1*, *qDTH3.2*, and *qDTH7.1* were designed (Table 1) and used to genotype two other populations. The first population comprised random F_3 individuals descended from F_2 lines with the earliest, medium, and latest DTH values. The second population was a sample of 105 F_2 individuals, including individuals not chosen for the early and late bulks.

InDel markers from *qDTH7.1* (ID13 and ID14) were more consistently associated with DTH variations (Figure 3). F_3 and F_2 lines with homozygous Rojolele alleles had significantly longer DTH than those with homozygous Rojolele Srinuk alleles in *qDTH7.1*. In contrast, homozygous Rojolele alleles had no significant effect on DTH in *qDTH3.2* (ID11 and ID12) and only a weak effect in *qDTH3.1* (ID08 and ID09). The flanking markers for *qDTH3.1* even showed opposite effects: the homozygous Rojolele allele shortened DTH in the ID08 marker but prolonged it in the ID09 marker. Therefore, markers from *qDTH7.1* were deemed more promising for further research.

Composite interval mapping revealed that the logarithm of the odds (LOD) values was highest at the tail end of *qDTH7.1*, where the ID14 marker is located (Figure 4). At this locus, the LOD value is 32, while the additive effect for Rojolele Srinuk allele is –10.05 and the dominant effect is –13.89 with R^2 value of 0.25. The loci for ID08 and ID11 also had LOD values higher than 2.5. ID08 has 10.06 additive effect, –11.97 dominant effect, and R^2 value of 0.06. ID11 has 3.02 additive effect, 9.35 dominant effect, and R^2 value of 0.01. In F_2 population, ID13 and ID14 have maximum the LOD value is 4.23, while the ad-

