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ORIGINAL PAPER

Stability and genetic control of morphological, biomass and biofuel traits under temperate maritime and continental conditions in sweet sorghum (*Sorghum bicolour*)

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Abstract

Key message **Eight morphological, biomass and biofuel traits were found with high broad-sense heritability and 18 significant QTLs discovered including one locus controlling the stem juice trait for sorghum grown in Denmark and China. Sweet sorghum with tall plant, fast maturation and high stem Brix content can be bred as a biofuel crop for Northern Europe.**

Abstract Sweet sorghum (*Sorghum bicolour*), a native tropical C_4 crop, has attracted interest as a bioenergy crop in northern countries due to its juice-rich stem and high biomass production. Little is known about the traits important for its adaptation to high altitude climatic conditions and their genetic controls. Recombinant inbred lines derived from a cross between a sweet and a grain *kaoliang* sorghum were used in five field trials in Denmark and in China to identify the stability and genetic controls of morphological, biomass and biofuel traits during three consecutive summers with short duration, cool temperatures and long days. Eight out of 15 traits were found with high broad-sense heritability. Strong positive correlations between plant height and biomass traits were observed, while Brix and juice content were under different genetic controls. Using newly developed PAV (presence and absence variant) markers, 53 QTLs were detected, of which 18 were common for both countries, including a locus controlling stem juice (LOD score = 20.5, r^2 = 37.5 %). In Denmark, the heading stage correlated significantly with biomass and morphology traits, and two significant maturity QTLs detected on chromosomes SBI01 and SBI02 co-localised with QTLs previously associated with early-stage chilling tolerance, suggesting that accelerating maturation might be a means of coping with low-temperature stress. Our results suggest that selection for tall and fast maturating sorghum plants combined with high Brix content represents a high potential for breeding bioenergy crop for Northern Europe.

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Anne Mocoeur, Yu-Miao Zhang and Zhi-Quan Liu have contributed equally to this work.

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Introduction

Sweet sorghum (*Sorghum bicolour*) has recently been promoted as an ideal biofuel feedstock. It is a natural variant of the common grain sorghum, which is the fifth most grown cereal worldwide, feeding over 500 million people in 98 countries (Pennisi 2009). Sweet sorghum has long been used as a forage crop in developing countries since it produces large quantities of juice-rich and sugar-rich stems and high biomass under harsh conditions during its three- to five-month growth cycle. In developed countries, sweet sorghum has considerable potential as forage, energy and biomass crop, while in developing countries the combination of biomass, biofuel and high yield could be used to tackle the growing and competing need for food, feed and fuel owing to the limited availability of arable land.

In contrast to maize, sorghum is regarded as a low-value crop and little effort has been made in breeding programmes towards its cold acclimation. Since sorghum originated from and has been domesticated in subtropical areas (Doggett 1988; Harlan and Dewet 1972), it is highly sensitive to low (below 15 °C) temperature stress. Besides, long days can affect the plant growth for photoperiod-sensitive sorghum cultivars. It has been shown that optimal temperatures for vegetative and reproductive growth are between 26–34 and 25–28 °C, respectively (Franks et al. 2006; Maiti 1996), while temperatures below 20 °C cause perturbation in all developmental processes (Franks et al. 2006; Prasad et al. 2008; Tiryaki and Andrews 2001; Yu and Tuinstra 2001). Diverse genetic resources have been identified as carrying chilling and low-temperature tolerance, mostly in grain sorghum. Chinese local grain *kaoliang* cultivars, adapted to temperate climates in Northeast China, have been described as potential germplasm harbouring transferable cold tolerance (Franks et al. 2006; Lu and Dahlberg 2001). However, such resources are still being poorly exploited in breeding programmes for sweet sorghum.

Responses to chilling, in sorghum, have mainly been investigated during the early developmental stages, characterised by poor performance in germination, seedling vigour, stand establishment and resistance to soil-borne pathogens which lead to reduced yield and biomass. Chilling episodes during early stages often delay flowering (Maulana and Tesso 2013) and induce male sterility (Downes 1972; Osuna-Ortega et al. 2003; Peacock 1982). Most of the sorghum genotypes are photoperiod sensitive, and long days prolong vegetative growth stages and inhibit flowering for cultivars sensitive to long days (Caddel and Weibel 1971). To date there have been no reports on the genetic controls of biomass and biofuel-associated traits of sorghum plants in Nordic climates and in long photoperiods, as found during summer seasons in northern latitudes such as Denmark. The identification of stable agronomic traits and an understanding of their relationships and genetic controls could help to understand sorghum acclimation to cooler conditions and to develop adapted varieties with a lower base temperature to expand the cultivation horizon.

In the present study, F_2, F_3 and F_5 populations were used from a cross between a *kaoliang* grain sorghum and a sweet sorghum for extensive phenotyping of biomass, plant morphology and biofuel-related traits in two countries: Denmark was used as a testing site characterising short and temperate summer with a long photoperiod, and in China, sorghum is a well-established crop. QTL analysis was performed to understand the stability of genetic controls for these traits and comprehend adaptive responses to cool nights and long-day conditions in order to develop breeding strategies to design sweet sorghum into a forage/energy crop in northern countries. The intention was also to test the use of small presence–absence variation (PAV)-based molecular markers (Shen et al. 2015) in QTL mapping in contrasting environments and on different mapping progeny.

Materials and methods

Plant materials

E-Tian, a sweet sorghum accession, was crossed with Ji2731, a representative Chinese *kaoliang*-type grain sorghum (Zheng et al. 2011), to develop mapping populations up to and including the F_5 generation, using the single seed descend method (Goulden 1939; Grafius 1965), at the Institute of Botany (IBCAS, Beijing). The descending F_2 ($n = 209$), F_3 ($n = 196$) and F_5 ($n = 175$) recombinant inbreed lines (RILs) were grown for the study. Ji2731 is well adapted to the temperate areas of northeast China, with good seedling vigour, non-tillering, a short growth period, a dry stem, a white dry midrib colour, and a good grain yield. E-Tian is a sweet sorghum which was introduced to China in the last century and is characterised by its tall juicy and sweet stem, low-tillering number, long growth cycle, green juicy midrib colour and lower grain yield. All three mapping populations of different generations (F_2, F_3, F_5 RILs) derived from the single cross, received seed quarantine treatment in greenhouses prior to the field trials.

Experimental design

Table 1 provides a summary of plant materials and locations used in this study. The F_2 was developed and grown in Jilin province, Northeast China, in 2010 (CN1). The

F_3 RIL families were grown for two consecutive years in Høje Taastrup, Denmark in 2012 and 2013 (DK1 and DK2 respectively). The F_5 RILs progeny were grown in 2013 in two locations in China, Beijing and Jilin (CN2 and CN3, respectively). The growing periods ran from late April-early May to late September-early October in all trials. The average temperatures in Chinese locations ranged from 16.3 to 25.9 °C, with varying annual rainfall, e.g. 13–224.5 and 7.1–174.8 mm per month in CN1 and CN2, respectively. In Denmark, monthly average temperatures ranged between 16.3–17.1 and 12.8–8.0 °C in DK1 and DK2, respectively, and constant rainfalls from 42 to 67 mm per month were observed both years. Due to its northern latitude (55°41′N), summers in Denmark are characterised by long days, with day length varying between 12.4 and 17.3 h between May and September. The Chinese locations have shorter days, with between 12.3 and 15.1 h of light a day.

Head-row sowing methods for individual RIL families were used in both countries. In China, three seeds were sown in holes 0.02 m in depth along 2.0 m-long rows with 0.20 m spacing between holes, with one row per RIL family. At the 5-leaf stage, the plants were thinned down to keep one plant per hole. Four hundred fifty kilograms of compound fertilisers and 225 kg urea per hectare were applied in these three trials. For DK1 and DK2, between 25 and 50 seeds were sown at 0.03 m in depth along 1 m-long rows spaced of 0.5 m, but low germination rates were obtained overall for both years, resulting in only 10-15 plants per row survived until the harvest time. In DK1 and DK2, 90 kg N per ha fertilisers was applied and weeding in the field was performed manually in both years. For each location, RILs were grown in rows and five tagged plants per RIL family were selected and harvested manually at the stem base for phenotyping within 2–3 h. However, for all locations, due to the low human force available for the extensive phenotyping labour, no replications were done. For obvious reasons, the 209 F_2 was phenotyped using single plants. Parental lines were grown in rows and replicated four to five times in each location and 10–15 plants were randomly selected and phenotyped for all each row replicate.

