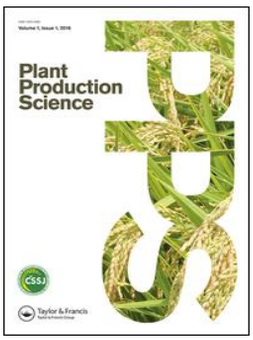

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Identification and validation of QTLs for cold tolerance at the booting stage and other agronomic traits in a rice cross of a Japanese tolerant variety, Hananomai, and a NERICA parent, WAB56-104

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ABSTRACT

In Africa, cold temperatures occur in the highlands of East and Southern Africa and in some areas of the Sahel region of West Africa leading to substantial rice yield losses. Cold tolerance (CT) at booting stage on basis of spikelet fertility after cold water irrigation was evaluated using F_2 population derived from a cross between temperate *japonica*, Hananomai, and tropical *japonica*, WAB56-104. Two Quantitative trait loci (QTLs) for CT were detected on chromosome 8 and 10 with enhanced effects on the trait coming from Hananomai and WAB56-104 allele, respectively. The QTLs explained 30% and 33% of phenotypic variation in spikelet fertility, respectively. CT was negatively correlated with panicle number ($r = -0.35$, $p < 0.01$) and positively correlated with panicle weight ($r = 0.61$, $p < 0.001$). Selected BC_1F_4 and BC_1F_5 genotypes having homozygous alleles for both CT QTLs exhibited higher spikelet fertility under cold stress. The identified QTLs will be useful in the development of cold-tolerant varieties for production in high altitude areas through marker-assisted selection.

Abbreviations: NERICA: New Rice for Africa; CT: Cold tolerance; QTLs: Quantitative trait loci; MAS: Marker-assisted selection; BC: Backcross; PCR: Polymerase chain reaction; CIM: Composite interval mapping

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CLASSIFICATION
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Introduction

Rice is one of the most important cereal food crop and it provides food to more than half of the world's population, particularly in many developing countries in Asia, Africa, and Latin America (Khush, 2005). Globally, rice is grown on approximately 163 million hectares of which an estimated 60% or more is affected by various abiotic stresses causing significant yield losses; 10% of rice crop area is subjected to low temperatures (Wu & Garg, 2003). In Africa, cold temperatures occur in the highlands of East and Southern Africa and in some areas of the Sahel region of West Africa leading to substantial yield losses (Zenna et al., 2010). The highlands of East Africa are unique agricultural zones with a huge potential for rice production. The most appropriate season for rice cultivation in the highlands of East Africa is during the long-rains (March–May). However, transplanting rice in April–May is associated with cold-induced sterility, since the reproductive phase of the crop coincides with the periods of low temperatures during the cold months of June and July (Sekiya et al., 2015). In this region, day average temperatures fall below 20 °C during June to July and minimum temperatures can fall to as low as 10 °C, for example in Zanzibar, Tanzania (Sekiya et al., 2015), and in Embu, Kenya (Nasuda et al., 2014).

In order to boost rice production in sub-Saharan Africa, New Rice for Africa (NERICA) varieties were developed at the Africa Rice Center (WARDA) (Jones et al., 1997). Due to their desirable characteristics, NERICA have gained considerable attention as a useful crop that could revolutionize the rice industry in many sub-Saharan Africa countries. The promotion of NERICA rice production, particularly under rain-fed conditions in high

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altitude regions of East Africa has been initiated to boost rice yields. Low temperatures, especially during panicle development and the booting stage, decrease the spikelet fertility of rice (Gunawardena et al., 2003; Shimono et al., 2002), resulting in considerable grain yield losses. In our previous study, we reported the suitability of NERICA 1 and NERICA 2 for production in the cold-prone regions of East Africa (Wainaina et al., 2015). Furthermore, we found out that WAB56-104 (*O. sativa* parent of NERICA1 to NERICA11) has greater cold tolerance based on spikelet fertility than the NERICAs and serves as a good genetic resource for breeding and improvement of cold tolerance of rice cultivars (Wainaina et al., 2015). WAB56-104 has also been reported to possess other beneficial traits such as salinity tolerance and striga weed pre-attachment resistance (Awala et al., 2010; Jamil et al., 2011). In this study, we tried to investigate the genomic regions associated with cold tolerance in WAB56-104 and further improve on its cold tolerance.

Some important agronomic traits such as culm length, heading date, panicle length, spikelets per panicle, spike length, grain weight, panicle number, panicle exsertion, and phenotypic acceptability are associated with cold tolerance and they may influence cold tolerance ability of rice (Jiang et al., 2011; Mackill & Lei, 1997; Zeng et al., 2009). These traits are also closely correlated with yield potential of rice thus important in determining yield outputs in rice. Here, we also evaluated other agronomic traits and their relationship with cold tolerance.

Materials and methods

Plant materials

The materials comprised of an F_2 population (108 individuals) derived from a cross between a cold-tolerant temperate *japonica*, Hananomai, and tropical *japonica*, WAB56-104. Hananomai is a Japanese variety with strong cold tolerance and is used as a standard check for cold tolerance evaluation at Aichi Prefecture Agricultural Research Institute, Japan. WAB56-104 is an upland improved variety (*O. sativa* ssp. *japonica)* and parent of NERICA 1 to NERICA 11 (Jones et al., 1997).

Field evaluation for cold tolerance and other agronomic traits

The F_2 population along with the parents were evaluated in a paddy field irrigated with water from a cold stream at Aichi Prefecture Mountainous Agricultural Research Institute (latitude: 35°13′N, longitude: 137°E, 505 m a.s.l). Spikelet fertility after cold water irrigation has been widely used as an effective parameter for determining the cold tolerance of rice at the reproductive stage (Jiang et al., 2011; Saito et al., 1995; Takeuchi et al., 2001). Germinated seeds were sown in seedling trays in early May, 2011 and 35 days old seedlings were transplanted (on June 6) in rows of 1.2 m length with a spacing of 30 cm × 15 cm between rows and between plants, respectively. Cold water irrigation started from the primordial stage to the completion of heading (July 12–September 2, 2011; Figure 1(a)). The depth of water was maintained at 20 cm. A control paddy field irrigated with normal water conditions (21–29 °C) was also planted at Nagoya University Farm for Science and Technology (latitude: 35°6′42′′N, longitude: 137°4′57′′E, 67 m a.s.l). As every genotype is genetically different in the F_2 population, a tiller originating from the same individual F_2 plant was transplanted in cold and normal water irrigation paddy field. Cold tolerance was evaluated based on mean spikelet fertility of three panicles from each individual plant.

