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## Abstract

With rising energy demands and costs for fossil fuels, alternative energy from renewable sources such as maize cobs will become competitive. Maize cobs have beneficial characteristics for utilization as feedstock including compact tissue, high cellulose content, and low ash and nitrogen content. Nitrogen is quantitatively the most important nutrient for plant growth. However, the influence of nitrogen fertilization on maize cob production is unclear. In this study, quantitative trait loci (QTL) have been analyzed for cob morphological traits such as cob weight, volume, length, diameter and cob tissue density, and grain yield under normal and low nitrogen regimes. 213 doubled-haploid lines of the intermated B73 × Mo17 (IBM) Syn10 population have been resequenced for 8575 bins, based on SNP markers. A total of 138 QTL were found for six traits across six trials using composite interval mapping with ten cofactors and empirical comparison-wise thresholds ( $P = 0.001$ ). Despite moderate to high repeatabilities across trials, few QTL were consistent across trials and overall levels of explained phenotypic variance were lower than expected some of the cob trait × trial combinations ( $R^2 = 7.3\text{--}43.1\%$ ). Variation for cob traits was less affected by nitrogen conditions than by grain yield. Thus, the economics of cob usage under low nitrogen regimes is promising.

## Disciplines

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## Abstract

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## Introduction

Limited supply of fossil fuels has caused high prices for energy and will likely continue to do so in the future. This makes alternative energy sources more competitive. With development of new conversion technologies to produce renewable energy from cellulosic biomass, feedstock such as maize cobs has become a valuable resource. Maize cobs have beneficial characteristics for utilization as feedstock including compact tissue, high cellulose content, and low ash and nitrogen content. Thus, possibly dual-purpose maize varieties with both, superior grain and cob biomass yield, will make maize cob utilization more attractive and economically feasible.

Nitrogen is quantitatively the most important nutrient for plant growth (George et al. 1995). However, increasing nitrogen prices and political decisions to protect the environment might urge farmers to limit nitrogen application (Power and Schepers 1989; Vitousek et al. 1997). Maize cobs have beneficial properties including low nitrogen content. Therefore, dual-purpose maize varieties were proposed combining high cob biomass and grain yield potential, especially under limited nitrogen supply (Jansen and Lübberstedt 2012). Exploring and understanding the genetic basis of cob biomass in relation to grain yield under varying nitrogen management regimes will help breeders to develop dual-purpose maize.

The ten times intermated B73 × Mo17 doubled haploid population (IBM10DH; Hussain et al. 2007) features alleles from two prominent inbred lines, differing substantially in their cob architecture. Moreover, IBM10DH has a high genetic resolution for fine mapping studies, due to ten generations of recombination. The resulting low linkage disequilibrium can be captured, as long as an adequate number of molecular markers are used. We achieved this using a genotyping-by-sequencing approach (Huang et al. 2009). The increase in both genetic resolution and marker density results in more precise mapping of QTL, and in turn it is expected to increase the efficiency of marker-assisted selection.

The objectives of this study were to (i) investigate cob biomass traits including cob weight, volume, length, diameter, and tissue density, as well as their interrelations and their relation to grain yield, (ii) identify genomic regions associated with these traits, and (iii) evaluate the effect of limited nitrogen supply on cob architecture traits and the effects of associated genomic regions.

## Materials and methods

245 doubled haploid lines (DHLs) of the IBM10DH population and their parental inbred lines B73 and Mo17 were evaluated in a total of six trials at two locations in Iowa in 2010 at Marion, Iowa, managed by Pioneer and at ISU Burkey Farm, Boone, Iowa and in 2011 again at ISU Burkey Farm. Genotypic data were available for a subset of 213 lines used for QTL mapping. Each year—location combination was considered an environment, each environment—nitrogen combination was considered a trial (Table 1).

In 2010, different levels of urea fertilizer application were used for low (LN) and normal nitrogen treatments (NN). LN treatments were established by applying 56 kg N/ha at Marion (MAR10LN), and no nitrogen (N) at Burkey Farm (BUR10LN) as this was a new LN location. For control, NN was applied with 250 kg N/ha at Burkey (BUR10NN) and

**Table 1** Means, standard deviations, minimum, maximum, and coefficients of variation at low and high nitrogen management for BUR10, MAR10, and BUR11

