QTL mapping and candidate gene analysis of low temperature germination in rice (*Oryza sativa* L.) by genome wide association study

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Low temperature germination (LTG) is a key agronomic trait in rice (*Oryza sativa* L.). However, genetic basis of natural variation for LTG is largely unknown. Here, a genome-wide association study (GWAS) was performed using 276 accessions from 3,000 Rice Genomes (3K-RG) project with 497 k single nucleotide polymorphisms (SNPs) to uncover potential genes for LTG in rice. In total, 37 quantitative trait locus (QTLs) from the 6th day (D6) to the 10th day (D10) were detected in full population, overlapping with 12 previously reported QTLs for LTG. One novel QTL, namely *qLTG1-2*, was found stably on D7 in two years. Based on two germination-specific transcriptome datasets, 13 seed-expressed genes were isolated within 200 kb interval of *qLTG1-2*. Combining with haplotype analysis, a functional uncharacterized gene *LOC_Os01g23580* and a seed germination-associated gene *LOC_Os01g23620* (*OsSar1a*) were identified as promising candidate genes, both of which were significantly differently expressed between high and low LTG accessions. Collectively, the candidate genes with favorable alleles may be useful for future characterization of LTG mechanism and improving LTG trait in rice breeding.

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Abstract

Low temperature germination (LTG) is a key agronomic trait in rice (*Oryza sativa* L.). However, the genetic basis of natural variation for LTG is largely unknown. Here, a genome-wide association study (GWAS) was performed using 276 accessions from the 3,000 Rice Genomes (3K-RG)

23   project with 497 k single nucleotide polymorphisms (SNPs) to uncover potential genes for LTG

24   in rice. In total, 37 quantitative trait loci (QTLs) from the 6th day (D6) to the 10th day (D10) were

25   detected in the full population, overlapping with 12 previously reported QTLs for LTG. One novel

26   QTL, namely *qLTG1-2*, was found stably on D7 in both 2019 and 2020. Based on two germination-

27   specific transcriptome datasets, 13 seed-expressed genes were isolated within a 200 kb interval of

28   *qLTG1-2*. Combining with haplotype analysis, a functional uncharacterized gene,

29   *LOC_Os01g23580,* and a seed germination-associated gene, *LOC_Os01g23620* (*OsSar1a*), as

30   promising candidate genes, both of which were significantly differentially expressed between high

31   and low LTG accessions. Collectively, the candidate genes with favorable alleles may be useful

32   for the future characterization of the LTG mechanism and the improvement of the LTG trait in rice

33   breeding.

34   **Subject** Agricultural Science, Population Biology

35   **Keywords** Genome wide association study, Low temperature germination, Haplotype analysis,

36   Rice

37   **Introduction**

38   Rice (*Oryza sativa* L.) is an important staple food that feeds nearly half of the world (Khush, 2005;

39   Sreenivasulu et al., 2021). Due to its tropical and subtropical origin, rice is susceptible to low

40   temperature at all phases of growth (Cheng et al., 2007). A temperature of 25-35℃ is optimal for

41   the growth of rice, and temperatures below 15℃ can cause poor seed germination and subsequently

42   bad seedling establishment (Fujino et al., 2004). However, more than 15 million hectares of rice

43   cultivated worldwide are threatened by low temperatures, especially in Japan, South Korea, North

44   Korea and Northeast China (Song et al., 2018). On the other hand, direct seeding has replaced

45   conventional transplanting as it is both labor-saving and lower in cost, which requires good

46   germination characteristics for rice seeds in low temperature, since the temperatures during the

47   sowing period in the spring planting season are frequently below 15℃ in temperate and high-

48   altitude regions (Fujino et al., 2004; Fujino and Matsuda, 2010; Sales et al., 2017; Yang et al.,

49   2020b). Therefore, it is important to uncover the genetic basis of LTG and apply the findings to

50   rice breeding in order to meet the challenges mentioned above.

51   In rice, LTG is a complex trait that is genetically controlled by multiple quantitative trait loci

52   (QTLs) (Fujino et al., 2008). One common method used to study genetic basis is QTL analysis

53   using bi-parental mapping populations (Huang et al., 2010). Generally, Japonica cultivars are more

54   cold-tolerant than Indica cultivars (Ma et al., 2015). Most bi-parental populations used in QTL

55   analysis have been derived from a cross between a cold-tolerance Japonica variety and a cold-

56   sensitive Indica group (Jiang et al., 2006; Ji et al., 2008; Li et al., 2013; Ranawake et al., 2014;

57   Jiang et al., 2017). Researchers identified 5 QTLs on chromosomes 2, 4, 5, and 11 in a Nipponbare

58   × Kasalath cross (Miura et al., 2001). Through USSR5 and N22, 11 QTLs for LTG were unveiled

59   on chromosomes 3, 4, 5, 9, 10 and 11 (Jiang et al., 2006). By crossing varieties Kinmaze and

60   DV85, two QTLs were found located on chromosomes 7 and 11 (Ji et al., 2008). Li et al. (2013)

61   detected 3 major QTLs for LTG and characterized *qLTG-9* to a region of ~72 kb which contained

62   5 potential genes explaining 12.12% of the phenotypic variation. A separate study used

63   recombinant inbred lines from a Japonica and Indica cross and found 5 QTLs for LTG that

64   explained 5.7% - 9.3% of the total phenotypic variance (Ranawake et al., 2014). Satoh et al. (2015)

65   reported 4 QTLs responsible for LTG on chromosomes 1, 3, and 11 in a European rice variety.

66   Borjas et al. found 49 QTLs related to LTG distributed on 10 chromosomes in US weedy rice

67   (2015). In addition, 6 QTLs distributed across chromosomes 1, 4, 8, and 11 were characterized for

68   LTG by crossing Changhui 891 and 02428 (Jiang et al., 2017). Among the identified QTLs, only

69   one QTL, *qLTG3-1*, has been cloned, encoding a protein with unknown molecular function that

70   may be involved in tissue weakening (Fujino et al., 2008).