Table 1 Summary of field trials and growing conditions

Year	RILs	Locations	Latitude longitude elevation	Growing months	Day temperatures (°C)	Rainfall (mm)	Day length (h)
2010	209 F_2	CN1, JAAS[a]	43°52′N	May	16.3	122.3	14.4
		Jilin Province	125°12′E	June	24.1	13.0	15.2
		China	250 m	July	23.4	215.8	15.1
				August	22.2	224.9	14.0
				September	16.9	17.8	12.3
2012	196 F_3	DK1, Høje Taastrup	55°41′N	May	12.6	42.0	16.2
		Denmark	12°15′58″E	June	13.3	52.0	17.2
			28 m	July	16.7	67.0	16.5
				August	17.1	63.0	15.0
				September	13.4	60.0	12.4
2013	196 F_3	DK2, Høje Taastrup	55°41′N	May	12.8	42.0	16.2
		Denmark	12°15′58″E	June	15.2	52.0	17.2
			28 m	July	18.0	67.0	16.5
				August	17.4	63.0	15.0
				September	17.1	60.0	12.4
2013	175 F_5	CN2, IBCAS[a]	39°55′N	May	20.7	7.1	14.2
		Beijing Province	116°18′E	June	22.6	114.3	15.0
		China	60 m	July	25.9	174.8	14.4
				August	25.6	95.7	13.4
				September	19.0	72.8	12.3
2013	163 F_5	CN3, JAAS[a]	43°52′N	May	NA	NA	14.4
		Jilin Province	125°12′E	June			15.2
		China	250 m	July			15.1
				August			14.0
				September			12.3

[a] JAAS, Institute of Agro-Food Technology, Changchun; IBCAS, Institute of Botany, Chinese Academy of Sciences, Beijing

Table 2 Summary of traits measured in each location and grouped by categories

Trait category	Traits		Locations
(1) Plant morphology	PHT	Plant height	All
	PeL	Peduncle length	All
	PaL	Panicle length	All
	NN	Number of stem nodes	All
(2) Biomass	FSW	Fresh stem weight	All
	FLW	Fresh leaf weight	CN1–DK2
	SD	Stem diameter	All
	PaW	Fresh panicle weight	CN1, CN2, CN3
	HSW	Hundred seed weight	CN1
(3) Biofuel-related traits	Brix	Stem sugar content	CN1, CN2, CN3, DK2
	Juice	Stem juiciness	CN1, CN2, CN3, DK2
		Midrib colour	CN1, CN2, CN3, DK2
(4) Plant development	HS	Heading stage	DK1–DK2
	FD	Flowering date	CN1
	Gsp	Growth speed	CN1–DK1

Ji2731 is a *kaoliang* landrace and is non-tillering, and E-Tian is a Russian inbred sweet sorghum line with low tillering

Phenotyping

Four traits categories were recorded, as summarised in Table 2. (1) Plant morphology: The plant height was measured from the base to the top of the ear (PHT). The final number of stem nodes was counted (NN). The peduncle length (PeL) was measured from the top of the stem to the base of the panicle and panicle length (PaL) was measured as the hear length. (2) Biomass: the fresh stem weight (FSW) was weighed after stripping leaves tissues. Fresh leaf biomass (FLW) was weighed only in DK2 and CN1. Stem diameter (SD) was taken at the middle of stem node position 6, from the base to the top. Fresh panicle weight and hundred seed weight (PaW, HSW) were only recorded in China as most of the F_3 RILs did not reach maturity in Denmark. (3) Biofuel-related trait: The Brix value was measured using a refractometer (MA871 Refractometer, Milwaukee, US) at the middle of stem node 5. "Juice" in RILs was visually determined as "dry" or "juicy" stems. The midrib colour was segregated among RILs with three observed phenotypes—"juicy green", "white dry" and "heterozygous"—when both phenotypes were found within a single RIL. (4) Plant development: The flowering date (FD) was counted as the number of days required after emergence for the F_2 in CN1. Heading stage (HS) was measured prior to harvest, for the F_3 RILs in DK1 and DK2, when 50 % of the plants in the RIL family had reached the same stage. HS scoring was based on the BBCH (Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie) scale for the growth stage of cereals, adapted to sorghum (Fig. S1) (Lancashire et al. 1991). The Growth speed (Gsp) was estimated in DK1 by scoring HS every weeks, from late August until harvest (late September) and the mean of the HS score differentials between each week was used to evaluate the Gsp. Gsp was evaluated in CN1 by measuring stem height every week for a total of seven weeks from June 2010 to late July 2010, with the means of the weekly increase in stem height used as Gsp score.

Genotyping

The 209 F_2 plants were genotyped with 325 novel small-size (40 bp–10 kbp) present and absent variant (PAV) markers (Shen et al. 2015) and 49 SSR markers (Bhattramakki et al. 2000; Kong et al. 2000; Yu et al. 2010) polymorphic between E-Tian and Ji2731. The development of PAV markers was described recently (Shen et al. 2015). Briefly, PAVs are structural variations present in one genome but missing in the other and have been uncovered using next-generation genome sequencing data (Zheng et al. 2011). After experimental validation of PAVs polymorphiic between parental lines, we selected 325 genic small-size PAVs for genotyping (Shen et al. 2015). DNA from the 209 F_2 plants was extracted from young leaves, using the CTAB extraction method (Doyle 1987) and a PCR mixture containing 1 µL of genomic DNA (80–120 ng/µL), 5 µL of MasterMix, 1 µL of 10× primers pairs designed 50–300 bp up- and downstream from the PAVs breakpoints and 3 µL of ultrapure water was used. A PCR programme, starting with 94 °C for 5 min, followed with 34 cycles of 94 °C for 30 s, 55–62 °C for 30 s, 72 °C for 30 s to 2 min and the final extension at 72 °C during 10 min. PCR products were revealed on a 2–5 % agarose gels and observed and monitored under UV light. The genetic map was constructed with JoinMap 4.x (Kyazma) using the Kosambi mapping function (Shen et al. 2015).

Data analysis

Phenotypic data were analysed with R version 3.0.2 software. An analysis of variance (ANOVA) was done for every trait using the following model (1):

$$Y_{ijk} \sim \mu + E_i + G_j + GE_{iij} + e_{ijk}, \quad (1)$$

where μ is the population mean, E_i the effect of the environment, G_j the genotype effect, GE_{iij} the genotype-by-environment effect and e_{ijk} the residual effect. Backward

modelling regression was done to fit the model for every phenotype, sequentially removing variables from the full model having the highest p values until all variables had a level of significance of $p < 0.001$. The models were validated using residual analysis. A Duncan test was conducted when significant differences were found between locations to classify RILs means.

Broad-sense heritability (H^2) was calculated for every phenotypes as indicated in model 2 (Fehr 1987), for the F_5 RILs grown in CN2 and CN3 in China, the F_3 RILs in DK1 and DK2, and between countries using the results of CN2, CN3, DK1 and DK2. Results from F_2 plants were not used in the H^2 estimation as only single plants were used for phenotyping without biological replications.

$$H^2 = \sigma_G^2/(\sigma_G^2 + \sigma_{GE}^2/E + \sigma_e^2/ER), \quad (2)$$

where σ_G^2 is the genetic variance, σ_{GE}^2 for variance explained by the genotype-by-environment effect, σ_e^2 for the residual variance, E for the number of environment and R the number of replicates. In this study, R was set to 1 as no replicates were done within locations.

Principal component analysis (PCA) was performed using country, population type and location as qualitative variables, and trait variables were scaled to unit variance using the FactoMineR R package (Lê and Husson 2008). The first two principal components were kept for graphical projections of RILs, and parent lines and the results plotted on several PCA representations using the *ggplot2* R package (Wickham 2009).

Pearson's correlation coefficients between phenotypes, separately for China and Denmark, were estimated ($p < 0.001$) by bulking trait mean values for CN1, CN2 and CN3 and for DK1 and DK2, respectively. Results were plotted on a heat map representation, superposing both correlation matrices using the *ggplot2* R package.