Trait	NN						LN						Red.%
	<i>n</i>	Mean	SD	Min	Max	CV	<i>n</i>	Mean	SD	Min	Max	CV	
<b>BUR10</b>													
WEI	419	10.06	3.02	2.53	19.61	30.04	418	5.6	1.83	0.82	12.11	32.61	44.34
VOL	419	51.01	13.34	9.36	103.32	26.15	418	31.5	9.24	4.01	62.42	29.32	38.25
LEN	419	12.49	1.93	6.13	19.53	15.47	418	9.67	1.8	4.95	16.07	18.64	22.56
DIA	419	2.19	0.21	1.21	2.7	9.79	418	1.93	0.2	0.87	2.37	10.11	11.62
DEN	419	0.2	0.03	0.12	0.28	15.3	418	0.18	0.03	0.11	0.28	16.11	8.8
GY	425	1.91	1.08	0	4.95	56.44	425	0.75	0.47	0	2.29	63.71	60.92
<b>MAR10</b>													
WEI	424	13.15	3.52	2.49	26.93	26.75	423	10.33	3.18	3.29	19.95	30.77	21.49
VOL	424	67.85	15.06	16.26	112.21	22.2	423	55.05	14.3	16.29	94.95	25.98	18.87
LEN	424	14.2	2.07	6.25	20.74	14.58	423	12.79	2.08	5.89	18.69	16.24	9.9
DIA	424	2.38	0.21	1.4	2.9	8.91	423	2.24	0.21	1.36	2.71	9.57	5.82
DEN	424	0.19	0.03	0.13	0.31	15.22	423	0.19	0.03	0.12	0.28	14.8	3.62
GY	424	4.45	1.46	0.37	7.41	32.75	424	3.08	1.27	0.18	6.17	41.34	30.72
<b>BUR11</b>													
WEI	417	10.85	3.71	1.31	23.29	34.23	426	11.64	3.36	2.97	21.06	28.88	-7.28
VOL	417	53.69	15.41	6.69	101	28.71	426	57.22	13.75	16.3	96.15	24.04	-6.58
LEN	417	12.42	2.14	6.46	19.32	17.2	426	13.25	2.03	5.84	20.77	15.29	-6.64
DIA	417	2.24	0.25	1	2.825	10.95	426	2.26	0.2	1.52	2.73	8.79	-0.99
DEN	417	0.2	0.03	0.12	0.289	14.77	426	0.2	0.03	0.12	0.28	14.55	-0.76
GY	425	2.68	1.54	0	6.484	57.2	426	3.4	1.43	0	6.49	42.06	-26.65

Traits include weight in g (WEI), volume in cm<sup>3</sup> (VOL), length in cm (LEN), diameter in cm (DIA), density in g/cm<sup>3</sup> (DEN), and grain yield in MT/ha (GY)

*n* number of observations, *SD* phenotypic standard deviation, *Min* minimum observation, *Max* maximum observation, *CV* coefficient of variation, *Red.%* reduction of mean under LN in % of NN mean (MEAN) for traits

269 kg/ha at Marion (MAR10NN). In 2011, a blend of 45 % urea, 40 % ESN<sup>®</sup> (Agrium), and 15 % AMS (ammonium sulfate) was used and 62 kg N/ha were applied at Burkey for LN (BUR11LN) and 250 kg N/ha for NN (BUR11NN). In all four location-years, both treatments were repeated twice in a randomized complete block design, with blocks nested within N-treatments. Plot size was  $5.5 \times 1.5 \text{ m}^2$  at Burkey in both years and  $5.3 \times 1.5 \text{ m}^2$  at Marion. Plant stands were thinned to a density of 69,187 plants/ha. At Burkey, all ears were hand harvested from each plot after a sample of four ears per plot was chosen for cob trait phenotyping from representative plants. At Marion, cob samples were taken prior to machine harvest. All ears were dried for 4 days to constant weight at 37.8 °C in an air blown commercial dryer. Dry ears were hand shelled and cob traits were obtained from the four sampled cobs and averaged for each entry. Grain yield (GY) was determined for all plants for each plot including sampled ears and corrected to 15.5 % moisture content for reporting in metric tons per ha (MT/ha).

### Image analysis

Image analysis was used to determine cob length, average cob diameter, and cob volume. From each cob, two pictures were taken and analyzed using MATLAB<sup>1</sup> (The Mathworks, Inc. Natick, MA, USA) (code 1; Appendix 1). Cob pixels were called, if color information of the pixel was different from the blue background as defined for each picture separately from color information of pixels in a reference area that only included blue background. Cob length (LEN) was derived from the average of both pictures for maximum number of cob pixels across multiplied by a constant accounting for the relation between pixel/cm. Average diameter (DIA) was calculated by averaging the length of all columns within the cob and multiplying with the constant accounting for the relation between pixel/cm. Cob radius at each column ( $0.5 \times \text{diameter}$ ) from both pictures were used to define an elliptical slice of one pixel width at a given position. Cob volume (VOL) was derived as the sum of all elliptical slices along the cob. Cob weight (WEI) was obtained for each cob on a fine scale and cob tissue density (DEN) was calculated as the ratio of cob weight and cob volume.

### Statistical analysis

Statistical analysis was carried out using mixed model procedure PROC MIXED in SAS software. The linear model

including all six trials, and 2010 trials can be written as  $Y_{ijkl} = \mu + E_i + N_j + N \times E_{ik} + B_{(ij)k} + L_l + E \times L_{il} + N \times L_{jl} + N \times L \times E_{ijl} + e_{(ij)kl}$ , where observation  $Y_{ijkl}$  is the plot-based phenotype as sum of the mean ( $\mu$ ), the random effect of the  $i$ th environment  $E$ , the fixed effect of the  $j$ th nitrogen level  $N$ , the random effect of the  $j$ th line  $L$ , their respective interactions  $N \times E_{ik}$ ,  $L \times E_{jk}$ , and  $N \times L \times E_{ijk}$  and the error  $e_{(ij)kl}$ . When analyzing each environment separately, the environment effect and interactions including environment were excluded from the model. When analyzing all six nitrogen  $\times$  environment combinations as six separate trials, only lines and blocks were fitted in the model. Repeatabilities for each trial and environment across nitrogen treatments were calculated on a plot and entry mean basis from variance components using PROC VARCOMP (SAS). Least square means were estimated by fitting lines as fixed effect, with block  $\times$  lines interactions as error term for each experiment. Genotypic and phenotypic correlations were derived for each nitrogen treatment separately, using PROC MIXED scripts as described by Holland (2006).