Agronomic evaluation of MAS backcross lines

BC_1F_2 population was genotyped using RM1376 and RM8271 for chromosome 8 and RM7217 and RM8207 for chromosome 10. Selected genotypes were grouped as +HW genotypes (with tolerant homozygous alleles for both Quantitative trait loci [QTLs]) and −HW genotypes (without the tolerant homozygous QTLs alleles). Selected genotypes (6 +HW and 16 −HW BC_1F_2) were subjected to cold water irrigation for CT evaluation along with WAB56-104. Two +HW BC_1F_2 lines and two −HW BC_1F_2 lines were further selected and advanced to produce BC_1F_4 lines. In year 2014, B_1F_4 plants were evaluated under natural weather conditions at Kenya Agricultural and Livestock Research Organization Mwea Centre, Kenya (KALRO-Mwea research farm, latitude: 0°40′35′′S, longitude: 37°18′06′′E, 1168 m a.s.l) during the long rains cropping season (March–July). 50 seedlings of BC_1F_4 plants and WAB56-104 were transplanted in the field at 4–5 leaf stage in single rows, five plants per row at a spacing of 30 cm × 15 cm between rows and between plants. Transplanting was done in early April in order to coincide with cold stress at the reproductive stage. In year 2015, BC_1F_5 plants were evaluated under cold water irrigation in Japan.

Agronomic traits measurements

Three to six panicles heading between August 1–12 in 2011 in Japan, June 22–30 in 2014 in Kenya and September 5–20 in 2015 in Japan were harvested from each plant and used for panicle traits measurements. Since water temperature in 2015 in Japan gradually decreased to below the critical water temperature zone, we categorized the panicles into two heading groups depending on heading dates of the panicles for each hill to investigate the effect

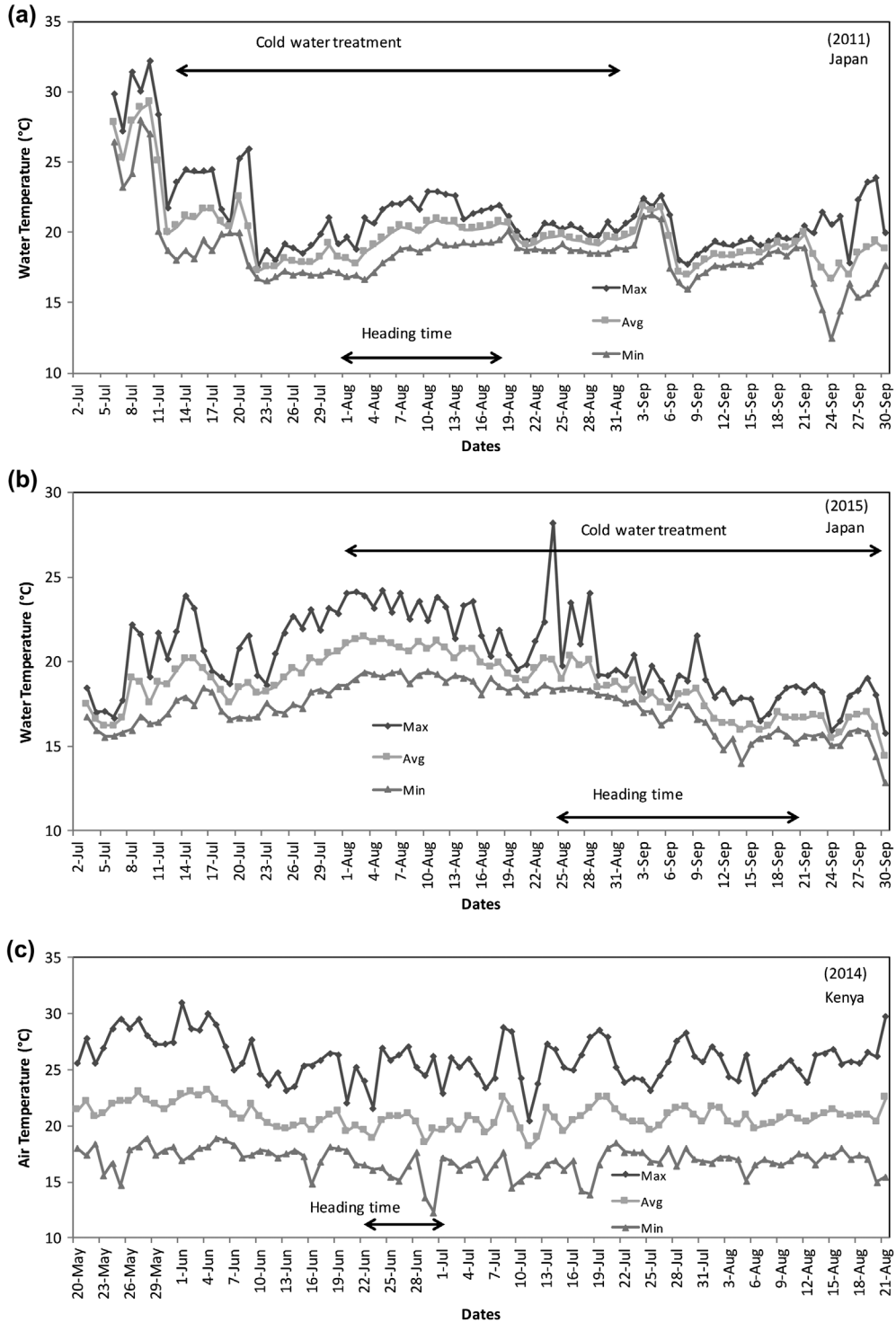

Figure 1. Water temperature in the cold water irrigation paddy field in 2011 (a) and 2015 (b) in Japan and air temperature under natural low temperature conditions in 2014 (c) in Kenya. Max, Avg and Min represent maximum, average and minimum temperatures, respectively.