QTL analysis

QTL analysis was performed with MapQTL software, using the linkage map constructed from screening the F_2 and trait means estimated for each RIL within each location. For the F_2, single plant trait values were used for QTL detection in CN1. Due to time and costs involved in such large QTL studies, trait means of every RIL families were regarded as traits value of the genotyped F_2. Therefore, QTL mapping was performed using $F_{2:3}$ for DK1 and DK2, and $F_{2:5}$ designs for CN2 and CN3, as described in previous studies (Chapman et al. 2003; Zhang and Xu 2004).

QTLs were initially detected using the Kruskal–Wallis test and interval mapping for all phenotypes and locations to uncover segregating QTLs with large effects (Bickel and Lehmann 1975a, b; Lander and Botstein 1989; Van Ooijen 1992; Van Ooijen et al. 2004). Markers underlying large-effect QTLs were put as co-factors for automatic co-factor selection analysis based on a backward modelling regression of the selected co-factors. The likelihoods of each of the subset models were compared to the likelihood of the full model at all co-factor loci. The outputs of automatic co-factor selection provided the set of co-factors that induced a significant change in likelihood ($p < 0.001$). The remaining markers with significant effects were selected as co-factors and thereafter QTL detection was performed using Multiple-QTL Model Analysis (MQM) (Jansen 1993, 1994; Jansen and Stam 1994). A LOD threshold of 3 was chosen for QTL detection.

Results

Phenotyping results

Four categories of morphological, biomass, biofuel and developmental traits were phenotyped in the field trials carried out in five locations in China and Denmark (Table 2), and the mean values for the parental lines and the RILs are presented in Fig. 1. Significantly lower mean values for most of the traits were recorded in Denmark as compared to those monitored in China. For instance, PHT values were lower in Denmark, with means of 119.9 and 175 cm in DK1 and DK2, respectively. In particular, the height of the sweet sorghum parent E-Tian was substantially suppressed. Furthermore, the sizes of PeL and PaL in Denmark were only half of those in China. Nonetheless, the sweet sorghum E-Tian had significantly longer PeL and PaL than Ji2731 in all locations, suggesting that although the traits were environment dependent, the differences between genotypes are with high genetic controls. FSW and FLW were also dramatically affected in Denmark, with less than 50 % of the aboveground vegetative biomass produced compared to China. E-Tian developed a heavier juicy stem in China (400–600 g), compared to 101.8–226.9 g in Denmark. RIL means of FSW were 108.5 and 166.6 g for DK1 and DK2, respectively, while they were 203.7 and 422 g, respectively, in China.

Interestingly, the F_3 RILs grown in DK2 had a Brox value mean reaching 15.6 % which was significantly higher than those in CN1 and CN2 ($p < 0.001$), suggesting that it is possible to reach high Brix content in short summers with long days. Stem juiciness was highly segregating in all locations, the highest ratio of RILs with juicy stems was found for the F_3 in DK2 (77 %), while in China the ratios were, 26.5, 36.5 and 51.9 % for the F_2 and the two F_5 RILS in CN2 and CN3, respectively. Furthermore, in all locations, the two parent lines consistently had the same stem juiciness and midrib colours as shown in Fig. 2, suggesting that stem juiciness trait is relatively stable.

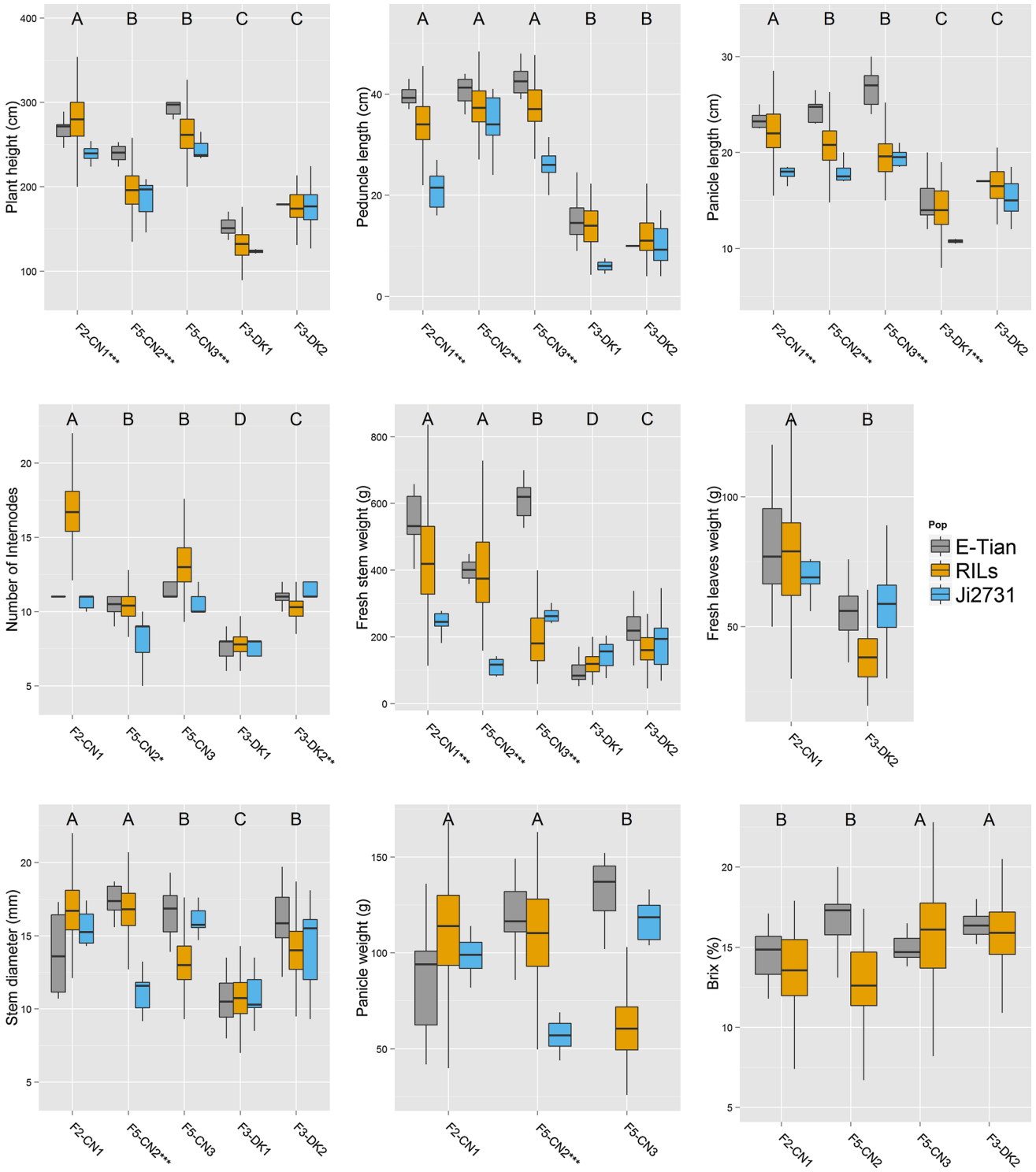


Fig. 1 Phenotypic results for all locations and populations. For all locations, results are grouped by trait category and illustrated by *box plots* for RILs and parent lines. *Grey boxes* for E-Tian, *orange* for RILs and *blue* for Ji2731; The *x* axis shows locations in China (CN) and Denmark (DK). Significant differences between parents within locations are indicated by the number of *stars* next to the locations ($p < 0.001$). Significant differences between RIL means across locations are given with the *output letters* from a Duncan test, and locations with *different letters* indicating significant differences ($p < 0.001$) (colour figure online)