### QTL analysis

Genotypic scores of 8575 SNP based recombination bins treated as markers were obtained from data derived by resequencing (Huang et al. 2009) carried out by the Beijing Genomics Institute (BGI; Beijing, China). After resequencing DHLs for candidate SNPs, the genotype of each DHL at a given locus was called based on the ratio between parental genotypes for all SNPs within a 15 SNP sliding window also defining recombination break points (Huang et al. 2009). Thirty lines indicated partial heterozygosity and were, thus, excluded from QTL mapping. All DHLs were aligned and compared for a minimum of 100 kb intervals, pooling multiple monomorphic intervals into larger bins, resulting into 8726 bins. The average bin size was 240 kb. Recombination rates were calculated for the remaining 213 lines and converted to cM using Haldane's mapping function (Haldane 1919). 140 markers of the initial 8726 markers were excluded because of colinearity. In addition, seven markers on chromosome 8 and four markers on chromosome 9 were excluded, as those markers were inconsistent with the location expected from the B73 reference genome sequence. Discrepancies between high-quality genetic maps and the current B73 assemblies are expected, based on findings from Ganai et al. (2011). Segregation distortion was found for several regions across the genome. Based on pair-wise similarities across all markers, the most likely positions of seven markers on chromosome 8 in the physical assembly belong to a well-covered region on chromosome 2, while the four chromosome 9 markers most likely belong

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**Table 2** ANOVA results 2010 and over all trials

Source	WEI	VOL	LEN	DIA	DEN	GY
Overall						
N	0.314	0.3147	0.4151	0.2836	0.3003	0.4572
E	0.2873	0.2486	0.3528	0.219	0.2996	0.1735
E × N	0.0031	0.0034	0.0011	0.0028	0.0018	0.0055
R(E × N)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
G	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
G × E	0.0007	<0.0001	0.0025	<0.0001	0.2439	0.0004
G × E	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
G × E × N	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
2010						
N	0.1344	0.1243	0.2029	0.1757	0.2518	0.0384
E	0.1202	0.095	0.1736	0.1314	0.7889	0.0143
E × N	0.2393	0.2688	0.0972	0.1261	0.0454	0.71
R(E × N)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
G	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
G × E	0.0038	0.0009	0.0242	0.0069	0.2091	0.0007
G × E	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
G × E × N	0.0007	0.0024	0.0003	0.0003	<0.0001	<0.0001

*P* values from ANOVA are shown for all trials (Overall) and trials of 2010 (2010) for each of the five cob traits including weight (WEI), volume (VOL), length (LEN), diameter (DIA), and tissue density (DEN), as well as grain yield (GY) for the effects of nitrogen management (N), environment (E), Block (R) nested in E × N combinations, genotype (G), and the respective interactions as indicated

at a different location on chromosome 9, around 100 cM apart. All remaining 8575 markers were used for QTL analysis.

QTL analyses were carried out with QTL Cartographer version 1.17 (Basten et al. 2003) using composite interval mapping (CIM; Zmap model 6), and the ten most significant cofactors identified with forward and backward regression. QTL were scanned at 1 cM intervals. During CIM, cofactor effects originating from positions mapping within 10 cM of the test position were excluded from the model. In order to limit the type I error rate, comparison-wise thresholds (CWT) based on 1000 permutations of the phenotype data were determined at significance levels of  $\alpha = 0.001$  (and  $\alpha = 0.01$ ) for each trait using scripts updated for CIM to include cofactor reselection for each permuted dataset (Lauter et al. 2008). QTL were treated as separate when their peaks were at least 20 cM apart.

LSmeans used for QTL analysis were correlated to and regressed on best linear unbiased predictors (BLUPs; Henderson 1975) for comparison. Due to close correlations (0.98–1) with an average of 0.999, and high average regression coefficients (0.997), results from LSmeans are similar to that from BLUPs.

QTL findings were compared to findings in the recombinant inbred lines of IBMSyn4 (Lee et al. 2002; Jansen and Lübberstedt 2012) using the estimated physical positions of associated markers in recombinant inbred lines (RILs) according to B73 reference sequence version 2 using the Locus Lookup tool (Andorf et al. 2010; <http://www.MaizeGDB.org>).

## Results

Analysis of variance in 2010, means for all six traits were higher under NN compared to LN conditions in the same location. Cob density (3.6 and 8.8 %) and cob diameter (5.8 and 11.6 %) decreased least and grain yield (30.7 and 60.9 %) and cob weight (21.5 and 44.3 %) decreased most under LN compared to NN (for MAR10 and BUR10, respectively, Table 1). In 2011, traits showed similar response relative to each other, but in opposite direction. CV was lowest (<11) for diameter in all six trials and highest for grain yield (32.8–62.7; Table 1).

The parental lines B73 and Mo17 differed significantly for cob traits in both NN and LN environments of 2010 (Supplementary Table 1). B73 had higher cob WEI and DEN as well as VOL and DIA, but lower LEN than Mo17 in all trials. Transgressive segregants both below and beyond parents were also observed for all investigated traits, suggesting that this population was suitable for QTL analysis of cob traits.