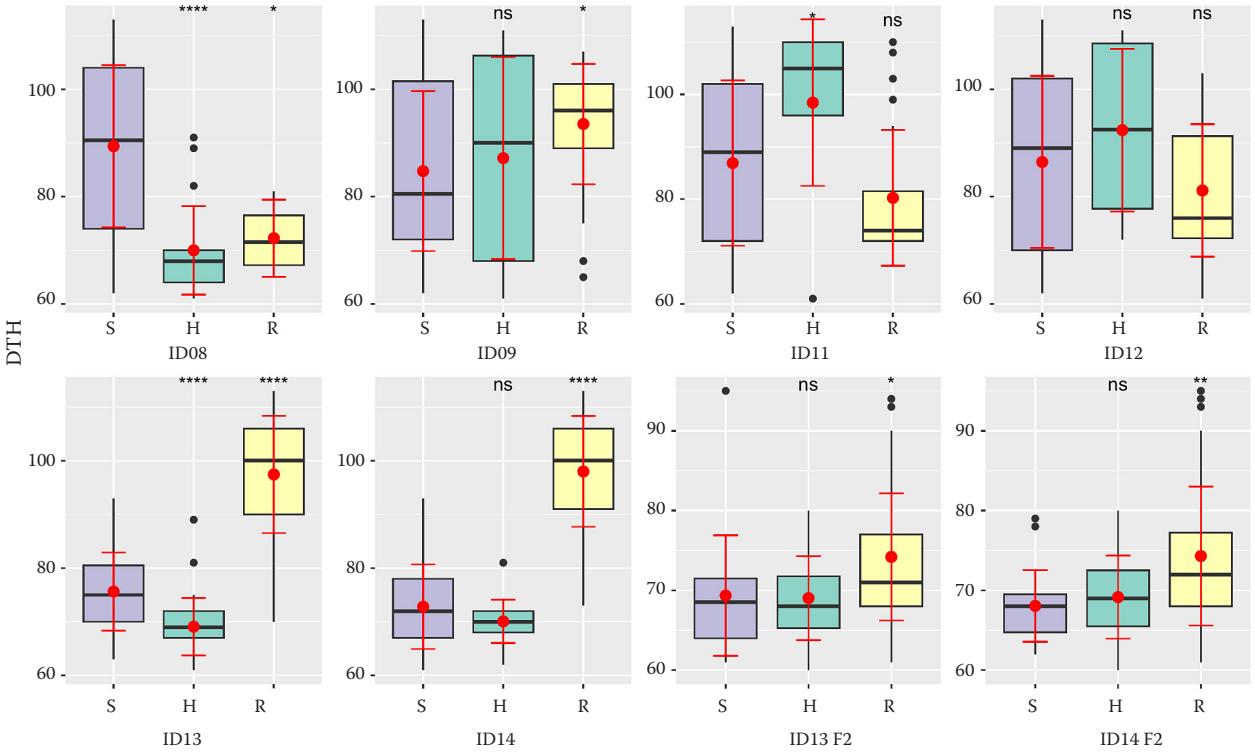

Figure 3. Days to heading (DTH) phenotypes of individuals carrying various combination of Rojolele and Rojolele Srinuk alleles, as evaluated using ID08, ID09, ID11, ID12, ID13, ID14 markers in F_3 population, as well as ID13 and ID14 markers in F_2 population

R – homozygous Rojolele allele; S – homozygous Rojolele Srinuk allele; H – heterozygous alleles; the lines in the middle of the boxes are median DTH values, while the upper and lower edges of the boxes are the upper and lower quartile of DTH values; red dots indicate the mean DTH while the red bars indicate the standard deviation

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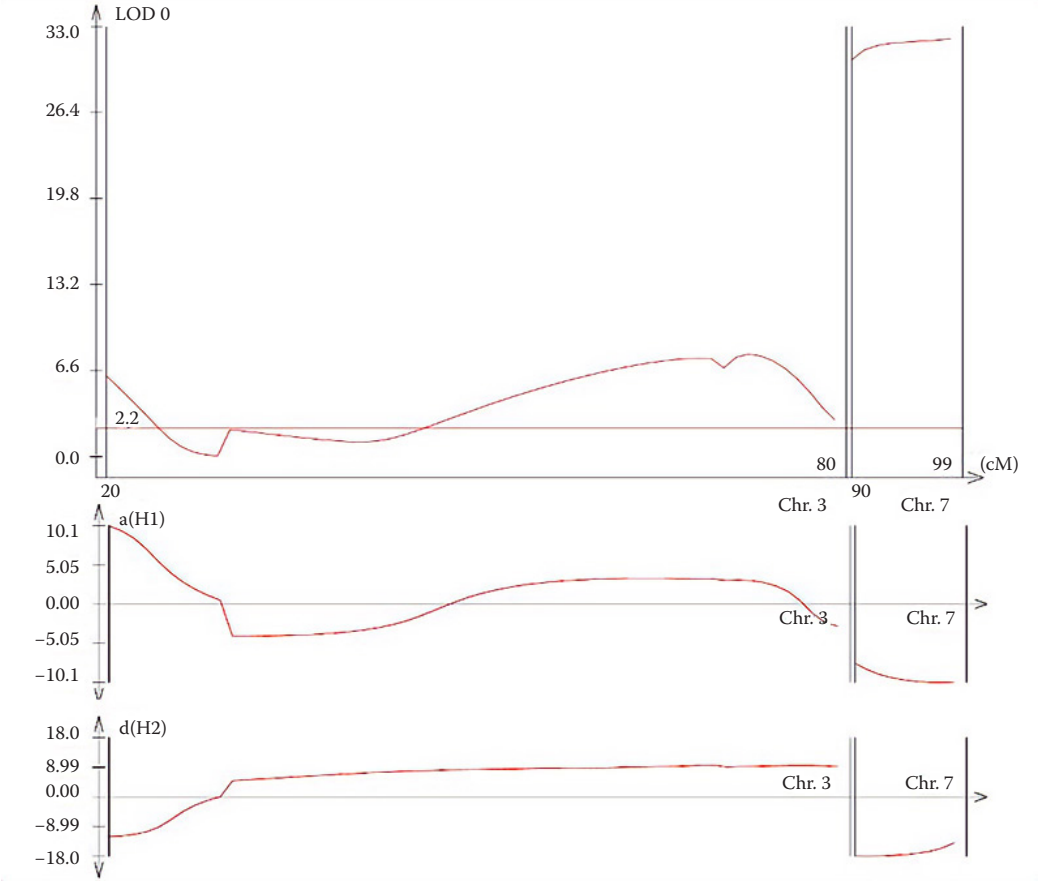

Figure 4. Composite interval mapping analysis of the six markers flanking the three candidate QTL intervals
Top chart – the logarithm of the odds scores; the middle chart – the additive effect; the bottom chart – the dominant effect

ditive effect for Rojolele Srinuk allele is –2.77 and the dominant effect is –4.02 with R^2 value of 0.07.