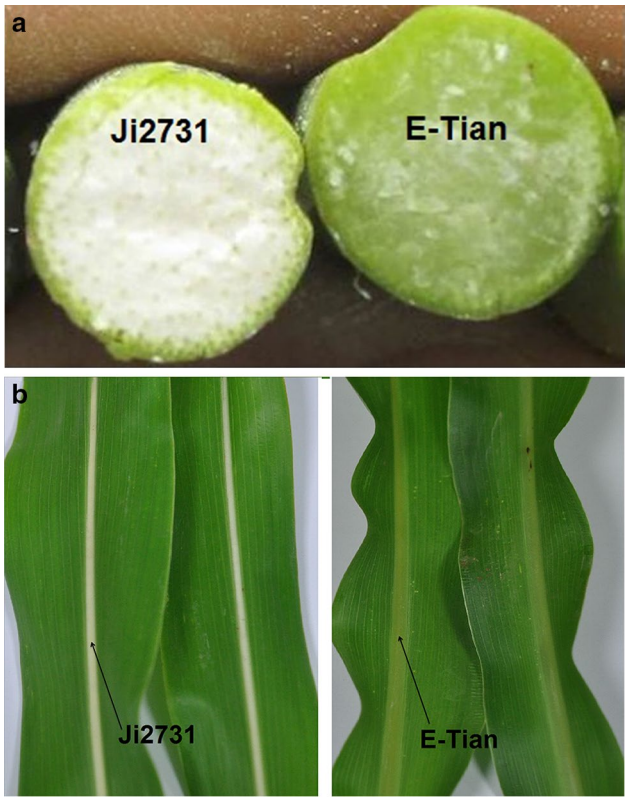


Fig. 2 Stem juiciness and midrib colour of the *parental lines*. Pictures of the dry stem of Ji2731 as compared to the juicy stem of E-Tian (**a**) and the *white* midrib colour observed for Ji2731 plants in contrast with the *green* midrib found in E-Tian plant leaves (**b**) as indicated by the *arrows* (colour figure online)

Table 3 Broad-sense heritability (H^2) estimated for each phenotype for F_3 RILs in Denmark (H^2_{DK}), F_5 RILs in China (H^2_{CN}) and the four trials ($H^2_{DK/CN}$)

Phenotypes	H^2_{DK}	H^2_{CN}	$H^2_{DK/CN}$
PHT	0.49	0.51	0.54
PeL	0.53	0.52	0.53
PaL	0.50	0.64	0.61
NN	0.46	0.62	0.58
FSW	0.41	0.65	0.60
SD	0.43	0.59	0.51
PaW	NA	0.58	NA
Brix	NA	0.49	0.49

Heading Stage (HS) reached for parents and RILs varied significantly in Denmark in both years. E-Tian reached late flowering (HS = 59) and Ji2731 early boot stage (HS = 41) in DK1, with an inverse maturity stage scored in DK2. However, the numbers of RILs observed with emerged inflorescences, in DK1 and DK2 (from HS = 57 onwards), represented a similar ratio among the F_3 RILs in both years, with 60 and 52 % of RILs reaching or passing HS = 57, respectively. These results suggest that in Denmark, maturation is achievable in high altitude northern countries.

Broad-sense heritability

Significant effects of genotype, environment, and genotype-by-environment components were detected for all the traits examined in China and Denmark ($p < 0.001$), except for the peduncle length trait (PeL) of the F_5 RILs grown in CN2 and CN3. The highest H^2 was obtained for the F_5 RILs, as advanced RILs are expected to be more homozygous (Table 3). Furthermore, the CN2 and CN3 locations had climate conditions which allowed for the full-plant development in contrast to Denmark with wider variation in temperatures. Calculated H^2 ranged from 0.41 to 0.53 for the F_3 in Denmark, from 0.49 to 0.65 for the F_5 in China, and from 0.49 to 0.61 using the F_3 and F_5. The highest H^2 values in Denmark were found for PeL and PaL and for the FSW and PaL for the F_5 RILs. The lowest H^2 values were obtained for FSW in Denmark and for Brix in China. Across countries, the lowest H^2 was calculated for Brix ($H^2 = 0.49$) and the highest was obtained for PaL.

Principal component analysis of growth performance

The first two principal components, PC1 and PC2, explained 46.0 and 12.7 % of total variance, respectively. E-Tian varied greatly between locations, as shown by the variation across locations for the traits means (Fig. 3a), indicating that traits in E-Tian were strongly affected by environment and were less stable. A clear separation was observed between RILs grown in Denmark and China on PC1 (Fig. 3b), with a larger phenotypic variation in China compared to Denmark. The F_2 had a largest phenotypic variation, for the studied traits, compared to the other RILs (Fig. 3c), which might be attributable to the fact that only single F_2 plants were phenotyped. The F_5 RILs, grown in CN2 and CN3, had similar phenotypic variation which was larger than that of the F_3 RILs grown in DK1 and DK2. Limited F_3 RILs were projected overlapping with the F_5 RILs projection grown in China, indicating similar trait values. RILs evaluated in the Chinese locations were projected overlapping the morphological and biomass trait variables (Fig. 3b, d), indicating that they had the highest values for these traits as compared to the F_3 grown in Denmark (DK1–DK2). Figure 3d also indicates that these traits explained similar contribution in the total variance, as seen by the similar arrow-length projection. However, Brix and juice variables contributed less to variation between RILs as observed by the smaller arrows, and both traits seemed to be independent from, or showed a negative correlation with other traits.