The ANOVA of all three environments uncovered no significant main effects of N and environment ( $\alpha = 0.05$ ). However, genotype effect, G × N-, G × E-, and G × E × N-effects were significant (Table 2). ANOVAs conducted by environment showed that the N-effect was significant ( $\alpha = 0.05$ ) at BUR10 for all traits, but non-significant at MAR10. However, the N-effect was significant at MAR10 for  $P = 0.20$ . At BUR11, the N-effect was significant for all traits but DIA and DEN, but effects were negative resulting in lower means under NN for all traits (Table 3). Genotype effects of lines and interaction of

**Table 3** ANOVA results for BUR10, MAR10, and BUR11

Source	WEI	VOL	LEN	DIA	DEN	GY
BUR10						
N	0.0005	<0.0001	<0.0001	0.0011	0.0048	0.0009
R(N)	0.005	0.0519	0.2906	0.0145	0.0159	<0.0001
G	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
G × N	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
MAR10						
N	0.1244	0.1231	0.1574	0.1252	0.1385	0.1186
R(N)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
G	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
G × N	0.0036	0.0006	<0.0001	0.0009	<0.0001	<0.0001
BUR11						
N	0.0004	0.0219	0.0005	0.06	0.3504	0.0079
R(N)	0.5141	0.1402	0.3975	0.2333	0.0826	0.0178
G	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
G × N	<0.0001	0.0008	0.0012	<0.0001	<0.0001	<0.0001

*P* values from ANOVA are shown for single environments including Burkey 2010 (BUR10), Marion 2010 (MAR10), and Burkey 2011 (BUR11) for each of the five cob traits including weight (WEI), volume (VOL), length (LEN), diameter (DIA), and tissue density (DEN) and grain yield (GY) for the effects of nitrogen management (N), Block (R) nested in N, genotype (G), and the respective interactions as indicated

**Table 4** Plot-based ( $r^2_{\text{plot}}$ ) and entry mean-based repeatability ( $r^2_{\text{mean}}$ )

Trait	BUR10NN	BUR10LN	MAR10NN	MAR10LN	BUR11NN	BUR11LN	BUR10	MAR10	BUR11	NN	LN
$r^2_{\text{plot}}$											
WEI	62.6	48.6	68.9	54.1	70.4	69.9	43.8	58.4	62.2	55.4	45.1
VOL	56.2	41.4	62.1	44.3	65.2	66.0	36.8	48.3	60.3	49.0	39.2
LEN	57.9	53.6	65.2	53.2	72.2	69.9	46.0	53.3	67.5	54.4	49.4
DIA	59.8	49.2	68.3	52.9	68.6	74.1	48.3	55.3	63.4	53.5	47.8
DEN	77.8	74.8	84.5	82.7	85.4	85.4	71.7	80.6	79.6	74.7	68.2
GY	79.6	75.1	73.7	45.1	85.5	76.0	57.2	52.1	77.3	58.2	44.1
$r^2_{\text{mean}}$											
WEI	77.0	65.4	81.6	70.2	82.7	82.3	75.7	84.9	86.8	88.2	83.2
VOL	72.0	58.5	76.7	61.4	78.9	79.5	70.0	78.9	85.9	85.2	79.4
LEN	73.3	69.8	78.9	69.5	83.9	82.3	77.3	82.0	89.3	87.8	85.4
DIA	74.8	66.0	81.1	69.2	81.4	85.1	78.9	83.2	87.4	87.4	84.6
DEN	87.5	85.6	91.6	90.5	92.1	92.1	91.0	94.3	94.0	94.7	92.8
GY	88.7	85.8	84.9	62.2	92.2	86.3	84.3	81.3	93.2	89.3	82.5

Traits are weight (WEI), volume (VOL), length (LEN), diameter (DIA), density (DEN), and grain yield (GY)

NN normal nitrogen management, LN limited nitrogen management

genotype × nitrogen (G × N) were significant for all three environments.

Plot-based repeatabilities were moderate to high (36.8–85.5; Table 4). On average, grain yield and cob density showed the highest and cob volume the lowest repeatabilities. While the repeatability for cob density was consistently high (68.2–85.4), moderate repeatabilities were found for grain yield in MAR10LN (45.1), BUR10 (52.1), and over all LN (44.1). Repeatabilities were generally lower under LN in 2010, but did not vary greatly between LN and NN at BUR11. Similar effects were found for entry mean-based repeatabilities (Table 4).

No significant negative correlations were found ( $p = 0.01$ , Table 5). Cob weight was closely correlated with cob volume ( $r_G = 0.79$ ) and showed moderate correlations with all other traits ( $r_G = 0.48$ – $0.66$ ). Grain yield was positively correlated with all traits ( $r_G = 0.37$ – $0.57$ ), but correlations with cob density were non-significant ( $r_G = 0.04$ ). Cob density was significantly correlated only with cob weight ( $r_G = 0.58$ ). Both the phenotypic and genotypic correlations reflect these values (0.50 and 0.58), which are not always the case. For example, the phenotypic correlation between length and diameter was significant ( $r_p = 0.38$ ), while the genotypic correlation was non-significant ( $r_G = 0.12$ , Table 5).

**Table 5** Genotypic and phenotypic correlations

$r_G/r_P$	WEI	VOL	LEN	DIA	DEN	GY
WEI		0.85	0.67	0.72	0.5	0.46
VOL	0.79		0.79	0.83	0.01 <sup>NS</sup>	0.52
LEN	0.52	0.68		0.38	-0.01 <sup>NS</sup>	0.39
DIA	0.66	0.8	0.12 <sup>NS</sup>		0.00 <sup>NS</sup>	0.48
DEN	0.58	-0.04 <sup>NS</sup>	-0.04 <sup>NS</sup>	0.01 <sup>NS</sup>		0.04 <sup>NS</sup>
GY	0.48	0.57	0.37	0.56	0.04 <sup>NS</sup>	

Genotypic correlation ( $r_G$ ) and phenotypic correlation ( $r_P$ ) are shown below and above the diagonal, respectively. Traits are weight (WEI), volume (VOL), length (LEN), diameter (DIA), density (DEN), and yield (GY)