71   Compared with a bi-parental QTL analysis, a genome-wide association study (GWAS) is a more

72   efficient way to identify the genes underlying a complex trait as it has the advantage of being able

73   to study abundant variations in natural populations (Huang et al., 2010). Recently, a GWAS has

74   been used to identify QTLs for LTG. Fujino et al. (2015) conducted a GWAS using 63 accessions

75   with 117 markers and discovered 17 QTLs associated with LTG, 9 of which were co-localized

76   with QTLs identified before. Using a core collection (Rice Diversity Panel 1, RDP1) of rice, a total

77   of 42 QTLs were identified as being associated with cold tolerance during the germination and

78   seedling stages (Shakiba et al., 2017). Through a GWAS, 11 QTLs were found to be associated

79   with LTG among Rice Diversity Panel 2 (RDP2) and two candidate genes were narrowed down

80   (Yang et al., 2020c). *OsSAP16* was cloned using 187 natural accessions by GWAS in rice (Wang

81   et al., 2018b). Yang et al. (2020b) found 159 LTG-related QTLs in Indica accessions, only 12 of

82   which were co-localized with previously reported cold tolerant QTLs. Consequently, a GWAS can

83   identify new QTLs for LTG and provide new insights in to the genetic basis of LTG in rice.

84   In this study, a collection of 276 rice accessions from the 3K-RG project with high density SNPs

85   were used to perform a GWAS in order to uncover potential QTLs and identify candidate genes

86 for LTG. The favorable haplotype and SNPs affecting gene expression from two candidate genes

87 for LTG were identified. These results provide a basis for molecular breeding to enhance LTG and

88 further elucidate the mechanisms in rice.

89 **Materials and methods**

90 **Plant materials**

91 In this study, a collection of 276 rice accessions were selected from the 3K-RG project. All rice

92 accessions were cultivated in the same geographical location in Huai'an (119°0'14" E, 33°38'43"

93 N), Jiangsu province in 2019 and 2020. Each accession was subject to the same field management

94 in 2019 and 2020. To eliminate error results caused by marginal effects, every rice accession was

95 planted in a 5 × 5 block within the 3 m × 3m square, and 5 plants of each accession were randomly

96 chosen from the middle of each square as the experimental subjects.

97 **LTG measurement**

98 The seeds of each rice accession were collected independently in a nylon bag with dense nets to

99 air dry seeds for two weeks. After that, air-dried seeds were placed in the oven at 50℃ for 7 days

100 to break primary dormancy. A total of 100 plumped seeds of each rice accession were extracted

101 and spread on a round wet filter paper and kept at 15℃ and in darkness for germination. The

102 number of germinated seeds was recorded daily from D6 to D10 with a seed shoot or root

103 exceeding 0.1 cm considered a germinated seed (Wang et al., 2018c; Akhtamov et al., 2020;

104 Najeeb et al., 2020). Seed germination rate = germinated seeds / 100. LTG was assessed according

105 to the germination rate of each recorded day.

106 **SNP filter analysis**

107   The genetic variations of 276 rice accessions are available publicly in the 3K-RG database and the

108   information for all SNPs can be downloaded from the website for free (https://snp-

109   seek.irri.org/_download.zul). In this study, the set criteria for selecting high-quality SNPs were

110   based on 1) minor allele frequency (MAF) ≥ 0.05 and 2) number of accessions with minor alleles

111   ≥ 6 (Yang et al., 2014). After filtering, only high-quality SNPs were retained. A slide window of

112   1Mb was adopted to demonstrate the distribution of variants in all 12 chromosomes to determine

113   the density of the SNPs. The detected SNPs were annotated and the possible effects were predicted

114   through ANNOVAR (Wang et al., 2010).

Population structure analysis

116   To analyze the population structure, a principal component analysis (PCA), a neighbor-joining

117   (NJ) tree and a K value analysis were applied. The phylogenetic tree was constructed using

118   MEGA7 (version 7.0) (Kumar et al., 2016) and the results were visualized using ggtree (version

119   1.7.10) (Yu et al., 2017). The PCA was conducted by PLINK (version v1.90) (Purcell et al., 2007).

120   According to the Bayesian Markov Chain Monte Carlo (MCMC) Program, the K value, ranging

121   from 2 to 7 in the full population was inferred using STRUCTURE (version 2.3.4) (Pritchard et

122   al., 2000). The optimal K value was determined by ΔK (Evanno et al., 2005). The result was

123   visualized and the relevant Q matrix was generated for further analysis.

Programs for GWAS analysis

125   Based on the factored spectrally transformed linear mixed model, two programs, FaST-LMM

126   (version 0.5.1) and GEMMA (version 0.98.1), adding different genetic similarities to analyze

127   random effects, were applied to perform the GWAS. The validated number of SNP markers (N)

128   was calculated using the Genetic type I Error Calculator (GEC) software (Turner, 2014) and

129   suggestive (1/N) *P* value threshold was adopted as the standard to control type I error.

130   **Quantitative real-time PCR assay**

131   Ten seeds of each accession were sampled at 15℃ and in darkness. Total RNA was extracted using

132   the TIANGEN RNAprep Pure kit (#DP441, TIANGEN, China) according to the manufacturer's

133   protocol. Complementary DNA (cDNA) was synthesized using a cDNA synthesis kit (#RR047A,

134   Takara, Japan). Quantitative real-time PCR (qRT-PCR) reaction was conducted using TB Green

135   Premix Ex Taq (#RR820A, Takara, Japan). The reaction was performed on the CFX Connected

136   Real Time System (Bio-Rad, USA). The expression level was calculated by $2^{-\Delta Ct}$ using the

137   expression level of *Ubiquitin* as reference. Each sample was tested three times to fulfill technical

138   replications. Relevant primer sequences are provided in the supplemental data (Table S1).

139   **Results**

140   **Phenotypic variation for LTG in natural rice accessions**

141   A collection of 276 rice germplasms was selected from the 3K-RG project for the LTG test. Rice

142   accessions in this study were from 17 different regions worldwide (Fig S1, Table S2). Previous

143   studies have applied different temperatures ranging from 12℃ to 15℃ to estimate LTG (Borjas et

144   al., 2015; Fujino et al., 2008; Li et al., 2013; Wang et al., 2011). Given the effect of secondary

145   dormancy induced in 12℃ (Miura and Araki, 1996), 15℃ was applied to evaluate LTG in this

146   study. Germination was defined as the seed shoot or root exceeding 0.1 cm from the seed coat (Fig

147   1a), and the evaluation of LTG was based on the germination rate from D6 to D10. The average

148   germination rate on 2019D6 was lower (3.5%) than that (13.6%) on 2020D6 (Fig 1b, Table 1),

149 suggesting that environmental factors have an impact on the phenotype of LTG. Furthermore, the

150 total number of germinated accessions was too small to draw any general conclusions (Fig S2).