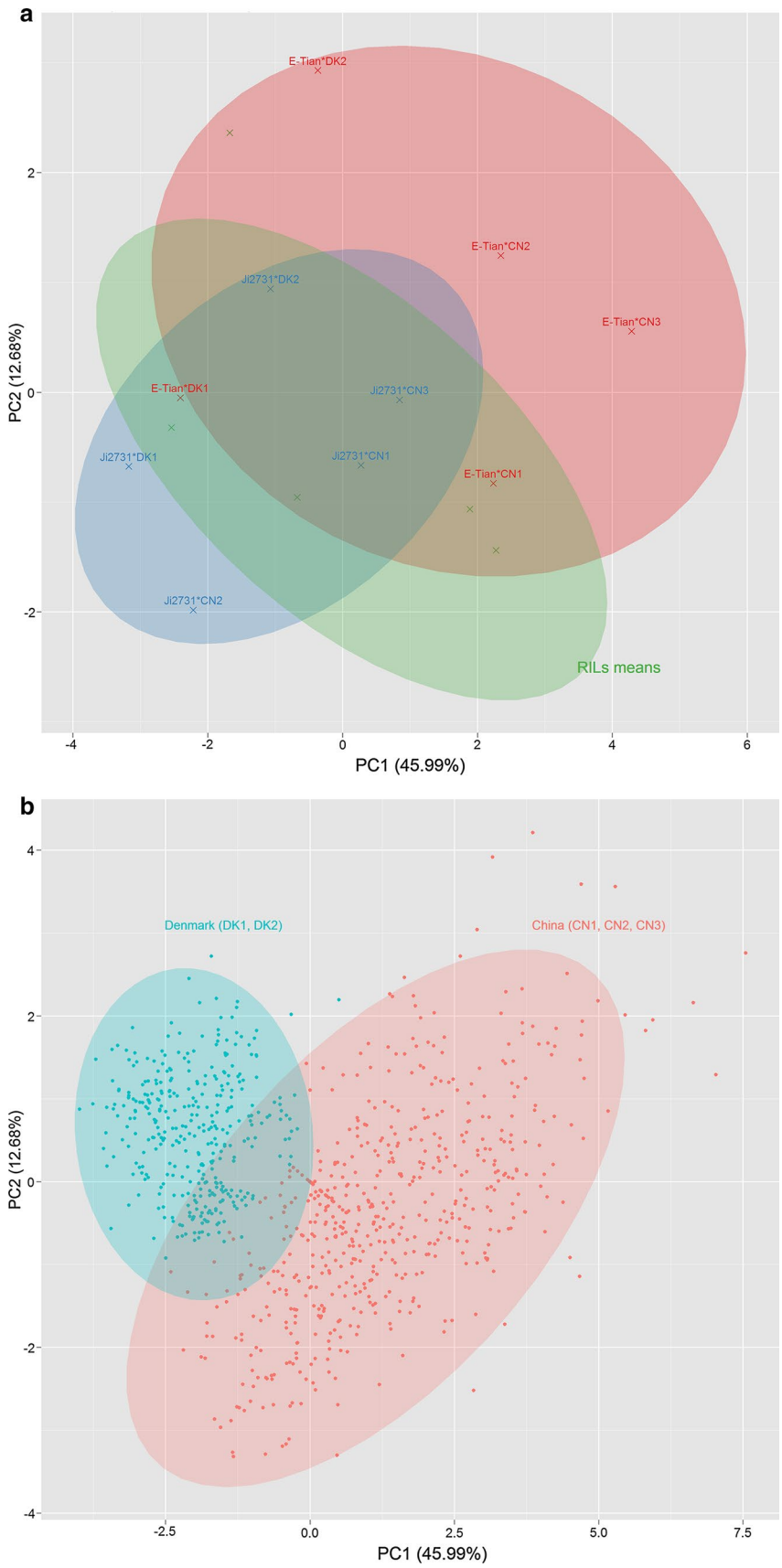
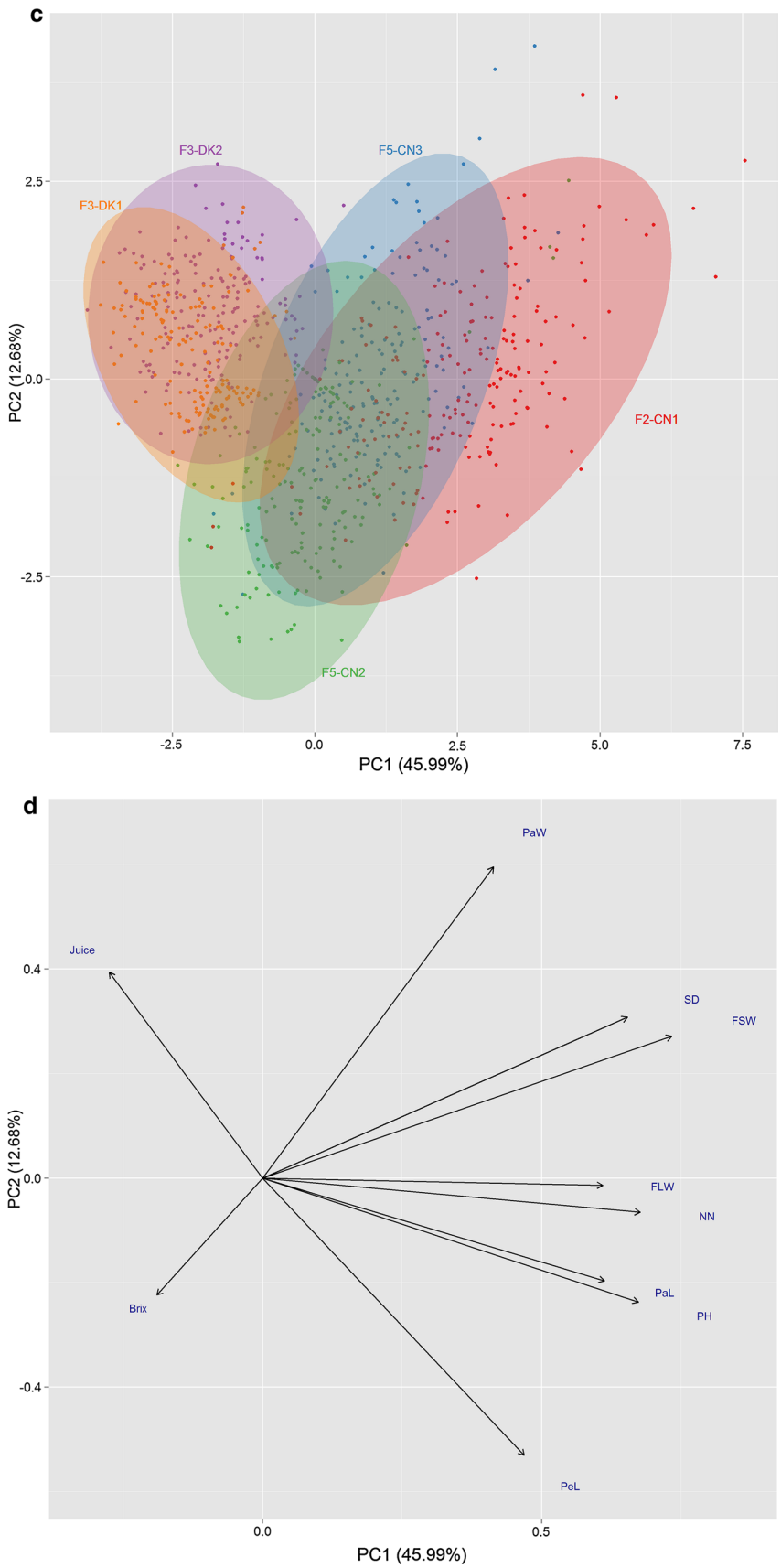


Fig. 3 PCA score plots of growth performance for various populations. The first and second principal components identified from PCA were used to make various plots including the performance of the two *parental lines* by field trial locations (**a**), the performance of the RILs by country with bulked locations within the country (**b**) or by locations (**c**). The variable factor map is shown to illustrate the coordinates of the 10 phenotypic variables used for constructing the PCA (**d**). See the main text for details

Fig. 3 continued



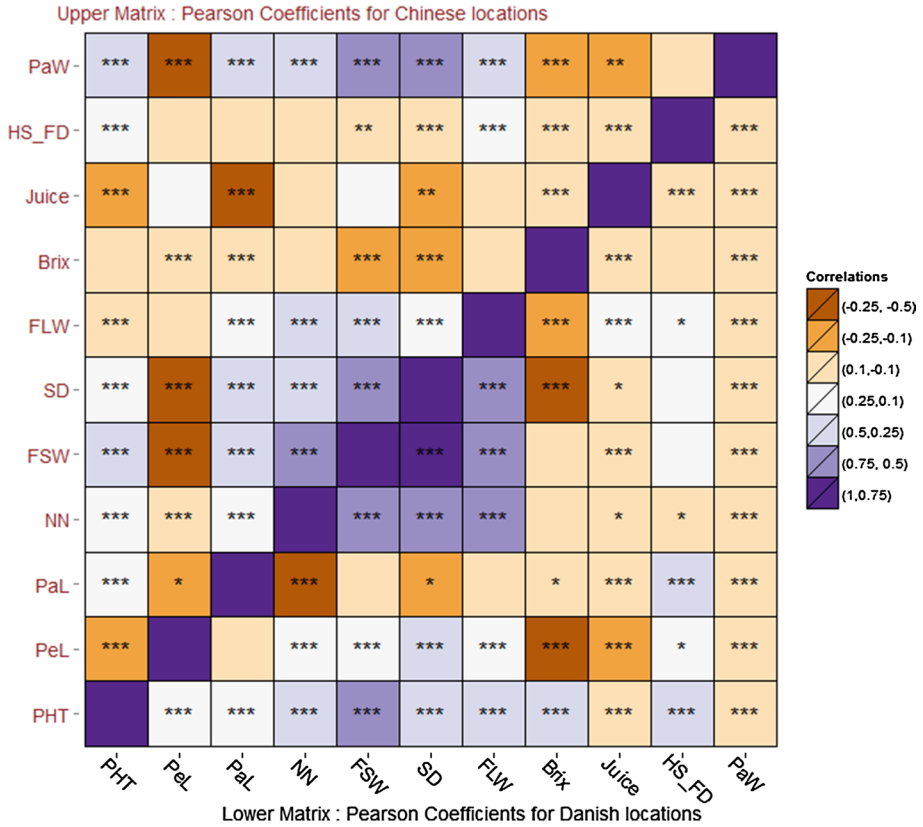


Fig. 4 Trait correlation matrixes by country. The matrixes were constructed with Pearson's coefficient-based heat map comparing trait relationships in China (*upper matrix*) and Denmark (*lower matrix*). The number of *stars* indicate the significant levels of the correlation with * for $p < 0.1$, ** for $p < 0.01$ and *** $p < 0.001$

Trait relationships in different climatic zones

The strongest correlation coefficients were found in Denmark between traits (Fig. 4), with biomass traits having strong positive correlations between each other in both countries. However, no correlation was found between FLW and other traits in China, in contrast to in Denmark where strong correlations were found with morphology traits. PeL and PaL had opposite correlation directions between countries. PeL was significantly negatively correlated to morphology and biomass traits in China, but was significantly positively correlated with these traits in Denmark. While PaL was significantly positively correlated with plant morphology and biomass traits in China, no significant correlations were estimated for PaL in Denmark, except for a negative correlation with NN. Only plants with an advanced heading stage (HS) could be phenotyped for PaL and PeL, explaining the positive correlation of HS with these traits. FSW was highly positively correlated to NN and SD in both countries. Brix was correlated negatively with biomass traits in both China and Denmark. Surprisingly, no correlations were found for Brix values and HS in Denmark.