NS not significantly different from zero at  $\alpha = 0.05$

**Table 6** Number of QTL and total sum of explained phenotypic variance  $R^2$  (in %) using CWT ( $\alpha = 0.001$ )

Trt	Trial	WEI		VOL		LEN		DIA		DEN		GY	
		QTL	$R^2$ (%)										
NN	BUR10	4	30.78	5	27.68	4	33.42	5	25.65	4	26.52	1	6.76
NN	MAR10	3	15.55	1	7.28	6	38.75	2	12.14	5	21.49	3	18.45
NN	BUR11	4	26	5	38.23	4	26.7	4	29.95	6	41.1	4	26.99
LN	BUR10	4	22.93	5	30.35	6	29.33	2	11.12	2	16.52	4	39.92
LN	MAR10	2	9.37	3	17.16	1	7.38	3	16.89	4	29.78	5	33.92
LN	BUR11	5	32.03	5	43.06	5	31.21	4	29.32	4	23.81	4	24.65

Traits are weight (WEI), volume (VOL), length (LEN), diameter (DIA), density (DEN), and yield (GY)

NN normal nitrogen management, LN limited nitrogen management

### QTL for cob traits

Summed over all six trials, 117 QTL were found for five cob architectural traits and 21 QTL for GY (Supplementary Table 2). One to six QTL were found at each trial  $\times$  trait combination. At BUR10, 22 and 21 QTL were found for cob traits at NN and LN, respectively. At MAR10, the lowest number of QTL was found for cob traits with 17 and 13 QTL at NN and LN, respectively. Most QTL were found at BUR11, with 23 QTL under each nitrogen treatment. For GY, this trend was inverted and 2 (1), 5 (3), and 4 (1) QTL were found at BUR10, MAR10, and BUR11 at NN (LN), respectively.

For cob weight (WEI), 22 QTL were found across the genome (Supplementary Table 2), with 11 QTL found for each nitrogen treatment level. For three genome regions, QTL peaks were found within 10 cM of one another across multiple trials (Supplementary Table 2). For example, on chromosome 1, QTL were found at similar positions in trials BUR10NN, BUR10LN, BUR11LN, and BUR11NN at positions 956.7, 964.8, 960.5, and 949.0 cM, respectively. On chromosome 4, QTL were found for BUR10NN, MAR10LN, and BUR11NN (617.5–627.5 cM), while for MAR10NN a QTL was found at position 640.8 cM. For

BUR10NN and MAR10LN, QTL were found on chromosome 10 at 710.3 and 700.3 cM, respectively. The lowest number of QTL (2) was found at MAR10LN and a maximum of five QTL at BUR11LN. Summing over all QTL within each of the six trials, the total explained phenotypic variance increased with number of detected QTL and was highest at BUR11LN ( $R^2 = 32.0\%$ ) and lowest at MAR10LN ( $R^2 = 9.3\%$ ; Table 6). The QTL with the highest  $R^2$  (11.8%, BUR10NN) was located on chromosome 1 at position 956.7 cM, and showed a positive additive effect of 1 g for the B73 allele. For the majority of QTL for cob weight, the B73 allele showed a positive effect (14 QTL with average allele effect of 0.80 g), while at 8 QTL the effects were negative (average  $-0.70$  g).

For cob volume (VOL) 11 and 13 QTL (total 24) were found at NN and LN trials, respectively.  $R^2$  was low for single QTL ( $<12.2$ , chromosome 1, 949 cM(MAR11LN), while total  $R^2$  for single trial analyses ranged between 7.3% (MAR10NN) and 43.1% (BUR11LN). Similar numbers of positive allele effects came from each of the parents B73 and Mo17 (for 11 and 13 QTL with an average effect of 3.34 and 3.41 cm<sup>3</sup>, respectively). For two genome regions around 370 cM apart on chromosome 1, QTL were found in multiple trials (Supplementary Table 2). In one of these

regions, QTL were found for BUR11NN and BUR11LN at positions 556.0 and 562.1 cM, respectively. In the second region, QTL were found for BUR10LN, BUR11NN, and BUR11LN at positions 946.2, 949.0, and 950.0 cM, respectively. For BUR10NN, a QTL was found at 930.6 cM on chromosome 1, too. In a third region on chromosome 10, two QTL were detected for BUR11NN and BUR11LN at positions 722.5 and 710.7 cM, respectively.

25 QTL were found for cob density (DEN) on all chromosomes, except chromosomes 6 and 10 (Supplementary Table 2). Fifteen of those were found at NN. For 14 QTL, the B73 allele showed a positive effect with an average of 0.0078 g/cm<sup>3</sup>. For 11 QTL, the effects were negative (average effect of -0.0076 g/cm<sup>3</sup>). Between two (BUR10LN) and six QTL (BUR11NN) were found in single trial analyses, where the total explained phenotypic variance was highest at BUR11NN ( $R^2 = 41.1\%$ ) and lowest at BUR10LN ( $R^2 = 16.5\%$ ; Table 6). On chromosome 9 at position 349.9 cM, the QTL with the highest  $R^2$  (11.1%) was found with a negative additive effect of -0.0097 g/cm<sup>3</sup> for B73 (BUR10LN). On chromosome 2, QTL were found at similar positions for MAR10NN and MAR10LN at 626.4, and 628.4 cM, respectively. On chromosome 4, QTL were found for trials BUR10NN, MAR10LN, and BUR11NN within a 4 cM interval at 639.4, 635.1, and 636.5 cM, respectively.