151 The average germination rates were 22% and 31% on 2019D7 and 2020D7, respectively (Fig 1b,

152 Table S3). On 2019D7, a total of 129 rice accessions had a germination rate that exceeded 10%

153 while on 2020D7 this number rose to 161 (Fig 1c). From D8 to D10 in both years, the average

154 germination rate almost reached or exceeded 40% (Fig 1b, Table 1). The investigation of

155 germination rate gaps between two adjacent days indicated that the gap between D6 and D7 in

156 both years was the largest at 18.5% in 2019 and 17.4% in 2020, respectively (Table 1). In contrast,

157 the smallest germination gap in 2019 was 13.8% between D8 and D9, while in 2020, the gap

158 between D9 and D10 was the smallest (10.3%) (Table 1). On D10 of both years, all rice accessions

159 had a relatively high germination rate ranging from 64.7% to 69.4%. Overall, the distribution of

160 germination rate followed similar trends in both years although the germination rate of D6 and D7

161 in 2020 was higher than that in 2019 (Fig 1b, Table 1).

162 **SNP density analysis**

163 The original version of the 3K-RG database contained 32million SNPs in total. Through filtering,

164 a total of 497,231 SNPs were detected. After the classification of SNPs, the density of SNPs in all

165 12 chromosomes were between 1033.3/1Mb and 1648.94/1Mb (Fig S3, Table S4). This indicated

166 that the filtered SNPs in this study were sufficient and distributed evenly in 12 chromosomes.

167 **Population structure and kinship**

168 Using the SNPs, we performed a PCA to quantify the population structure of these 276 accessions.

169 The total variance explained by PC1 and PC2 was 35.60% and 16.79%, respectively (Fig 2a).

170   Based on the Nei's genetic distance (Nei, 1972), the NJ tree was plotted separating the full group

171   into two groups (Fig 2b). Meanwhile, using STRUCTURE, the peak of ΔK appeared when K=2,

172   suggesting that the full population could be divided into two subgroups (Fig 2c and 2d). These two

173   subgroups corresponded to Japonica and Indica (Table S2), which is consistent with the findings

174   of Wang et al (2018a).

175   **GWAS for LTG in rice**

176   A total of 136,276 validated SNPs (MAF ≥ 0.05) were used for the GWAS through the FaST-

177   LMM and GEMMA models. The GEC was used to calculate the indicator p value, which gave

178   7.41E-6 as the suggestive p value (-log (p value) = 5.13). According to a previous study, the

179   distance of two adjacent lead SNPs within 200 kb was considered one QTL (Lv et al., 2016). A

180   total of 37 QTLs with 54 SNPs were found using FaST-LMM for LTG from D6 to D10 in both

181   years whereas 107 QTLs with 159 SNPs were detected using GEMMA (Table 2, Table S5-7).

182   Nearly half of the QTLs identified in FaST-LMM (15/37) were also identified in GEMMA,

183   suggesting FaST-LMM had stricter criteria in controlling false positive association (Table S6).

184   Using FaST-LMM, there were 26, 8, 5, 4 and 4 QTLs detected from D6 to D10, respectively, in

185   2019 and 2020 (Table 2). There were 7 QTLs characterized repeatedly in the total (Table 2).

186   Compared with QTLs reported before, 13 QTLs were co-localized within the interval of 1Mb in

187   this study (Table 2). Among these QTLs, 12 QTLs were associated with LTG, 5 QTLs were

188   associated with cold tolerance at the seedling stage, and 4 QTLs were associated with both LTG

189   and cold tolerance at the seedling stage (Table 2). These results confirmed that the GWAS results

190   in this study were reliable for further candidate analysis. The remaining 24 QTLs that had been

191   uncharacterized before were considered novel QTLs for LTG. Among the novel QTLs, it was

192   notable that *qLTG1-2* was repeatedly detected on D7 in both years using FaST-LMM and

193   GEMMA (Fig 3a-3c). Moreover, this QTL was detected on 2020D6 as well using FaST-LMM and

194   GEMMA (Fig S4). Therefore, further analysis was focused on *qLTG1-2* with this repeated lead

195   SNP (Chr.1_13340259).

196   **Haplotype and expression analysis of the candidate genes**

197   To further locate the candidate genes of *qLTG1-2*, two public germination-related transcriptome

198   datasets (SRP277875, GSE115371) were adopted. Dataset SRP277875 contained the expression

199   data at different time points of two rice accessions for germination (Yang et al., 2020a) and Dataset

200   GSE115371 provided the expression data of one rice accession under aerobic conditions for

201   germination at various time points (Narsai et al., 2017). According to the published transcriptome

202   data, 13 expressed genes located in the *qLTG1-2* interval were found, which were then used for

203   further comparison analysis of the expression levels in low temperature between high and low

204   germination accessions (Fig S5). *LOC_Os01g23600*, *LOC_Os01g23705* and *LOC_Os01g23850*

205   failed to be amplified, suggesting they exhibit very low expression levels in the seeds. Eight genes,

206   including *LOC_Os01g23590*, *LOC_Os01g23610*, *LOC_Os01g23630*, *LOC_Os01g23640*,

207   *LOC_Os01g23680*, *LOC_Os01g23710*, *LOC_Os01g23740* and *LOC_Os01g23870*, did not show

208   obvious differences between high and low germination accessions (Figure S6).

209   *LOC_Os01g23580* was located 90 kb from the lead SNP and was associated with abiotic stress in

210   a GO analysis. Furthermore, the homolog of *LOC_Os01g23580* in *Arabidopsis* has been shown to

211   be involved in the regulation of auxin transport and response to several abiotic stresses (Li et al.,

212   2005; Wijewardene et al., 2020). One non-synonymous SNP (Chr.1_ 13243045, base G-C, amino

213   acid Ser-Thr) and one upstream SNP (Chr.1_ 13236390, base A-G) were identified within the

214   sequence of *LOC_Os01g23580*, which generated three haplotypes in the full population (Fig 4a).

215   Haplotype I of *LOC_Os01g23580* displayed better performance for LTG than the left two

216   haplotypes (Fig 4a and 4b). Moreover, accessions of high germination rates were usually ones with

217   G allele whose transcriptional levels were much lower than accessions of low germination rates

218   with an A allele in the upstream region (Fig 4c). *LOC_Os01g23620*, namely *OsSar1a*, was located

219   50 kb from the lead SNP and *OsSar1abc* RNAi mutants led to pre-harvest sprouting (Tian et al.,

220   2013). Based on one upstream SNP (Chr.1_ 13285882 base A-G), the full population was divided

221   into two haplotypes (Fig 4d). Haplotype I of *OsSar1a* showed a higher germination rate than

222   haplotype II which was negatively associated with transcriptional level (Fig 4e and 4f).