of different levels of cold water stresses. Spikelet fertility was determined as the number of fertile grains in percentage (%) and was calculated based on the number of filled spikelets divided by the total number of spikelets per panicle. Panicle length was measured in centimeters from the panicle neck to the tip of the top most rachis-branch on the main axis. Culm length was measured in centimeters from the base of the culm to the panicle neck. Heading date were recorded on waterproof plastic tags for each tiller when a panicle was exserted from the sheath of the flag leaf. Panicle numbers were counted at harvest. Panicle weight was measured as weight of all panicles per plant

in grams at harvest and weight per panicle was calculated by dividing by number of panicles.

Genotyping, linkage map construction, and QTL analysis

DNA was extracted from leaves of each plant and their parents by TPS method as described by Hattori et al. (2007). A set of 252 SSR markers (McCouch et al., 2002) spanning all 12 chromosomes were screened for parental polymorphism between Hananomai and WAB56-104, of which 68 were polymorphic with an average marker interval of 21.5 cM, and were used to analyze the mapping population. PCR conditions, gel electrophoresis of PCR products, visualization, and genotype scoring methods were as described by Hattori et al. (2007). A linkage map was constructed using the 68 SSR markers that were polymorphic between Hananomai and WAB56-104 (Figure 2) using Windows QTL Cartographer version 2.5 (Wang et al., 2011). Composite interval mapping (CIM) was employed to detect QTLs affecting cold tolerance at the booting stage and other agronomic traits. Data for mean spikelet fertility percentage were transformed by arcsine transformation and were used as an index of cold tolerance for CIM. Significance threshold values of LOD scores for QTL detection were determined by 1000 permutations (Churchill & Doerge, 1994) and an experiment-wise error threshold of 0.05 was retained. The critical threshold value of LOD at a genome-wide significance level of $p = 0.05$ was 2.5. Naming of QTLs followed QTL nomenclature system defined by McCouch et al. (1997).

Statistical analysis

For the differences in spikelet fertility and other agronomic traits between genotypes, analysis of variance (ANOVA) was performed using GLM procedure and correlation analysis between the agronomic traits was performed using the correlation procedure in SAS program (SAS version 9.1, SAS Institute Inc., Cary, NC, U.S.A, 2002). Data for mean spikelet fertility were arcsine-transformed and means are reported after back transforming. Means were separated by least significant difference test at $p < 0.05$.

Results

Water temperature in the cold water irrigation paddy field in Japan and heading dates

The water temperature during the treatment period in 2011 (July 12–September 2) had ranges of 17.5–25.9, 17.2–22.5, and 16.5–20.1 °C for the maximum, average, and minimum temperatures, respectively (Figure 1(a)). The mean temperatures in 2011 were 21.1, 19.6, and 18.4 °C for the maximum, average, and minimum, respectively (Figure 1(a)). The water temperature during the treatment period in 2015 (August 1–September 30) had ranges of 15.8–28.5, 14.4–21.5, and 12.9–19.4 °C for the maximum, average, and minimum temperatures, respectively (Figure 1(b)). The mean temperatures in 2015 were 20.3, 18.6, and

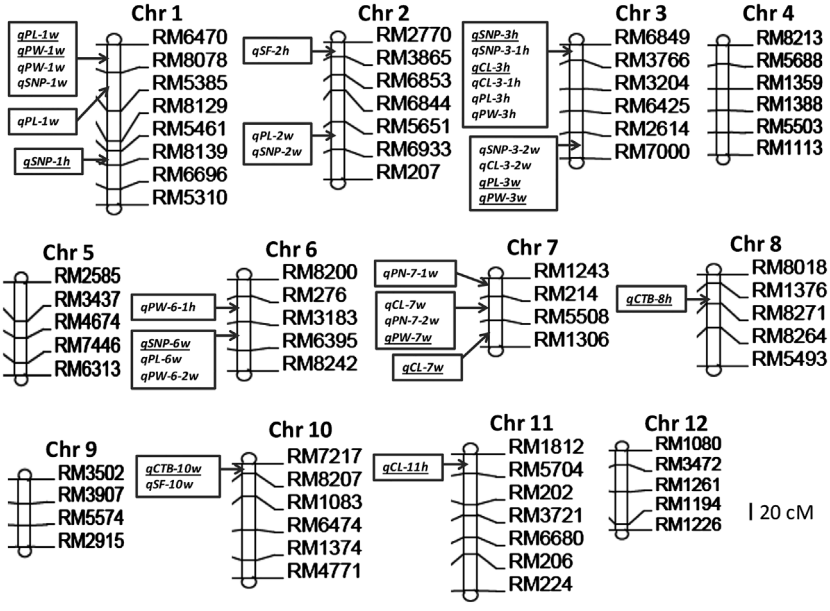

Figure 2. Linkage map showing quantitative trait loci (QTLs) and polymorphic SSR markers in the F_2 population between Hananomai and WAB56-104. QTLs detected under cold water irrigation are underlined. h and w at end of QTL names indicate allele with enhanced effect is from Hananomai and WAB56-104, respectively. Scale bar = 20 cM.

17.3 °C for the maximum, average, and minimum, respectively (Figure 1(b)). Heading dates were between August 1–19 in 2011 (Figure 1(a)) and August 25–September 20 in 2015 (Figure 1(b)) under cold water irrigation. Cold water irrigation started at least 20 days before heading time and thus, the treatment periods were within the critical stage for cold injury to occur in the reproductive stage, especially in the booting stage (Satake, 1976).