For cob length and diameter 26 and 20 QTL were found, respectively. For both traits similar numbers of QTL were found in NN and LN (14 and 12 for LEN and 11 and 9 for DIA, respectively).  $R^2$  for single QTL was small with a maximum of 10.4% for LEN and 9.8% for DIA. Both, negative and positive B73 allele effects were found for both traits for LEN (17 and 9 QTL, respectively) and DEN (8 and 12 QTL, respectively). In three genome regions, different for each of the two traits, QTL were found in multiple trials. For LEN, QTL were found on chromosome 3 for BUR10NN and BUR10LN at 354.1 cM; for MAR10NN at 363.0 cM and on chromosomes 4 and 5, QTL were found in trials BUR10LN and MAR10LN at 1047.2 and 1049.6 cM; and for MAR10NN and BUR11NN at 1004.1 and 1010.8 cM, respectively. For DIA, QTL were found for BUR10NN and BUR11LN on chromosome 1 at 963.4 and 966.3 cM, respectively, and on chromosome 7 for BUR10NN and BUR10LN at 64.5 and 60.1 cM, respectively. On chromosome 10, QTL were found for DEN at 695.0 and 693.0 cM for BUR10NN and BUR11LN, respectively.

A total of 21 QTL (8 and 13 QTL at NN and LN, respectively) were found for grain yield on all chromosomes except chromosomes 1, 5, and 7 (Supplementary Table 2). In single trial analyses, 1 to 5 QTL were found (BUR10NN, MAR10LN, respectively) with total explained phenotypic variance between 6.9% (BUR10NN) and

39.9% (BUR10LN, Table 6). Single QTL explained up to  $R^2 = 15.7\%$  (chromosome 9, 473.0 cM, BUR10LN). For 12 QTL for GY, the B73 allele showed a positive effect with an average of 0.36 MT/ha, while for 10 QTL the effects were negative (average effect of -0.27 MT/ha). QTL were found in multiple trials within 10 cM intervals for three genome regions on chromosomes 2, 3, and 4 (Supplementary Table 2).

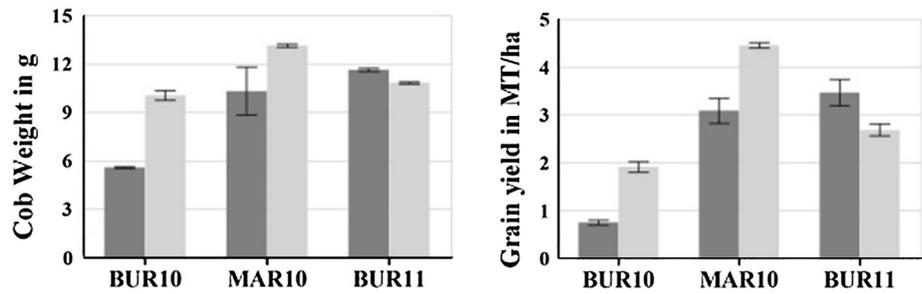
#### Co-location QTL for cob traits

57% of all QTL (138) were located in clusters (Fig. 3). Large clusters were found on chromosomes 1, 4, and 10. On chromosome 1, eleven QTL with positive B73 allele effects for VOL, DIA, and WEI (for LN as well as for NN) clustered within 36 cM between 930.6 and 9.66 cM (Fig. 3). On chromosome 4, nine QTL for GY, DEN, and WEI (for LN as well as NN) mapped within 25.2 cM between 617.5 and 642.7 cM, all with positive B73 allele effect. On chromosome 10, eight out of nine QTL for VOL, DIA, WEI, and GY mapped within 41 cM between 681.1 and 722.5 cM, all showing negative B73 allele effects (Fig. 3, supplementary Table 2). In 11 regions of the genome, QTL of different traits co-located within a trial. In all cases of co-locating QTL, the B73 allele effects showed the same direction for all involved traits. WEI QTL co-located with QTL for all traits, but LEN and GY. On chromosome 4, a co-location between DEN and GY was found for BUR11NN (636.5–642.7 cM). In addition, GY QTL co-located with QTL for VOL on chromosome 10 (712.6–722.5 cM) for BUR11NN and with LEN on chromosome 9 (465.9–473.0 cM) for BUR10LN. Other co-locations were found between LEN and VOL on chromosome 3 (132.8–132.9 cM) for BUR10NN and between DIA and DEN on chromosome 4 (134.8–135.2 cM) for MAR10LN (Fig. 3; Supplementary Table 2).

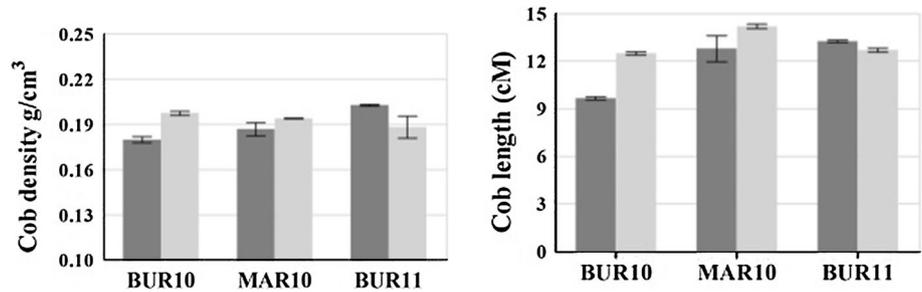
Additional 38 pair-wise co-locations of QTL for different traits were found in 14 regions, when QTL were compared across trials (Fig. 3). In only two of these regions, the allele effects of co-locating QTL from different trials showed opposite allele effects. In the first case on chromosome 2, a DEN QTL found for BUR11NN mapped to position 929.7 cM with a negative B73 allele effect, while two GY QTL found for MAR10LN and BUR11LN at positions 937.3 and 934.4 cM, respectively, showed positive allele effects. In the other case, QTL for DIA and DEN were found for MAR10LN and BUR11LN on chromosome 3 at 748.7 and 754.1 cM (Supplementary Table 2).