223   **Discussion**

224   The genetic variation of rice cultivars provides a resource for trait improvement via breeding

225   (Breseghello and Coelho, 2013). The 3K-RG project provides a foundation for finding potential

226   candidate genes associated with quantitative traits (Wang et al., 2018a). Using rice accessions from

227   the 3K-RG project, several genes for crucial agronomic traits were identified (Anacleto et al.,

228   2019; Kumar et al., 2020; Lu et al., 2021).

229   LTG is an essential agronomic trait for direct seeding rice in high altitude regions (Li et al., 2013).

230   In previous studies, LTG was measured using two parameters: low temperature germination index

231   (LTGI) and low temperature germination potential (LTGP) (Ji et al., 2009; Wang et al., 2018b).

232   Since germination varies greatly in natural accessions, LTG was generated according to daily

233   germination rates (Fujino et al., 2004). Although accessions in both years of this study had similar

234   patterns of germination rates, a few of them differed in the early days of germination, indicating

235   that environmental factors could not be ignored (Fig 1b).

236   In both study years, four accessions, 'IRIS_313-7728', 'B199', 'B077' and 'IRIS_313-9886'

237   showed an extremely high germination rate on D6 in low temperatures. Thus, these four accessions

238   could be considered potential donors for rice breeding with regard to LTG.

239   A GWAS was also performed in Japonica and Indica subgroups, separately, using FaST-LMM. A

240   total of 21 and 33 QTLs with 49 and 37 SNPs were mapped in Japonica and Indica, respectively,

241   for LTG in both years (Table S8-S11). For both subgroups, the GWAS results for LTG were

242   consistent with those in the full population. In the Japonica group, 11 QTLs overlapped within the

243   interval of QTLs mapped previously (Fujino et al., 2015; Najeeb et al., 2020; Shakiba et al., 2017;

244   Wang et al., 2016) (Table 2, Table S10), of which 4 QTLs were also detected in the full population.

245   Coincidentally, in the Indica group, there were also 11 QTLs that had been mapped previously and

246   4 of them were also found in the full population (Table 2, Table S11) (Fujino et al., 2015; Najeeb

247   et al., 2020; Shakiba et al., 2017; Wang et al., 2016). These analyses confirmed the GWAS results

248   in the full population in this study.

249   The repeatedly detected QTL (*qLTG1-2*) in the full group was also found in the Japonica (*qLTG-*

250   *1-1-2*) and Indica (*qLTG-2-1-1*) subgroups (Table 2, Table S10-S11). Two candidate genes showed

251   different expression levels in contrast with germination rate varieties (Fig 4c and 4f). *OsSar1a*

252   (*LOC_Os01g23620*) was functionally identified to be involved in seed germination (Tian et al.,

253   2013). According to the haplotype analysis, accessions with a G allele variant located within the 1

254   kb upstream region of *OsSar1a* showed higher germination rates (Table S2). Through a *cis-*

255   element analysis (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/), accessions with A

256   allele had complete CAAT-box functions as an enhancer motif in the promoter region (Fig 4d, Fig

257   S7). In agreement with these results, the SNP variant A is associated with high transcriptional

258   levels (Fig 4f). These results indicate *OsSar1a* could be a promising candidate gene for LTG in

259   rice breeding. So far, few reports have clarified the function of *LOC_Os01g23580*, but its homolog

260   in *Arabidopsis* is involved in the regulation of auxin transport and confers tolerance to various

261   stresses (Li et al., 2005). Further elucidating the biological function of *LOC_Os01g23580* may be

262   important for rice breeding application.

263   **Conclusion**

264   A set of 276 rice accessions from the 3K-RG project with 497 k re-sequenced SNPs was used for

265   a GWAS to uncover candidate genes regulating LTG. Combined with the phenotypes from two

266   consecutive years, a total of 37 QTLs were identified in the full population, co-localizing with 12

267   QTLs reported before for LTG. Among all QTLs, one novel QTL, *qLTG1-2* was detected

268   repeatedly in both study years by both the FaST-LMM and GEMMA programs. Based on two

269   published transcriptome datasets, a total of 13 seed-expressed genes were identified for a haplotype

270   analysis and expression analysis. Eventually, two promising candidate genes, *OsSar1a*

271   (*LOC_Os01g23620*) and *LOC_Os01g23580*, which both showed differential expression levels in

272   the accessions of contrast LTG traits, were explored as favorable haplotypes of LTG for rice direct

273   seeding. These results may be helpful for further developing rice varieties with high LTG for rice

274   direct seeding through marker-assisted breeding.

Author contributions

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276 • Feng Mao performed the experiments, analyzed the data, prepared figures and/or tables,

277 authored or reviewed drafts of the paper, and approved the final draft.

278 • Depeng Wu analyzed the data, authored or reviewed drafts of the paper, and approved the

279 final draft.

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281 and/or tables, and approved the final draft.

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289 data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved

290 the final draft.

291 • Lilian Ji conceived and designed the experiments, authored or reviewed drafts of the paper,

292 and approved the final draft.

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426 **Figure Legends**

427 **Fig 1. Description of LTG.** (a) Variations of low temperature germination in D7. Bar = 200 μm.

428 (b) Germination rate from D6 to D10 for two different years. (c) Germination rate distribution on

429 D7 in 2019 and 2020.

430 **Fig 2. Description of population structure.** (a) Principal component analysis. (b) NJ tree based

431 on Nei's genetic distance. (c) Delta K values plotted as the number of subgroups. (d) Subgroups

432 inferred using STRUCTURE.

433 **Fig 3. Manhattan plot and Q-Q plot for LTG using 2 programs in D7.** (a) A GWAS performed

434 on 2019D7 using FaST-LMM. (b) GWAS performed on 2020D7 using FaSTL-MM. (c) A GWAS

435 performed on 2019D7 using GEMMA. (d) A GWAS performed on 2020D7 using GEMMA. An

436 orange arrow represents QTLs detected previously. A black arrow represents novel QTLs detected

437 in this study. A yellow dotted box represents the repeated identified QTLs. A dashed horizontal

438 line represents the suggestive threshold (P= 7.34×10^{-6}, $-\log_{10}$P = 5.13).