Compared with 2015, the water temperature at the booting stage (around middle to end of July) in 2011 was lower (Figure 1(a) and (b)). The water temperature at the booting stage (around early to late August) in 2015 gradually decreased to below the critical water temperature zone (Figure 1(b)). Therefore, we categorized the panicles into two heading groups depending on heading dates of the panicles for each hill to investigate the effect of different levels of cold water stresses.

Air temperature in Mwea, Kenya field, and heading dates

Heading dates under natural weather conditions in 2014 were between June 22–30 (Figure 1(c)). The air temperature in early to the end of June (including both of the booting and heading stages) in 2014 had ranges of 21.5–26.0, 18.1–22.0, and 12.3–18.9 °C for the maximum, average, and minimum temperatures, respectively (Figure 1(c)). The mean air temperatures for the same period were 24.3, 20.2, and 15.8 °C for the maximum, average, and minimum, respectively (Figure 1(c)). Cold stress during this period in 2014 was considered as mild cold stress since the mean average air temperature (20.2 °C) was slightly higher than the sub-optimal temperature (18–20 °C) for cold injury to occur (Shimono et al., 2007). Nevertheless, minimum temperatures were low enough to cause cold injury to rice. The air temperatures during the panicle initiation stage (around late May to early June) in 2014 were higher than the critical temperature (Figure 1(c)) (Satake et al., 1987), indicating that there was minimal damage by cold temperature in the panicle initiation stage.

Phenotypic variation

The parental cultivars, Hananomai, and WAB56-104 showed phenotypic variation in spikelet fertility and other agronomic traits after cold water irrigation which largely reflected the genetic variation of cold tolerance among the F_2 population (Table 1). Hananomai had significantly higher spikelet fertility and reached heading time earlier than WAB56-104 under cold water irrigation whereas WAB56-104 was superior in other traits except for panicle number which was comparable between the two parents (Table 1). All the traits were reduced and heading delayed in F_2 population and the parents by cold water treatment indicating that the treatment was successful for evaluating the effects of cold stress, except for panicle number in F_2 population. The distribution curves for most traits were mesokurtic, with kurtosis values close to zero (Table 1). Spikelet fertility and other agronomic traits showed continuous distribution, ranging between and over the parental values, indicating quantitative inheritance of these traits.

Spikelet fertility of Hananomai and WAB56-104 under cold water treatment was 72.3 and 50.7%, respectively. Under normal temperature conditions (Table 1), the spikelet fertility of Hananomai and WAB56-104 was 92.7 and 89.5%, respectively. Spikelet fertility of the F_2 population ranged from 0% to 95.7%, with a mean of 35.4% under cold water treatment. Spikelet fertility of the F_2 population under normal temperature condition ranged from 57.2% to 96.9% with a mean of 84.8%.

Correlation analysis between the agronomic traits in the F_2 population

Correlation analysis was performed to establish the relationship between the traits and the results are shown in Table 2. Spikelet fertility was not significantly correlated with most of the traits except panicle number ($r = -0.35$) and panicle weight ($r = 0.61$) under cold water irrigation and with culm length ($r = 0.25$) under normal water irrigation. Spikelet fertility may be negatively affected by panicle numbers under cold stress and this panicle numbers may influence cold tolerance ability of rice. Positive and significant correlations were observed between the traits spikelet number per panicle, panicle length, and culm length, with coefficients (r) of 0.37–0.75 under cold water irrigation and 0.37–0.76 under normal water irrigation (Table 2). Panicle weight and panicle number showed significant but weak negative correlations under both water conditions ($r = -0.22$ and -0.25). Heading time showed positive significant correlations with culm length ($r = 0.18$ and 0.33) under both water conditions and a significant weak negative correlation with panicle number ($r = -0.17$) under normal water irrigation (Table 2).

QTLs identified under cold water irrigation

QTL analysis for cold tolerance (spikelet fertility) and other agronomic traits under cold water irrigation are described below.

Spikelet fertility

Two QTLs for cold tolerance (CT) on the basis of percent spikelet fertility were detected on chromosome 8 (*qCTB-8*) and 10 (*qCTB-10*) with additive effects from Hananomai and WAB56-104, respectively (Table 3). The QTLs explained

Table 1. Trait mean values of 108 F_2 plants and their parents under cold and normal water irrigation in 2011, Japan.

	Cold water irrigation								Normal water irrigation							
	Hananomai		WAB56-104		F_2 population		Skewness	Kurtosis	Hananomai		WAB56-104		F_2 population		Skewness	Kurtosis
Trait					Mean	Range							Mean	Range		
SF (%)	72.3	a	50.7	b	35.4	0.0–95.7	0.4	−1.4	92.7	a	89.5	a	84.8	57.2–96.9	−1.3	2.0
SNP	114.0	b	152.0	a	107.0	29.0–212.0	0.6	0.3	130.0	b	177.0	a	142.0	53.0–238.0	0.2	0.0
PN	5.0	a	5.0	a	7.6	2.0–17.0	0.6	0.4	7.0	a	6.0	a	5.4	1.0–16.0	1.1	2.0
PL (cm)	15.8	b	20.6	a	18.1	13.0–25.3	0.5	0.5	21.5	a	24.3	a	21.8	15.4–30.2	0.4	0.6
PW (g/panicle)	1.0	b	2.3	a	1.5	0.4–3.2	0.6	−0.5	1.8	b	3.8	a	2.3	0.4–4.6	0.5	1.1
CL (cm)	71.6	b	80.5	a	67.7	39.0–93.9	−0.3	0.4	79.4	b	98.9	a	71.4	52.0–86.8	−0.3	−0.3
HD (DAT)	47	b	70	a	61	37–74	−1.1	0.9	44	a	46	a	46	44–47	−0.6	−0.7

Notes: Means followed by the same letter along the rows are not significantly different (GLM procedure in SAS, $p < 0.05$). SF, Spikelet fertility; SNP, Spikelet number per panicle; PN, Panicle number; PL, Panicle length; PW, Panicle weight; CL, Culm length, HD, Heading date (Days after transplanting).