Excluding GY QTL, 26 pairs were found each of two QTL for the same trait found in different trials within 10 cM. Five pairs were found between NN and NN, three pairs between LN and LN, and 18 pairs across nitrogen treatments between LN and NN trials. However,

**Fig. 1** Average cob weight (g; *left*) and grain yield (metric ton/hectare (MT/ha); *right*) at normal (*light gray*) and limited (*dark gray*) nitrogen management for BUR10, MAR10, and BUR11 environments



**Fig. 2** Average cob density (g/cm<sup>3</sup>; *left*) and cob length (cm; *right*) at normal (*light gray*) and limited (*dark gray*) nitrogen management for BUR10, MAR10, and BUR11 environments



treating BUR11LN as NN trial, pairs between NN and NN increased to nine, pairs between LN and LN to two, and pairs between LN and NN dropped to 15 (Supplementary Table 3). However, based on  $X^2$  tests (unpublished results) QTL were no more likely to be consistent within than across the same nitrogen management.

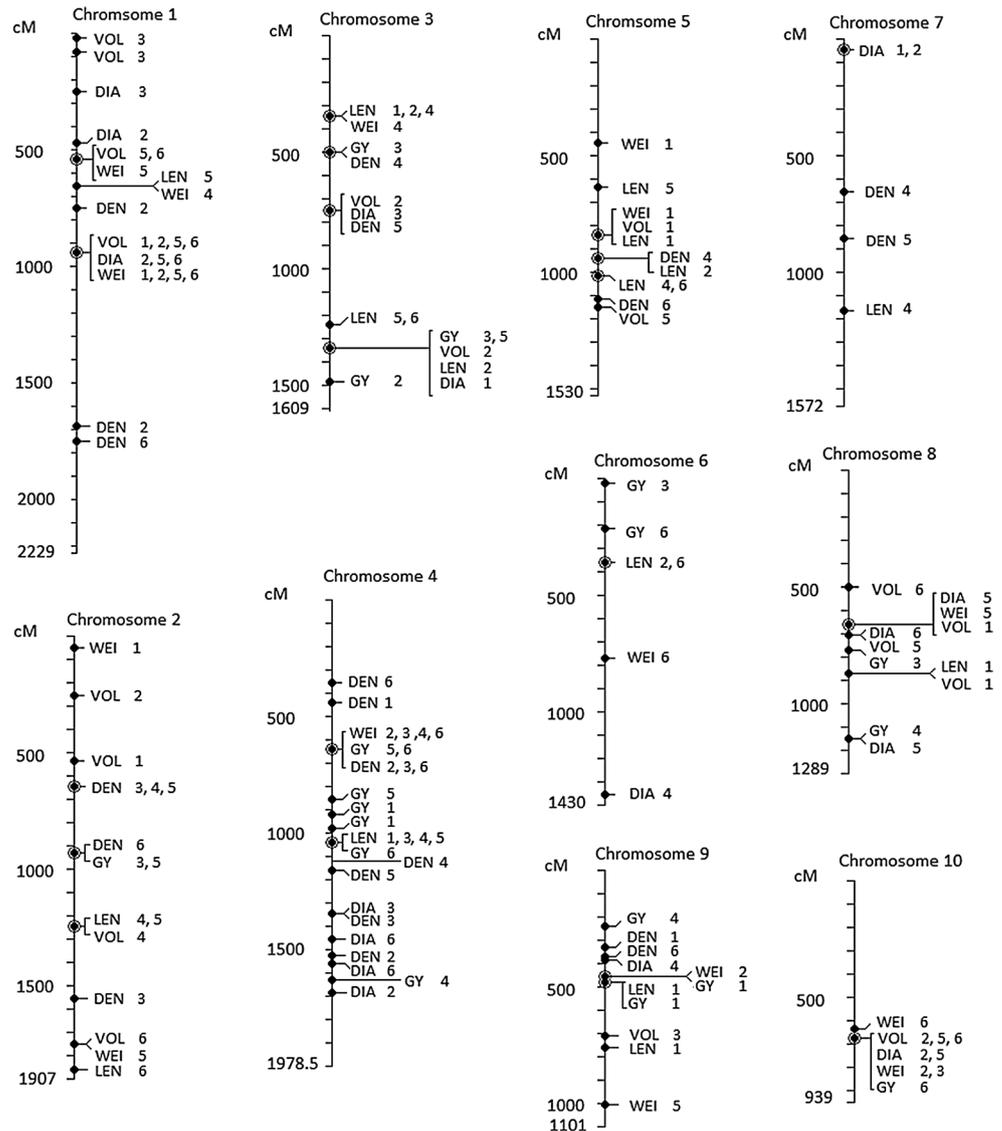
Consistent QTL can be found for density and diameter comparing QTL locations with QTL locations found in recombinant inbred lines (RILs) of IBM, after five generations of intermating (Lee et al. 2002; Jansen et al. 2013). One of six QTL for pith diameter and one of seven QTL for total diameter found with RILs were consistent with two DIA IBMSyn10 QTL (out of 20) on chromosomes 1 and 4, respectively. For density, four QTL (out of 9) were confirmed among the 25 QTL on chromosomes 1, 2, 5, and 7. On chromosome 5, a tissue density QTL was found in RILs at marker umc1752 with an estimated location between 192,727.68 and 195,590.49 kb and at about 194,050 kb in IBMSyn10 for BUR11NN (Supplementary Table 2). On chromosome 7, the tissue density QTL with the largest additive effect in RILs was found at marker umc2092 with an estimated location between 109,977.32 and 114,759.47 kb, according to B73 reference sequence version 2 ([www.MaizeGDB.org](http://www.MaizeGDB.org)) and at bin 111,800.00 kb in IBMSyn10 for BUR11LN (Supplementary Table 2). In IBMSyn10, those regions on chromosome 5 and 7 were covered with 20 and 22 markers, respectively. One QTL was consistent for GY on chromosome 10. No other traits including cob weight, length, and volume showed consistent QTL for the nine other locations.