439 **Fig 4. Candidate genes analysis.** (a) Gene structure and haplotype analysis for

440 *LOC_Os01g23580.* (b) Comparison of germination rate among *LOC_Os01g23580* haplotypes in

441 full population (**, $p < 0.01$; ***, $p <0.001$).   (c) Expression level of *LOC_Os01g23580* in

442 contrast accessions after three days soaking in water in 15°C and darkness for germination. Black

443 bars represented expression levels of rice accessions with high germination rate under low

444 temperature. Grey bars represent the expression levels of rice accessions with low germination rate

445 under low temperature. (d) Gene structure and haplotype analysis for *LOC_Os01g23620.* (e)

446 Comparison of germination rate among *LOC_Os01g23620* haplotypes in full population (**, $p <$

447 0.01).   (f) Expression level of *LOC_Os01g23620* in contrast accessions after three days soaking

448 in water in 15°C and darkness for germination.

449 **Supplementary Figure Legends**

450 **Fig S1. The origin of 276 rice accessions in the study.** (a) The distribution of accessions from

451 different regions worldwide. (b) Specific information for abbreviations of regions.

452 **Fig S2. Description of germination rate on D6 of 2019 and 2020.** (a) The distribution of

453 germination rate on D6 of 2019. (b) The distribution of germination rate on D6 of 2020.

454 **Fig S3.** The distribution of SNPs on 12 chromosomes. The SNP density was demonstrated using

455 a 1Mb slide window.

456 **Fig S4. Manhattan plot and Q-Q plot for LTG using 2 programs on D6.** (a) A GWAS

457 performed on 2020D6 using FaST-LMM. (b) A GWAS performed on 2020D6 using GEMMA.

458 The red arrow represents the repeatedly identified *qLTG1-2* in the study. The dashed horizontal

459 line represents the suggestive threshold (P= 7.34×10^{-6}, $-\log_{10}$P = 5.13).

460 **Fig S5. Heat maps of candidate gene transcriptional level within 200k interval around the**

461 **lead SNP Chr.1_13340259.** (a) Transcriptomic data from Narsai et al (2017). (b) Transcriptomic

462 dataset from Yang et al (2020a).

463 **Fig S6.** The results of expression level analysis for the left 8 genes using contrast rice accessions.

464 **Fig S7.** SNP variant on *OsSar1a* in the upstream region. Blue block represents CAAT-box; red

465 box represents Chr.1_13285882 which located within 1 kb region ahead of the CDS region of

466 *OsSar1a.*

467   **Supplementary Table Captions**

468   **Table S1.** Primers used for qRT-PCR

469   **Table S2.** Rice accessions origin & classification

470   **Table S3.** Germination rate of all accessions

471   **Table S4.** SNP density

472   **Table S5.** SNPs detected using GEMMA

473   **Table S6.** QTLs summarized using GEMMA

474   **Table S7.** SNPs identified in full population using FaST-LMM

475   **Table S8.** SNPs identified in Japonica group

476   **Table S9.** SNPs identified in Indica group

477   **Table S10.** QTLs summarized in Japonica group

478   **Table S11.** QTLs summarized in Indica group

Figure 1

Description of LTG.

(a) Variations of low temperature germination in D7. Bar = 200 μm. (b) Germination rate from D6 to D10 for two different years. (c) Germination rate distribution on D7 in 2019 and 2020.

(a)

(b)

(c)

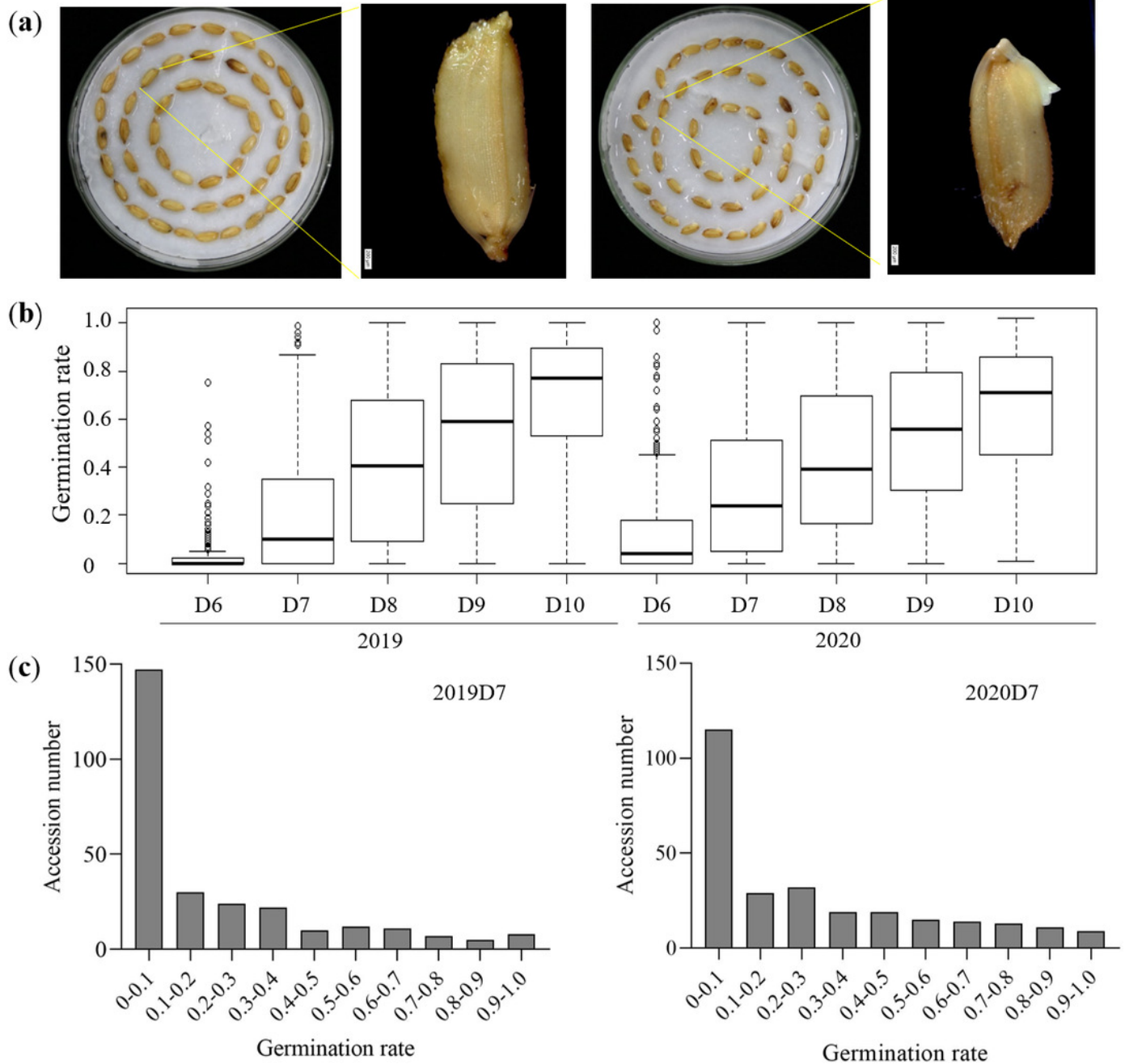



Figure 2

Description of population structure.