Table 2. Correlation coefficients for the agronomic traits in F_2 population under cold and normal water irrigation in 2011, Japan.

Trait	SF		SNP		PL		CL		PN	
					r (Cold water irrigation)					
SNP	0.10	ns								
PL	0.12	ns	0.66	***						
CL	0.17	ns	0.75	***	0.46	***				
PN	−0.35	**	−0.02	ns	−0.13	ns	0.04	ns		
PW	0.61	***	0.53	***	0.44	**	0.37	**	−0.25	*
HD	0.04	ns	0.17	ns	0.04	ns	0.33	***	−0.04	ns
					r (Normal water irrigation)					
SNP	0.10	ns								
PL	0.06	ns	0.76	***						
CL	0.25	*	0.69	***	0.43	***				
PN	−0.06	ns	0.12	ns	0.10	ns	0.01	ns		
PW	0.10	ns	0.54	***	0.48	***	0.37	**	−0.22	*
HD	0.14	ns	0.13	ns	0.06	ns	0.18	*	−0.17	*

Notes: *, **, *** significant at $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively; ns, not significant. SF, Spikelet fertility; SNP, Spikelet number per panicle; PL, Panicle length; CL, Culm length; PN, Panicle number; PW, Panicle weight/panicle; HD, Heading date.

Table 3. QTLs detected for cold tolerance (spikelet fertility) and other agronomic traits under cold water irrigation by composite interval mapping.

QTLs	Chr.	Flanking markers	Marker interval (cM)	Site (cM)	LOD	AE	R^2 (%)	DPE
Cold tolerance at booting (percent spikelet fertility, %SF)								
qCTB(SF)-8	8	RM1376-RM8264	43.8	13.2	5.04	8.7	30	H
qCTB(SF)-10	10	RM7217-RM1083	21.2	4	9.77	−10.5	33	W
Panicle length (PL)								
qPL-1	1	RM6470-RM8078	27.9	0.7	3.45	−1.08	11	W
qPL-3	3	RM2614-RM7000	31.7	17.2	2.62	−1.05	11	W
Spikelet number (SN)								
qSNP-1	1	RM8139-RM6696	22.6	7.2	2.57	14.3	60	H
qSNP-3	3	RM6849-RM3766	19.6	0.4	4.63	24.1	20	H
qSNP-6	6	RM3183-RM8242	61.7	25.9	3.05	−15.3	8	W
Culm length (CL)								
qCL-3	3	RM6849-RM3766	19.6	4.2	3.14	4.55	9	H
qCL-7	7	RM5508-RM1306	35.1	28.1	4.82	−4.28	20	W
qCL-11	11	RM1812-RM202	38.8	18	2.84	3.71	5	H
Panicle weight (PW)								
qPW-1	1	RM6470-RM8078	27.9	0.4	4.08	−0.45	19	W
qPW-3	3	RM6425-RM7000	57.2	33	3.73	−0.53	25	W
qPW-7	7	RM214-RM5508	31.7	8	2.54	−1.04	41	W
Heading date (Days after transplanting, DAT)								
qHD-4	4	RM5503-RM113	8	20	13.9	18	70	H
qHD-7	7	RM1243-RM214	9	20.2	13.8	17	71	H
qHD-11	11	RM6680-RM206	4.7	7.3	7.7	1	3	H

Notes: Chr., chromosome number; AE, additive effect of the Hananomai allele; R^2, percent of phenotypic variation explained; DPE, direction of phenotypic effect to which an allele enhances a trait; H and W indicate Hananomai and WAB56-104, respectively. LOD experiment-wise $p = 0.05$ was equivalent to critical LOD score threshold of 2.5. Site is the genetic distance between the peak of putative QTL and the left-side marker.

30 and 33% of the phenotypic variation, respectively. The *qCTB-8* showed a positive additive effect ($a = 8.7$), indicating that allele from Hananomai could increase spikelet fertility in F_2 population by 8.7%. The *qCTB-10* showed negative additive effect ($a = -10.5$), indicating an increased effect from WAB56-104 allele and could increase spikelet fertility in F_2 population by 10.5%.

Panicle length

Two QTLs for panicle length were detected on chromosome 1 (*qPL-1*) and 3 (*qPL-3*) with additive effects from WAB56-104 (Table 3). Each of the QTL explained 11% of the phenotypic variation and showed negative additive effects ($a = -1.08$ and -1.05), indicating that allele from WAB56-104 could increase panicle length in F_2 population by 1.08 and 1.05 cm on these chromosome loci, respectively.

Spikelet number per panicle

Three QTLs for spikelet number per panicle were detected on chromosome 1 (*qSNP-1*), 3 (*qSNP-3*) and 6 (*qSNP-6*). The additive effects of QTLs on chromosome 1 and 3 were from Hananomai whereas that of the QTL on chromosome 6

Table 4. QTLs detected for agronomic traits under normal water irrigation by composite interval mapping.

QTLs	Chr.	Flanking markers	Marker interval (cM)	Site (cM)	LOD	AE	R^2 (%)	DPE
Spikelet fertility (%)								
qSF-2	2	RM2770-RM6853	37.4	16.9	2.79	2.53	88	H
qSF-10	10	RM7217-RM1083	21.2	4.2	3.08	−0.16	7	W
Panicle length (PL)								
qPL-1	1	RM8078-RM8129	66	32.1	2.69	−1.57	14	W
qPL-2	2	RM5651-RM6933	30.4	14.1	3.01	−0.94	5	W
qPL-3	3	RM6849-RM3766	19.6	0.4	6.3	1.92	24	H
qPL-6	6	RM3183-RM8242	61.7	30.8	3.49	−1.31	17	W
Spikelet number (SNP)								
qSNP-1	1	RM6470-RM5385	63.9	20.1	3.43	−20.5	15	W
qSNP-2	2	RM5651-RM207	62.8	42.8	2.87	−14.7	9	W
qSNP-3–1	3	RM6849-RM3766	19.6	0.4	7.62	26.3	27	H
qSNP-3–2	3	RM6425-RM7000	57.2	28.9	4.18	−21.7	27	W
Culm length (CL)								
qCL-3–1	3	RM6849-RM3766	19.6	0.4	5.04	3.06	13	H
qCL-3–2	3	RM6425-RM7000	57.2	16.4	3.49	−3.95	13	W
qCL-7	7	RM214-RM1306	66.7	24.4	5.1	−4.56	14	W
Panicle number (PN)								
qPN-7–1	7	RM1243-RM214	24	22.1	3.75	−5.61	34	W
qPN-7–2	7	RM214-RM5508	31.6	2.1	2.72	−6.01	34	W
Panicle weight (PW)								
qPW-1	1	RM6470-RM8078	27.9	2	5.4	−0.4	13	W
qPW-3	3	RM6849-RM3766	19.6	5.5	3.1	0.24	8	H
qPW-6–1	6	RM276-RM3183	31.4	25.2	3.5	0.46	19	H
qPW-6–2	6	RM6395-RM8242	24.4	1.5	5.3	−0.44	19	W