DISCUSSION

QTL-seq is a cost-effective method for identifying rice chromosome segments linked to specific traits. WGS data from QTL-seq is also useful for the design of new markers and identification of underlying genes based on allelic variations. Studies have shown that QTL-seq results align well with linkage map-based QTL detection for major QTLs, though pinpointing the exact gene and allele responsible remains challenging due to limited resolution from the number of recombination events in the bulked population.

In this study, QTL-seq identified three candidate loci for DTH: *qDTH3.1*, *qDTH3.2*, and *qDTH7.1*, which collocate with the flowering regulation genes *PHYB*, *PHYA*, and *OsPRR37*, respectively (Sun et al. 2022). Validation with six new InDel markers flanking these loci showed that only markers from *qDTH7.1* (ID13 and ID14) were consistently associated with DTH variations in F_2 and F_3 populations. Homozygous Rojolele alleles at these markers resulted in significantly later DTH compared to homozygous Rojolele Srinuk alleles. F_3 populations with homozygous Rojolele alleles flowered on average 100 days after transplanting, while Rojolele Srinuk alleles flowered after 72 days. In the F_2 population, the difference was smaller at 72 vs 68 days, but still significant. The greater variation in days to heading (DTH) observed in the F_3 population compared to the F_2 population can be attributed to both genetic and environmental factors. We observed a large additive effect in the F_3 population compared to the F_2 population, it often indicates that the genetic contribution to the trait has become more consistent and pronounced due to increased homozygosity and selection. In the F_3 population, increased genetic recombination and segregation lead to a broader range of phenotypic expressions, especially in traits influenced by multiple genes, such as DTH. Genetic effect of *qDTH7.1* had

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a large effect on DTH, while other genes, including *Hd1*, *Hd3a*, *Ghd7*, *DTH8*, *OsGI, OsLHY*, and *OsELF3*, possibly contribute smaller effects (Kim et al. 2018; Wu et al. 2020; Cao et al. 2021b; Sun et al. 2022). These genes interact, which can promote or inhibit flowering (Sun et al. 2022), further contributing to the variation in DTH within the F_3 population. ID14 was more significant than ID13, indicating its closer proximity to the causal gene.

Composite interval mapping corroborated ID14 as the best-performing marker for predicting DTH in the F_3 population. The consistent performance of ID14 highlights its potential for marker-assisted selection in tropical japonica backgrounds. This marker could help expedite the backcrossing of early heading date alleles from Rojolele Srinuk into other economically valuable traditional tropical japonica varieties in Indonesia. The use of InDel markers is also advantageous since they can be visualized using ordinary agarose gel electrophoresis, simplifying marker analysis and reducing costs (Bommisetty et al. 2020; Cao et al. 2021a). Since this study was conducted in one location, one replication and one F_2 population, the next study needs more location, replication and use advanced populations such as backcross or recombinant inbred lines (RILs).

The *qDTH7.1* region, where the ID14 marker is located, also contains the *OsPRR37* gene, which is known to regulate flowering in rice, though its activity in tropical environments is less documented. Previous studies on tropical rice flowering have emphasized the role of *Hd1* locus on chromosome 6 in delaying flowering in short-day conditions (Kim et al. 2018; Wu et al. 2020), but those studies compared rice varieties from temperate and tropical regions. Both Rojolele and Rojolele Srinuk are native to tropical Indonesia and have longer DTH than introduced temperate japonica rice. It is plausible that other loci in the flowering regulation pathway, like *PHYB, PHYA,* and *OsPRR37* have more impact in DTH differences between these cultivars.

CONCLUSION

The *qDTH7.1* locus in rice chromosome 7, which co-located with the *OsPRR37* gene, has significant association with DTH in tropical japonica background. Validation using InDel markers from *qDTH7.1* locus, particularly ID14, confirmed its potential utility in marker-assisted selection for DTH. Further gene expression studies are needed to elucidate the precise regulatory mechanisms of DTH in tropical japonica rice.

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