(a) Principal component analysis. (b) NJ tree based on Nei's genetic distance. (c) Delta K values plotted as the number of subgroups. (d) Subgroups inferred using STRUCTURE.

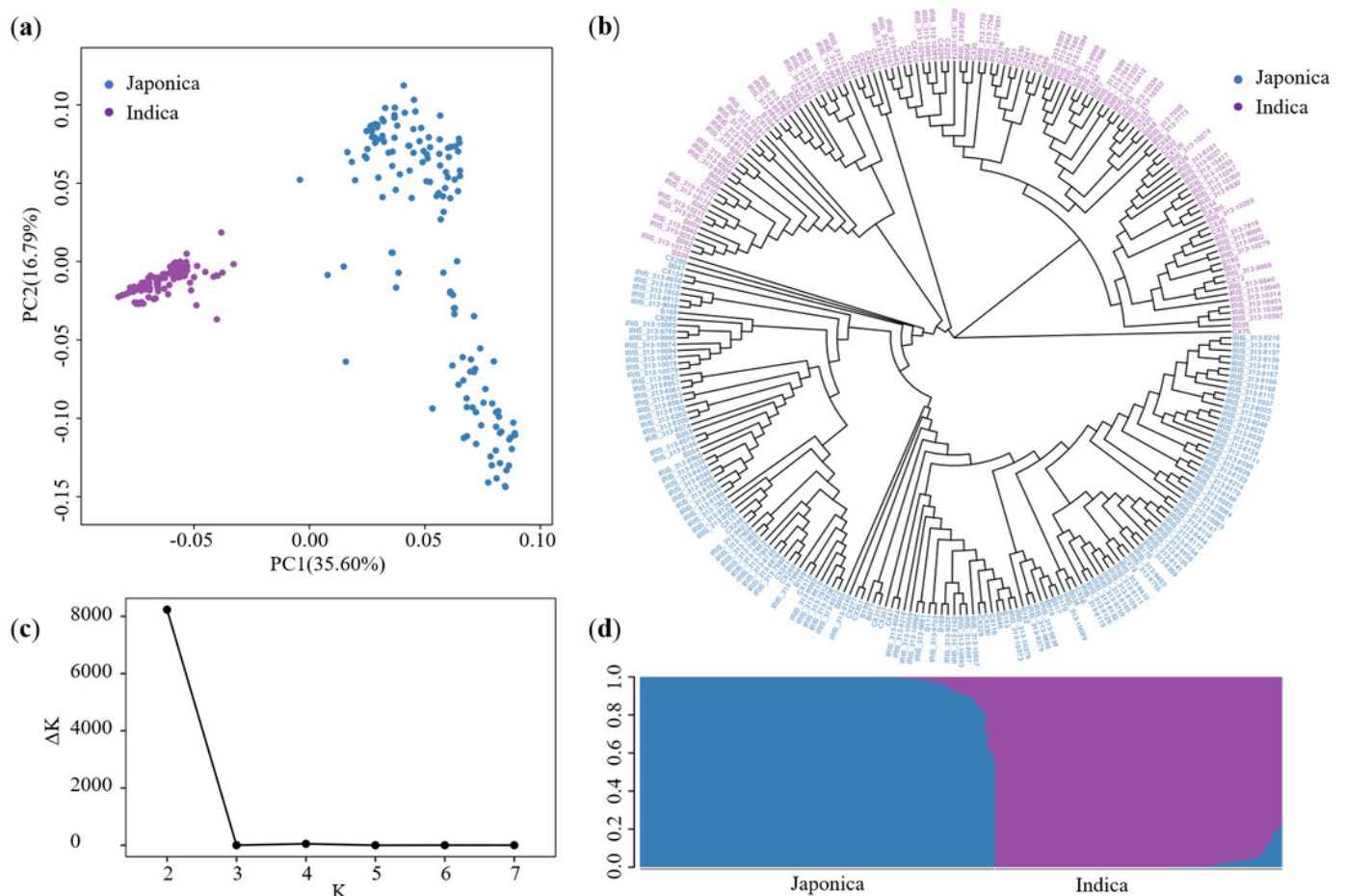



Figure 3

Manhattan plot and Q-Q plot for LTG using 2 programs in D7.

(a) A GWAS performed on 2019D7 using FaST-LMM. (b) GWAS performed on 2020D7 using FaSTL-MM. (c) A GWAS performed on 2019D7 using GEMMA. (d) A GWAS performed on 2020D7 using GEMMA. An orange arrow represents QTLs detected previously. A black arrow represents novel QTLs detected in this study. A yellow dotted box represents the repeated identified QTLs. A dashed horizontal line represents the suggestive threshold (P= 7.34×10^{-6}, -$\log_{10} P = 5.13$).