Notes: Chr., chromosome number; AE, additive effect of the Hananomai allele; R^2, percent of phenotypic variation explained; DPE, direction of phenotypic effect to which an allele enhances a trait; H and W indicate Hananomai and WAB56-104, respectively. LOD experiment-wise $p = 0.05$ was equivalent to critical LOD score threshold of 2.5. Site is the genetic distance between the peak of putative QTL and the left-side marker. Marker nearest to QTL peak are underlined.

was from WAB56-104 (Table 3). The QTLs explained 60, 20, and 8% of the phenotypic variation, respectively. The QTLs on chromosome 1 and 3 showed positive additive effect (a = 14.3 and 24.1), indicating that allele from Hananomai could increase spikelet number per panicle in F_2 population by 14.3 and 24.1 on these chromosome loci, respectively. The QTL on chromosome 6 showed negative additive effect (a = −15.3), indicating that allele from WAB56-104 could increase spikelet number per panicle by 15.3 on this chromosome locus.

Culm length

Three QTLs for culm length were detected on chromosome 3 (*qCL-3*), 7 (*qCL-7*), and 11 (*qCL-11*). The additive effects of QTLs on chromosome 3 and 11 were from Hananomai whereas that of the QTL on chromosome 7 was from WAB56-104 (Table 3). The QTLs explained 9, 20, and 5% of the phenotypic variation, respectively. The QTLs on chromosome 3 and 11 showed positive additive effect (a = 4.55 and 3.71), indicating that allele from Hananomai could increase culm length in F_2 population by 4.55 cm and 3.71 cm on these chromosome loci, respectively. The QTL on chromosome 7 showed negative additive effect (a = −4.28), indicating that allele from WAB56-104 could increase culm length by 4.28 cm on this chromosome locus.

Panicle number

No QTLs for panicle number were detected in the F2 population under cold water irrigation

Panicle weight

Three QTLs for panicle weight were detected on chromosome 1 (*qPW-1*), 3 (*qPW-3*), and 7 (*qPW-7*) with additive effects from WAB56-104 (Table 3). The QTLs explained 19, 25, and 41% of the phenotypic variation, respectively. All the QTLs showed negative additive effect (a = −0.45, −0.53 and −1.04), indicating that allele from WAB56-104 could increase panicle weight in F_2 population by 0.45, 0.53, and 1.04 g on these chromosome loci, respectively.

Heading date

Three QTLs for heading date were detected on chromosome 4 (*qHD-1*), 7 (*qHD-3*), and 11 (*qHD-7*) with additive effects from Hananomai (Table 3). The QTLs explained 70, 71, and 3% of the phenotypic variation, respectively. All the QTLs showed positive additive effect (a = 18, 17, and 1), indicating that allele from Hananomai could promote early heading in F_2 population by 18 days, 17 days, and 1 day on these chromosome loci, respectively.