Overview of QTL results

Fifty-three QTLs were detected spreading across the entire genome (Fig. 5; Table 4), with each chromosome harbouring a minimum of two QTLs and SBI01, SBI04 and SBI06 with the highest QTL numbers. LOD scores ranged from 3.0 to 20.5 with r^2 ranging from 5.1 to 37.5 %. The highest LOD score and r^2 were obtained for a locus linked to the juice trait mapped on SBI06 (LOD score = 20.5, $r^2 = 37.5$ %). Eighteen QTLs were only detected in a single location or within one country, e.g. PaW could only be assessed in China or HS in Denmark. A total of 16 QTLs were common between the two countries and 18 QTLs were detected in a minimum of two locations. The highest number of QTLs found in a single population was from the F_2 followed by the F_3 grown in DK2. However, fewer QTLs were detected in DK1 when the same F_3 population was grown in the same geographical locations. The environmental conditions were found to be more extreme in DK1, with cooler temperatures during the growing period. Apart from two QTLs, all the QTLs identified from the F_5 population were detected in the earliest populations (F_2 and F_3).

Common and distinct QTLs between climatic zones

The QTLs associated with juice and midrib colour on SBI06 were consistently uncovered in all locations. Analyses for plant morphology-related traits led to the detection of several QTLs, most of which were common in both countries. For example, five QTLs were found for

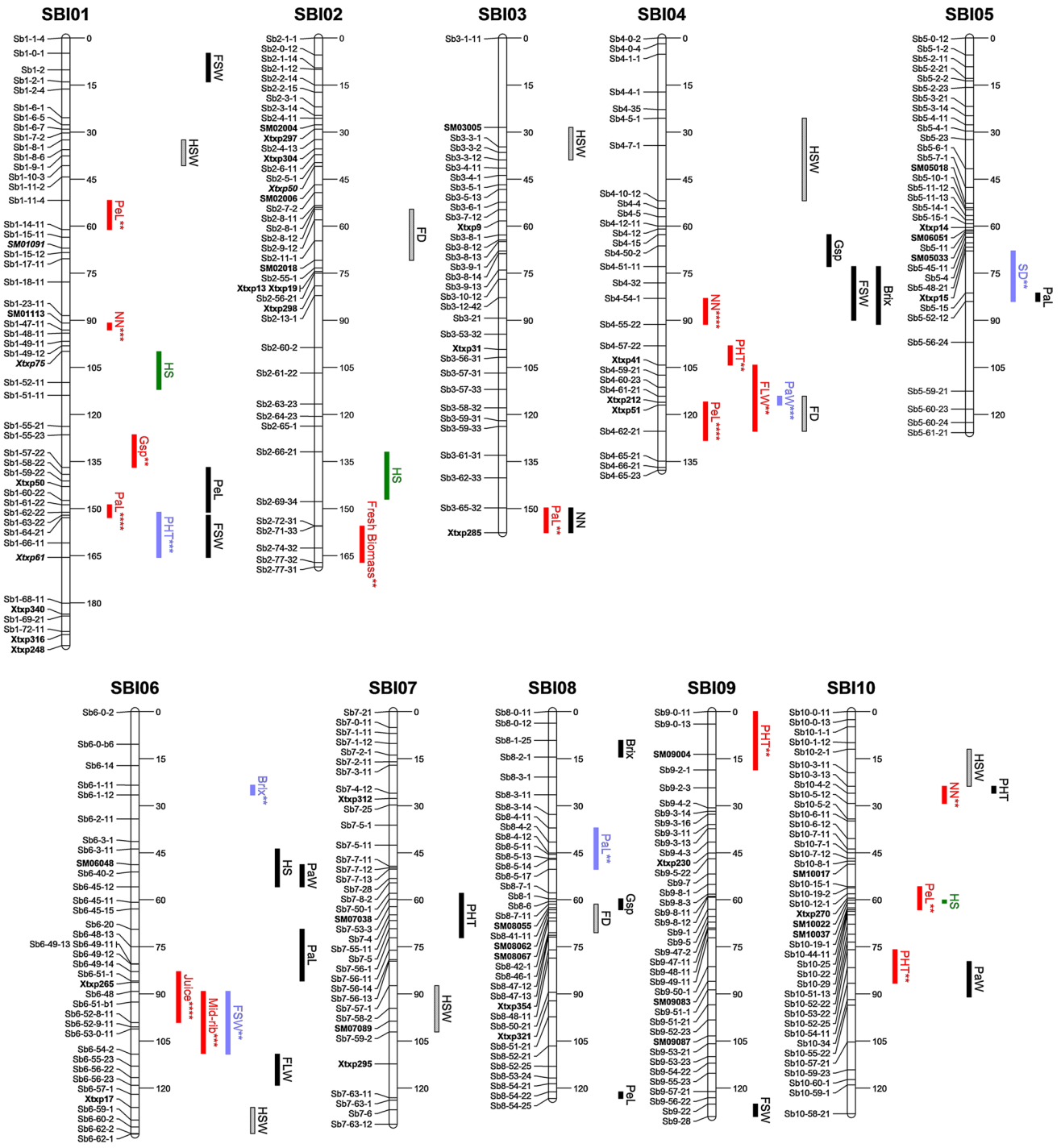


Fig. 5 A diagram showing the locations of 53 QTLs for plant morphological, biomass and biofuel traits identified in this study on the genetic map from E-Tian × Ji2731 populations. The linkage map is from PAV (*Sb numbers*) and SSR markers (*Italic-bold font*). QTLs detected from a single environment are in *black*, QTLs for phenotypes measured in a single environment are in *grey*, QTLs detected in both years in Denmark are in *green*, QTLs detected in more than one location in China are in *purple* and QTLs commonly detected in China and Denmark are highlighted in *red*. The number of *stars* indicates the number of locations where the QTLs have been detected (colour figure online)

PeL and three QTLs were detected in both countries, with the most significant one mapped on SBI04 and detected in four locations. PaL was linked to five QTLs, three of which were detected in both countries as well as in multiple locations in China. Similarly, four QTLs were detected for NN, with three shared between countries. PHT was linked to five QTLs, of which three were common between countries. Finally, FLW and fresh biomass