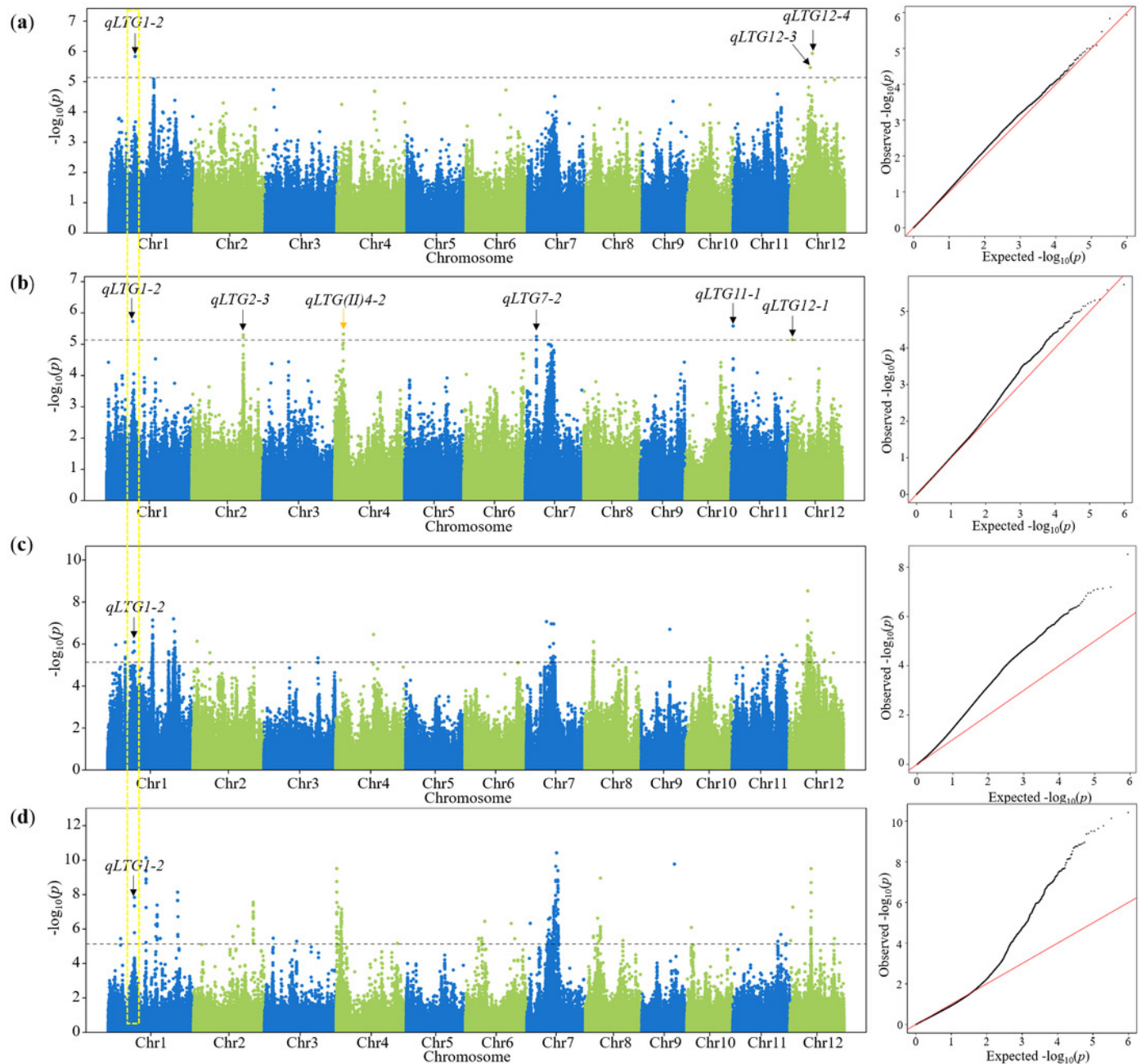



Figure 4

Candidate genes analysis.

(a) Gene structure and haplotype analysis for *LOC_Os01g23580.* (b) Comparison of germination rate among *LOC_Os01g23580* haplotypes in full population (**, $p < 0.01$; ***, $p < 0.001$). (c) Expression level of *LOC_Os01g23580* in contrast accessions after three days soaking in water in 15℃ and darkness for germination. Black bars represented expression levels of rice accessions with high germination rate under low temperature. Grey bars represent the expression levels of rice accessions with low germination rate under low temperature. (d) Gene structure and haplotype analysis for *LOC_Os01g23620.* (e) Comparison of germination rate among *LOC_Os01g23620* haplotypes in full population (**, $p < 0.01$). (f) Expression level of *LOC_Os01g23620* in contrast accessions after three days soaking in water in 15℃ and darkness for germination.

(a)

Hap.	upstream 13236390	CDS 13243045	2019 rate	2019 num	2020 rate	2020 num
hap I	G	G	0.318	86	0.398	82
hap II	G	C	0.189	38	0.268	37
hap III	A	G	0.171	123	0.259	111

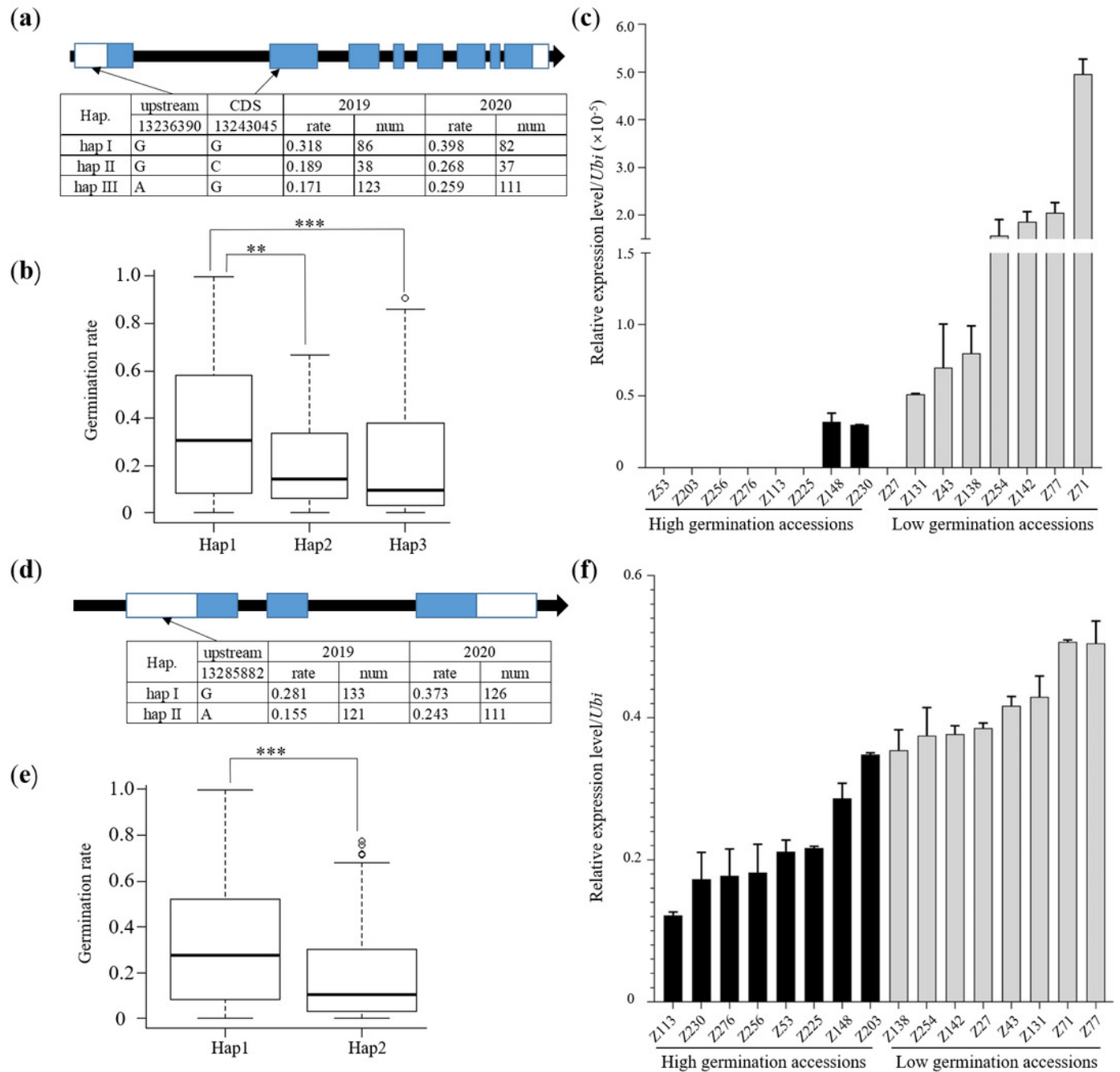
(b)