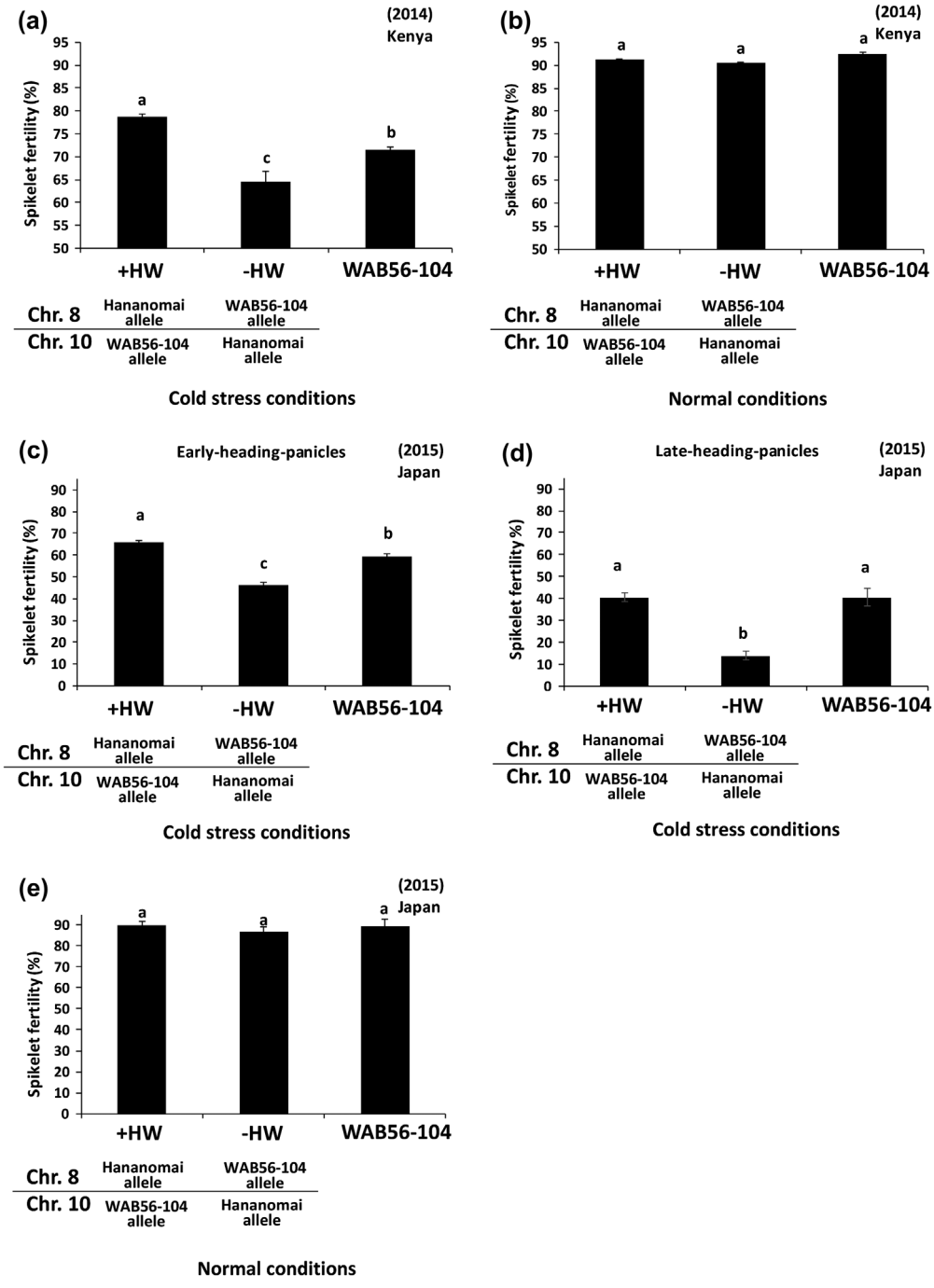

Figure 3. Genetic effects of quantitative trait loci (QTLs) on chromosome 8 and 10 on spikelet fertility in BC_1F_4 (a,b) and BC_1F_5 (c,d) genotypes under cold stress (a,c) and normal conditions (b,d). H and W indicate homozygotes of Hananomai and WAB56-104, respectively. Each bar represents mean trait value for each genotype, and error bars represent standard errors. Letters above the bars indicate significant differences between genotypes (GLM procedure in SAS, $p < 0.05$). The number of individuals for each genotype is shown in parentheses.

QTLs identified for the agronomic traits under normal water conditions

QTL analysis for spikelet fertility and other agronomic traits under normal water conditions are shown in Table 4. A total of 19 QTLs were identified as follows: 2 for spikelet fertility, 4 for panicle length, 4 for spikelet number per panicle, 3 for culm length, 2 for panicle number, and 4 for panicle weight.

QTL effects validation in selected backcross genotypes

To elucidate the genetic effects of the CT QTLs on spikelet fertility, we evaluated performance of backcross genotypes with homozygous alleles for the two CT QTLs on chromosome 8 and 10 (+HW genotypes) and those lacking the alleles for the QTLs (–HW genotypes) under

natural weather conditions in Kenya in 2014 (BC_1F_4 generation) and cold water irrigation in Japan in 2015 (BC_1F_5 generation). For effective evaluation of spikelet fertility, genotypes with comparable panicle numbers were used, as spikelet fertility exhibited a negative correlation with panicle number (Table 2).

Spikelet fertility was significantly higher in +HW BC_1F_4 genotypes (78.6%) than –HW BC_1F_4 genotypes (64.5%) and WAB56-104 (71.5%) under natural low temperature conditions in Kenya (Figure 3(a)). On the other hand, spikelet fertility was comparable between all genotypes under normal weather conditions (90.5–92.4%) (Figure 3(b)).

As mentioned above, since water temperature in 2015 in Japan gradually decreased to below the critical temperature zone (Figure 1(b)), BC_1F_5 genotypes grown under cold water irrigated conditions in Japan were categorized into two heading groups depending on heading dates of the panicles for each hill as follows: panicles heading between September 5 and 9 (early heading group), panicles heading between September 10 and 14 (late-heading group). The corresponding mean 5-day moving average temperature at 11 days before heading were 19.5 and 18.4 °C, respectively. For the early heading group of panicles, spikelet fertility was significantly higher in +HW BC_1F_5 genotypes (66%) than in -HW BC_1F_5 genotypes (46.2%) and WAB56-104 (59.3%) (Figure 3(c)). For late-heading group of panicles, spikelet fertility was much lower in both +HW genotypes and WAB56-104 (40.5%) but it was greatly reduced in –HW genotypes (13.8%) (Figure 3(d)). On the other hand, spikelet fertility of +HW, –HW BC_1F_5 genotypes, and WAB56-104 ranged from 86.7 to 90% and were not significantly different under non-stress conditions (Figure 3(e)).

Discussion

In this study, we identified two QTLs for cold tolerance at the booting stage on chromosome 8 (*qCTB-8*) and chromosome 10 (*qCTB-10*). We also found three QTLs for heading date (*qHD-4, 7, and 11*). Since it was impossible to keep the water temperature completely constant even though we treated cold water to the paddy field, we predicted that there would be some kind of relation between *qCTB* and *qHD*. However, no correlation was observed between these two traits (Table 2) and the positions of these QTLs were not overlapped. In this aspect, the water temperature at booting stage in 2011, when QTL analysis was done, was comparatively constant over more than 10 days (around July 22 to August 2), suggesting that *qCTBs* were detected by QTL analysis with little impact on the difference of heading date among F_2 population.

The QTL *qCTB-8* was identified on the short arm of chromosome 8 in a 26.1 cM interval flanked by markers RM1376 and RM8264. This QTL explained 30% of the phenotypic variation in spikelet fertility and its additive effect ($a = 8.7$) was from Hananomai. QTLs for cold tolerance have been mapped on all 12 chromosomes but very few QTLs have been mapped on chromosome 8. Kuroki et al. (2007) mapped a QTL (*qCTB8*) on the short arm of chromosome 8 in a 1.7 cM interval which explained 26.6% of the variation and was further narrowed down to a 193-kb interval between RM5647 and PLA61. The interval of *qCTB-8* detected in this study is very near that of *qCTB8* identified by Kuroki et al. (2007) and they may be similar QTLs controlled by the same gene for cold tolerance in *japonica* subspecies of rice. Liu et al. (2003) mapped a QTL (*qSLT8–2*) on the long arm of chromosome 8 which explained 5% of the variation near marker RM223 and is different from *qCTB-8* detected in this study because they are far apart on the chromosome.