Table 4 Information about QTLs detected in more than one location

Phenotypes	Flanking markers	LG	Position (cM)	LOD score	r^2 (%)	Additive effects	Locations where QTLs were detected
China & Denmark							
Gsp (3)	Sb1-49-12–Xtxp56	SBI01	98.1–99.8	5.6	17.8	230.80	DK1, CN1
PHT (6)	Sb1-62-22–Xtxp61	SBI01	151.2–165.5	7.7	15.5	−0.2	CN1, CN2, CN3
	Sb9-0-11–Sb9-2-3	SBI09	0.0–24.3	4.0	10.0	−0.1	CN1, DK2
	Sb4-57-22–Xtxp41	SBI04	98.1–104.3	3.4	8.6	−0.1	CN1, DK1
	Sb10-53-22–Sb10-34	SBI10	75.9–91.8	3.4	13.6	11.2	DK2, CN2
PaL (5)	Sb8-4-11–Sb8-5-17	SBI08	37.1–50.3	5.7	16.0	−1.3	CN2, CN3
	Sb1-61-22–Sb1-64-21	SBI01	148.8–152.9	4.2	17.4	0.4	DK2, DK1, CN1, CN3
	Sb3-65-32–Xtxp285	SBI03	149.8–157.7	4.1	9.3	−101.2	DK2, CN1
PeL (5)	Xtxp41–Sb4-62-21	SBI04	104.3–120	4.6	29.2	2.3	DK1, DK2, CN2
	Sb10-15-1–Sb10-44-11	SBI10	56.2–63.2	4.5	17.1	−2.5	DK2, CN2
	Sb1-11-4–Sb1-14-11	SBI01	51.8–61.1	3.2	9.0	−3.3	CN2, DK2
NN (4)	SM0113–Sb1-47-11	SBI01	91.6–93.1	4.1	11.0	0.4	DK1, DK2, CN2
	Sb4-54-1–Sb 4-55-22	SBI04	82.9–91.3	3.9	10.2	0.1	DK1, DK2, CN1, CN2
	Sb10-3-13–Sb 10-5-12	SBI10	23.8–29.3	4.1	13.0	0.5	DK1, CN1
SD (1)	Xtxp15–Sb5-52-12	SBI05	67.9–84.0	4.2	14.6	0.9	CN1, CN3
FSW (5)	Xtxp265–Sb6-54-2	SBI06	86.3–109.2	19.5	37.1	−119.4	CN1, CN3
FLW (2)	Xtxp41–Sb4-61-21	SBI04	104.3–114.2	5.4	12.5	−100.3	DK2, CN1
Fresh biomass (FSW + FLW) (1)	Sb2-71-33–Sb2-77-32	SBI02	155.6–167.2	4.2	9.3	−71.2	DK2, CN1
Midrib colour (1)	Sb6-48–Sb6-56-22	SBI06	89.2–116.7	9.2	33.3	−0.5	DK2, CN1, CN3
Brix (3)	Sb6-1-11–Sb6-1-12	SBI06	23.5–26.6	3.0	17.2	−0.2	CN2, CN3
Juice (1)	Sb6-49-14–Sb6-52-8-11	SBI06	82.9–99.1	20.5	37.5	−0.5	CN1, CN2, CN3, DK2
Denmark							
HS (4)	Xtxp75–Sb1-55-21	SBI01	99.9–123.8	5.6	17.0	315.5	DK1, DK2
	Sb2-69-34–Sb2-74-32	SBI02	147.8–162.6	5.1	15.7	1.8	DK1, DK2
	SM10022–SM10037	SBI10	60.1–61.1	4.1	11.5	330.2	DK1, DK2
China							
PaW (3)	Sb4-61-21–Xtxp51	SBI04	114.2–116.1	3.9	9.9	−13.0	CN1, CN2, CN3
HSW	Sb4-5-1–Sb4-10-12	SBI04	25.6–51.9	4.8	11.4	−0.1	CN1
	Xtxp17–Sb6-62-2	SBI06	126.2–134.5	3.5	8.0	−0.1	CN1
	Sb7-57-1–Sb7-59-2	SBI07	87.4–102.1	3.4	8.2	0.2	CN1
	SM03005–Sb3-3-12	SBI03	28.5–38.9	3.4	8.2	−0.1	CN1
	Sb10-2-1–Sb10-3-13	SBI10	12.0–23.8	3.3	7.5	0.1	CN1
	Sb1-8-1–Sb1-10-3	SBI01	32.5–40.7	3.3	7.8	0.3	CN1
FD	Sb2-8-12–SM02018	SBI02	54.6–70.9	4.6	12.2	1.3	CN1
	Sb8-7-11–Sb8-46-1	SBI08	61.4–70.5	3.9	10.6	−0.5	CN1
	Sb4-61-21–Sb4-62-21	SBI04	114.2–125.4	3.3	8.3	−1.2	CN1

The first part indicates QTLs commonly detected in China and Denmark and locations in which the QTLs were detected. The second part indicates QTLs found for phenotypes recorded only in Denmark or China. The numbers in the brackets next to the phenotypes are the total number of QTLs detected for each trait throughout the study

(FLW + FSW) were associated with QTL on SBI04 and SBI02, respectively, and both were detected in CN1 and DK2.

The most significant QTLs for PaL and PHT were only detected for the Chinese locations and mapped on SBI08 and SBI01, with a LOD score of 7.7 and 5.7, respectively. Interestingly, only a few QTLs for biomass traits and Brix were found in multiple locations. As anticipated, the most significant QTL for FSW, detected in two locations, overlapped with the juice QTL on SBI06 (r^2 = 37.1 % and LOD score = 19.5). A single QTL was found for SD on SBI05 in two locations. Three QTLs associated with Brix

were detected, but only one, on SBI06, was shared from results in CN2 and CN3. The Brix results for DK2 led to the detection of a single QTL on SBI08. As included in the supplementary material in Table S2 and shown on Fig. 5, a large number of QTLs were only mapped in single locations, and, therefore, even regarding their significance based on LOD score and r^2 values, it is suspected that some could result from genotype-by-environment interactions.

QTL for maturity and flowering time

The results of Gsp in CN1 and DK1 showed the same region on SBI01, explaining 17.8 % of the total variance and making it the most significant QTL for this trait. Four QTL were detected for HS with three of them consistently in DK1 and DK2. The most significant locus was mapped on SBI02, explaining 15.7 % of the total variance. HS affected the detection of QTL for others traits and markers underlying HS QTL were selected as co-factors for a few traits. FD, recorded in CN1, led to the detection of three QTL, but none overlapped with the HS QTL. HSW, only recorded for CN1, was under the control of six additive QTL, the most significant one resided on SBI04 (LOD score = 4.8, $r^2 = 11.4$ %). PaW was under the control of three QTLs, the most significant one mapped to SBI10. An additive QTL on SBI04 was linked to PaW and detected in all trials.

Discussion

In the current report, sorghum recombinant inbred lines of various generations were used for the intensive phenotyping of morphological, biomass, biofuel and developmental traits in five locations in both Denmark and China. The aim was to identify traits stable across different climatic conditions and the associated QTLs. Our results uncovered 53 QTLs for 15 important traits and the findings are useful for further genetic studies and molecular breeding of biofuel sorghum in high altitude Northern Europe.

Fast maturation as a breeding target for Northern Europe

In Denmark, the heading stage (HS) segregated greatly among RILs, most likely in response to the photoperiod. HS is strongly linked to and influences biomass and morphological traits. Indeed, strong positive correlations were found between HS and other traits recorded in this study, and for the detection of most of the QTLs, a significant influence of markers underlying HS QTLs was found. However, in China, HS/FD did not influence QTL detection for other traits. Hence, QTLs for heading stage is specific to Denmark, which may allow us to breed for fast maturation varieties.

Photoperiod-sensitive genes have been discovered on chromosome 7 in rice (Quarrie et al. 1997; Yano et al. 1997; Yano and Sasaki 1997), which is collinear to SBI02 (Kresovich et al. 2005; Paterson et al. 1995). QTLs controlling photoperiod responses have previously been found on SBI01, SBI02 and SBI10 (Clerget et al. 2004; Shiringani et al. 2010). Thus far, six flowering/maturity genes, *Ma1* to *Ma6*, have been detected by studying photoperiod. *Ma1*, mapped on SBI06, is shown to have the greatest impact, followed by *Ma3* mapped on SBI01 (Childs et al. 1997; Klein et al. 2001; Lin et al. 1995; Murphy et al. 2011; Murray et al. 2008b; Pao and Morgan 1986; Quinby and Karper 1954; Rooney and Aydin 1999). QTLs controlling HS in Denmark were very stable in both years. Two QTLs on SBI01 and SBI06 were mapped for HS, respectively, closely resideed to the known maturity genes. The QTL on SBI06 was estimated with negative additive effects and with alleles from E-Tian, the late-flowering parent. This QTL could overlap *Ma3*, described as a flowering repressor. Two other significant QTLs, resided on SBI01 and SBI10, were consistent with previous studies (Murphy et al. 2011; Srinivas et al. 2009). QTLs detected for FD on SBI02, SBI04 and SBI08 have previously been reported (Feltus et al. 2006; Murray et al. 2008a; Ritter et al. 2008) and positive additive effects found from the fast-growing *kaoliang* parent, which is more chilling tolerant and photoperiod insensitive. Interestingly, QTLs on SBI02 and SBI01 linked to HS, as with the QTLs for Gsp, were closely located to QTLs detected for early chilling tolerance (Bekele et al. 2014; Burow et al. 2011). Although these studies were focused on field emergence and seedling vigour, these genomic regions might harbour genes either with pleiotropic effects for low-temperature tolerance from early to late developmental stage or playing a role in cooler temperature acclimation which could be involved in a faster development. Furthermore, very strong correlations were estimated between plant morphology and biomass traits with HS in this study in Denmark, consistent with what has been observed when comparing similar traits under long and short photoperiods (Zou et al. 2012). Thus, as early-flowering plants had a better agronomic performance in Denmark, it suggests that early flowering might be a potential target for directional breeding and adaptation to new climates. Further close examination of growth speed from emergence to flowering under low-temperature conditions would be helpful to define the role of these genomic regions in cold tolerance during sorghum development.