(c)

(d)

Hap.	upstream 13285882	2019 rate	2019 num	2020 rate	2020 num
hap I	G	0.281	133	0.373	126
hap II	A	0.155	121	0.243	111

(e)

(f)

Description of germination rate in full population.

1 Table 1. Description of germination rate in full population.

Days	Germination rate in 2019			Germination rate in 2020		
	Range	Mean ± SD	Median	Range	Mean ± SD	Median
D6	0-0.75	0.035±0.100	0	0-1	0.136±0.204	0.04
D7	0-0.99	0.220±0.265	0.1	0-1	0.310±0.285	0.24
D8	0-1	0.399±0.315	0.405	0-1	0.429±0.302	0.39
D9	0-1	0.537±0.311	0.59	0-1	0.544±0.286	0.56
D10	0-1	0.694±0.258	0.77	0.01-1	0.647±0.259	0.71

Summary of detected QTLs using FaST-LMM in the full population.

1   Table 2. Summary of detected QTLs using FaST-LMM in the full population.

QTLs	Trait ID	Chromosome	Peak SNP	P-value	reported QTLs overlapped
qLTG1-1	2019D6	Chr1	12,153,951	5.51	*qCTGERM1-5* (Shakiba et al. 2017)
qLTG1-2	2019D6,2019D7,2020D6,2020D7	Chr1	13,340,259	5.83	
qLTG1-3	2020D6	Chr1	19,239,470	6.09	*qCTS1-2* (Wang et al.2016)
qLTG1-4	2019D6	Chr1	22,886,860	6.85	*qCTGERM1-6* (Shakiba et al. 2017)
qLTG1-5	2020D6	Chr1	24,833,598	5.60	
qLTG1-6	2020D10	Chr1	29,923,602	5.88	
qLTG1-7	2019D6	Chr1	35,250,579	5.23	*qLTG1b* (Fujino et al. 2015)
qLTG2-1	2019D6	Chr2	4,583,247	5.73	*OsWRKY71,qCTS2-2* and *qLTGS(III)2* (Kim et al., 2016;Wang et al.2016;Najeeb et al. 2020)
qLTG2-2	2019D6	Chr2	20,749,806	6.10	*qLTG(III)2* (Najeeb et al. 2020)
qLTG2-3	2020D6,2020D7,2020D8	Chr2	26,062,949	5.54	
qLTG2-4	2019D6	Chr2	30,309,540	7.91	*OsMADS57*(Guo et al., 2013)
qLTG2-5	2019D6	Chr2	30,974,975	8.54	
qLTG3-1	2019D6	Chr3	24,070,502	6.09	
qLTG4-1	2020D10	Chr4	2,756,738	5.28	*qCTGERM4-3* (Shakiba et al. 2017)
qLTG4-2	2020D8,2020D9	Chr4	3,566,435	5.35	
qLTG4-3	2020D8,2020D9,2020D10	Chr4	4,192,136	6.74	
qLTG4-4	2020D6,2020D7	Chr4	4,527,433	5.14	*qLTG(II)4–2* (Najeeb et al. 2020)
qLTG4-5	2019D6	Chr4	20,867,550	5.20	
qLTG4-6	2019D6	Chr4	23,131,460	5.27	
qLTG6-1	2019D6	Chr6	20,322,237	6.07	
qLTG7-1	2020D6	Chr7	1,702,699	6.14	*qLTG7* and *qCTS7-1* (Fujino et al. 2015; Wang et al.2016)
qLTG7-2	2020D7	Chr7	5,701,029	5.25	
qLTG7-3	2020D6	Chr7	11,338,200	5.47	
qLTG7-4	2020D8	Chr7	13,267,244	5.21	*qCTGERM7-2* (Shakiba et al. 2017)
qLTG7-5	2020D6	Chr7	14,587,580	6.83	
qLTG7-6	2019D6	Chr7	28,676,190	6.33	*qCTS7-5* and *qCTGERM7-5* (Wang et al.2016; Shakiba et al. 2017)
qLTG8-1	2020D6	Chr8	6,167,751	5.66	

qLTG8-2	2020D6	Chr8	7,601,891	5.80	
qLTG9-1	2019D6	Chr9	7,410,218	6.22	
qLTG10-1	2019D6	Chr10	23,066,742	6.61	*qCTGERM10-4* (Shakiba et al. 2017)
qLTG11-1	2020D7,2020D8	Chr11	1,170,653	5.58	
qLTG11-2	2019D6	Chr11	17,712,316	5.55	*qCTS11-5* and *qCTGERM11-4* (Wang et al.2016; Shakiba et al. 2017)
qLTG12-1	2019D6	Chr12	1,512,598	5.40	
qLTG12-2	2020D7,2020D9,2020D10	Chr12	2,084,623	5.14	
qLTG12-3	2019D7	Chr12	10,140,027	5.47	
qLTG12-4	2019D7	Chr12	11,182,503	5.93	
qLTG12-5	2019D9	Chr12	23,640,519	5.56	□