The QTL *qCTB-10* was identified in a 17.3 cM interval in the region flanked by markers RM7217 and RM1083. This QTL explained 33% of the phenotypic variation in spikelet fertility and its additive effect ($a = -10.5$) was from WAB56-104. In a similar overlapping interval as *qCTB-10* in this study, a major QTL for cold tolerance (qLTSPKST10.1) was identified on the short arm of chromosome 10 in a 3.5 cM interval which explained 20.5% of the phenotypic variation (Ye et al., 2010). Xu et al. (2008) identified a QTL (*qCTB-10-2*) in another cross population in the same interval which explained 14.9% of the total phenotypic variance. Jiang et al. (2011) detected a QTL (QTL 10.1) between markers S10001B and S10019 in the same interval which explained 15.9% of the phenotypic variation. The QTL *qCTB-10* reported here explains more variation (33%) than qLTSPKST10.1, *qCTB-10-2,* and QTL 10.1 reported before in other studies. The interval of *qCTB-10* in this study overlaps with that of qLTSPKST10.1, *qCTB-10-2,* and QTL 10.1 identified in other studies. Thus, *qCTB-10* in this study may be similar to qLTSPKST10.1, *qCTB-10-2,* and QTL 10.1 and thus could be controlled by the same cold tolerant gene. Kuroki et al. (2009) detected a QTL for cold tolerance (*qCT-10*) on the long arm of chromosome 10 between markers RM3510 and RM333 which explained 20.4–35.4% of the phenotypic variation and this QTL is different from *qCTB-10* reported in this study.

We also evaluated other agronomic traits such as spikelet number per panicle, panicle number, panicle length, panicle weight, and culm length and their relationship with cold tolerance (spikelet fertility). These traits are important in determining yield potential of rice and have been reported to be associated with cold tolerance ability of rice (Jiang et al., 2011; Mackill & Lei, 1997; Zeng et al., 2009). Spikelet fertility was only significantly correlated with panicle number, panicle weight, and culm length (Table 2). Spikelet fertility was negatively correlated with

panicle number ($r = -0.35$, $p < 0.01$) especially under cold water irrigation. It has been reported that plants with few panicles produce increased number of engorged pollen grains available for fertilization at anthesis which could increase spikelet fertility (Gunawardena et al., 2003). Moreover, the panicles are larger with more spikelet number per panicle resulting in improved grain weights per panicle (Gunawardena et al., 2003). Results of correlation analysis in this study concur with this hypothesis as spikelet fertility and panicle weight were higher in plants with fewer panicles (Table 2). The relationship between spikelet fertility and panicle number should be an important consideration while progressing crop improvement for cold tolerance involving WAB56-104 and its descendants such as the NERICAs. We have previously observed such a relationship to contribute to relatively high grain weights per plant under cold stress in NERICA 3 and 4 due to production of few panicles with high number of spikelets per panicle coupled with relatively high filled grain ratio (Wainaina et al., 2015).

No correlation was observed between spikelet fertility and spikelet number per panicle, panicle length and culm length under cold water irrigation (Table 2). Besides, QTLs for spikelet number per panicle, panicle length, panicle weight, and culm length were detected in different regions compared with the QTLs for cold tolerance (Figure 2). In addition, the results of QTL analysis showed that no QTLs for the agronomic traits were detected in the same region with those of cold tolerance (spikelet fertility) under both cold and normal water irrigation. These results suggest that effects of these QTLs for the agronomic traits might not be associated with the effect of QTLs for cold tolerance.

A total of 35 QTLs for the different agronomic traits were detected under both cold water irrigation (16 QTLs) and normal water irrigation (19 QTLs). Only 5 of these QTLs were detected under both water treatment conditions. These are *qCTB-10/qSF10*, *qSNP-3*, *qCL-3*, *qCL-7*, and *qPW-1* (Tables 3 and 4). This indicates that most of the QTLs (genes) controlling the agronomic traits are influenced by the genotype by environment interaction and the effects of these loci are more greatly enhanced under a specific environment.

Under natural low temperature conditions in Kenya,+HW BC_1F_4 genotypes showed significantly higher spikelet fertility than –HW BC_1F_4 genotypes and WAB56-104 by an increment of 14.1 and 7.1%, respectively (Figure 3(a)). Besides, under cold water irrigation in Japan, +HW BC_1F_5 genotypes showed significantly higher spikelet fertility than -HW BC_1F_5 genotypes and WAB56-104 by increments of 19.8 and 6.7%, respectively (Figure 3(c)). When cold stress intensity increased to below 19 °C water temperature, spikelet fertility of all genotypes was greatly depressed from 66% to 40.5% in +HW genotypes and 46.2% to 13.8% in –HW genotypes (Figure 3(c) and (d)). However, there was still a significant difference on spikelet fertility between +HW and –HW genotypes (Figure 3(d)). These findings suggest that both QTLs play a significant role on cold tolerance and can be utilized for marker assisted selection in breeding rice for cold-prone environments such as in the highland regions of East Africa where upland NERICA production is being disseminated. In addition, we identified 8 QTLs for yield related traits under a cold stress environment (Table 3) of which 6 QTLs exhibited QTL by environment interaction with enhanced effects only under cold stress. The QTLs for these traits can be used for improvement of yields of rice subjected to cold-prone environments through QTL pyramiding without negatively affecting spikelet fertility of rice. Such a breeding approach could lead to the development of mega varieties with high cold tolerance and high yields.

Disclosure statement

The authors declare that they have no conflict of interest.

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