Genetic control of biofuel-related traits and trade-off between stem sugar content and grain yield

The main potential of sweet sorghum as a biofuel crop lies in the accumulation of fermentable sugars in

the stem during a short growth season. High Brix values were obtained in Denmark independent of heading stage variation, and most of other traits. Only limited numbers of Brix QTLs were detected in this study, compared to studies conducted on populations developed from contrasting parents for Brix concentration (Jo et al. 2011; Murray et al. 2008a; Ritter et al. 2008; Shiringani and Friedt 2011). A novel QTL detected on SBI08 from DK2 has been reported in a region thus far associated with biotic stress resistance (Klein et al. 2001; Parh et al. 2008; Rami et al. 1998). The major QTL associated with juice on SBI06 overlapped with the QTL for the midrib colour. These phenotypes were associated and stable across environments. As expected, both QTLs had negative additive effects coming from the sweet sorghum parent allele's inheritance with a juicy stem and green midrib. Two morphological markers controlling the midrib juicy properties have previously been mapped on SBI06 close to the position of the QTL in this study, the so-called gene *D* and *Mrco* (Srinivas et al. 2009; Xu et al. 2000). It appears that this major QTL is stable and could be explored for breeding high Brix content cultivars.

On the other hand, the fresh biomass yield is highly dependent on and interacts with the environment, as indicated by the lowest h^2. Particularly, biomass yield was reduced by half for E-Tian and RILs grown in Denmark, with FSW and FLW strongly repressed under northern climate conditions. However, since biomass traits in Denmark were strongly linked to morphological traits, breeding strategies targeting taller plants with accelerated maturation could be a way forward to produce reasonable biomass. A new QTL for FSW was presented, mapped to SBI06, and two QTLs for FLW and the total fresh biomass (FSW + FLW) were detected between the climatic zones. Thus, FLW seemed more stable and more genetically controlled compared to FSW.

Many reports have investigated QTLs for grain yield in sorghum, but, depending on populations, trade-offs between stem sugar content and grain yield have been reported (Murray et al. 2008a). In this study, no significant trade-off was observed in China, showing the potential to breed these traits together with PaW, SD and total fresh biomass. QTLs for PaW were mapped to all sorghum chromosomes. QTLs responsible for grain yield were found on SBI01, SBI03, SBI04, SBI06, SBI07 and SBI10, with the most significant one residing on SBI04. However, only the QTL found on SBI04 was detected across Chinese locations and resided on a key hub responsible for biomass and plant morphology traits stable between countries. Therefore, this region appeared to be highly responsible for biomass and plant morphology traits.

Genomic regions controlling plant height and biomass

Plant height and associated traits were some of the most affected traits in Denmark. In plant species, PHT QTLs are well documented as being controlled by dwarf. In sorghum, four dwarf genes have been discovered, annotated from *Dw1* to *Dw4* (Quinby and Karper 1954), with *Dw2* and *Dw3* mapped to SBI06 and SBI07, respectively, (Brown et al. 2008; Paterson et al. 1995). The combination of these genes can dramatically decrease PHT. In this report, six QTLs were revealed for PHT, with three detected across countries and mapped on SBI04, SBI09 and SBI10, respectively. Another plant height QTL on SBI01 was detected in all trials in China and was consistent with other studies (Feltus et al. 2006; Hart et al. 2001; Klein et al. 2001; Lin et al. 1995; Natoli et al. 2002; Pereira and Lee 1995; Rami et al. 1998; Srinivas et al. 2009). Similar to previous reports, the QTL on SBI07 in this study was found with negative additive effects, the contribution of the tall sweet sorghum allele and therefore might not be related to *Dw3* (Brown et al. 2006; Hart et al. 2001; Srinivas et al. 2009). The QTL detected on chromosome SBI09 in this study overlapped the recently discovered locus, annotated "*Sb-HT9.1*", described together with *Dw3* as the most important region for PHT determination in sorghum (Brown et al. 2008). PeL and PaL were estimated to have the highest H^2 and, as expected, strong genetic controls for these traits were detected across environments. Furthermore, phenotypic correlations are known to uncover co-localisation of genes (Hemamalini et al. 2000; Hittalmani et al. 2002; Lebreton et al. 1995). As predicted from the correlation coefficient and their high H^2, PeL and PaL QTLs overlapped the PHT QTL region and were shared between countries. The QTL for PaL on SBI06 was found with positive additive effects from the *kaoliang* parent, characterised with a shorter PaL. It is believed that this QTL is conditioned by *Dw2*, as described previously (Lin et al. 1995; Rami et al. 1998; Srinivas et al. 2009). Two novel QTLs controlling panicle length (PaL) on SBI08 and SBI05, respectively, were reported. The QTL on SBI08, detected in CN2 and CN3, was the most significant for PaL and the QTL on SBI05 was detected in Denmark with negative additive effects. Unlike PaL, the genetic control of PeL has been studied less, with only a few reported QTLs similar to the QTLs in this study (Klein et al. 2001; Parh et al. 2008; Zou et al. 2012).

The stability of the plant morphology QTLs between countries illustrated the great potential for breeding stable sorghum lines with predictable performances. Even with a smaller number of RILs phenotyped for PaL and PeL in Denmark, a strong genetic expression was detected. Moreover, in consideration of the overlapping, QTL regions found on SBI01, SBI04 and SBI10 regrouping the major stable QTLs for PHT-dissected phenotypes, as well as

co-localising with genetic regions responsible for HS, breeding sorghum in warmer climates by choosing easy-to-score traits such as PHT could facilitate gain selection by simultaneous introgression of other desirable traits, as they appeared to be genetically linked or co-segregating.

Uses of PAV markers for breeding

With the rapid advances in NGS technologies, broad sources of genetic variations can be uncovered, such as sub-microscopic structural variations like presence–absence variants (PAVs). In our previous paper, we reported the development of molecular markers based on small-size PAVs and the construction of a genetic map using these markers together with previously published SSR markers (Shen et al. 2015). In this study, we aim at testing these PAV markers for (1) QTL mapping applications, (2) their transferability across RIL progeny and (3) their robustness in detecting QTLs in contrasting environments. Most of the crop QTL studies rely on genotyping and phenotyping data from a same population but designs where these data are acquired from different population progenies (F_u/F_v, $u < v$) are also popular as they are time and cost saving. Designs such as F_2/F_4 and even F_6/F_7 have been reported in maize (Sala et al. 2006; Veldboom et al. 1994) and soybean (Chapman et al. 2003). We used, in this study, a simple QTL mapping model in which phenotypic mean values from early RILs ($F_{2:3}$) to advanced inbreeding RILs ($F_{2:5}$) have been considered as F_2 phenotypic values. A considerable number of QTLs were detected consistently across RILs progeny and locations. Furthermore, the number of QTLs detected for every RIL was consistent with expectations, as the F_2 results led to the highest number of QTL detected as genotyping was carried on the F_2 plants. The uses of the F_5 RILs, an advanced mapping population with increased homozygous level, allowed the confirmation of the highlighted genetic regions responsible for phenotype segregation and increased the reliability of the QTL analysis by improving precision of the QTL positions and additive effects. This molecular marker system presents a useful tool for crop genetics and breeding.

In conclusion, we revealed a number of stable QTLs across countries for most of the traits of economic importance and paved the way to define a sorghum ideotype for temperate climatic regions which will combine fast growing and tallness features within one plant. Furthermore, the stable QTLs discovered are promising genomic regions for more extensive fine mapping. Dissecting traits such as heading stage and seedling vigour could provide a better genetic understanding of the complex mechanisms involved in low-temperature and photoperiod abiotic stress responses in sorghum.

Author contribution statement A.M performed the field experiments and undertook QTL analysis in Denmark. Y.M. Z, Z.Q.L and X. S. developed the mapping populations, designed the field experiments, undertook the phenotyping and QTL analysis in the three locations in China. H.C.J and S.K.R conceived the project. A.M and H.C.J wrote the first and the final draft.

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Conflict of interest The authors declare that they have no conflict of interest.

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