## Discussion

### Interpretation of response to nitrogen treatments

The objective at each of the three locations was to provide one environment with less than optimal nitrogen supply (LN), and one environment with optimal nitrogen supply (NN). The three environments BUR10, MAR10, and BUR11 differed in their potential to distinguish trait performance under low and normal nitrogen management (Figs. 1, 2). In 2010, heavy rainfall in June (277 and 233 mm at BUR10 and MAR10, respectively) likely reduced nitrogen availability in all four trials. Plants showed severe N stress in 2010, and, therefore, nitrogen application was increased in 2011. Lower precipitation in 2011 likely caused oversupply of N at NN and satisfactory N levels at LN for inbred lines (Supplementary Table 4). High performance for all traits in the LN trial of BUR11 suggests that our attempt to create differentiating environments failed in this location. Observations in both trials at BUR11 should, therefore, be interpreted as for sufficient nitrogen management. At MAR10, N effects were not significant, which was likely due to large differences between blocks at LN. However, plants under LN at MAR10 did suffer from lower N availability, and performance of traits was substantially reduced under LN (4–30 %, Table 3). Even though reduction in MAR10 was only about half as strong as in BUR10, MAR10LN can still be interpreted as trial with limited N supply. We, therefore, interpreted results from BUR10 and MAR10 with respect to their susceptibility to limited nitrogen supply.

**Fig. 3** QTL localization of cob traits in maize chromosomes 1–10. QTL positions are shown for single QTL (black dot) and clusters (circled dot). Cob traits are weight (WEI), volume (VOL), length (LEN), diameter (DIA), density (DEN), and yield (GY). Numbers indicate the trial where QTL were detected (1 BUR10LN, 2 BUR10NN, 3 MAR10LN, 4 MAR10NN, 5 BUR11LN, 6 BUR11LN)



DEN, with the lowest relative reduction under LN (3.6–8.8 % of NN for MAR10 and BUR10, respectively), was least affected by N management. Relative reduction of cob weight (21.5–44.3 %) was similar to reductions of cob volume (18.9–38.3 %) and, therefore, probably mainly due to reduction in cob size under LN. Reduction in cob volume was due to reduction in length and diameter. Relative reductions in length (9.9–22.6 %) were about twice as high as for diameter (5.8–11.6 %). Reductions in diameter, however, will affect volume in a quadratic fashion, while reduction in length reduces volume linearly. The observed reduction in MAR10 and BUR10 are, therefore, likely equally caused by length and diameter reductions under LN. Genotypes with high cob weights and high cob tissue density, rather than high volume will, therefore, likely show more stable cob biomass under low nitrogen management.

Density and volume were measured using image analysis for cob volume. In the past, cob volume traits such as cob length and diameter were measured using yard sticks or caliper. While cob or ear length is comparably simple to measure, cob diameter will vary along the cob and depends on the position where it is measured. Common procedures in archeology or for inbred line patenting follow, measuring at mid-cob (Adams 1999; Bohning 2000; Vattikonda 2000). For cob volume and consecutively cob tissue density, estimation based on the average diameter (and length) assuming cylindrical shape can be erroneous. In most cases, cob volume is overestimated due to the pointed cob tip which results in underestimation of density using conventional methods. Using image analysis, misestimating of cob volume and density was minimized. In addition cob measurements were taken about three times faster than with traditional methods (data not shown).

## Genetic characterization of cob trait inheritance

Moderate to high repeatabilities and significant genetic effects indicate that variances for trait observations differentiate lines mainly due to genetic effects. However, few QTL explaining with maximum total  $R^2$  of 43.1 % were found in any of our analyses. Low total explained variance within each environment ( $R^2$  ranges from 7.3 to 43.1 %) suggests additional QTL with smaller effects and insufficient power to detect those (Melchinger et al. 1998, Openshaw and Frascaroli 1997). In each of the six trials for each trait, on average four QTL were found, which together explained about 25 % of the phenotypic variance. Finding only a small number of QTL for cob traits under stringent thresholds such as empirical comparison-wise thresholds ( $\alpha = 0.001$ ) is in agreement with earlier findings in IBM-Syn4 (Jansen et al. 2013). When using  $\alpha = 0.01$ , the average number of detected QTL per trait in each trial increases to 20 and average total  $R^2$  to 66.5 % with a maximum of 93.2 % for VOL for BUR10LN (Supplementary Table 5). However, decreasing the thresholds increases the risk of false positive QTL detection (van Ooijen 1999) and does not influence the chance of detecting QTL with large effects which would be of interest for breeders.

Using markers with segregation distortion can increase type I error, due to sampling within a smaller genotype set for the underrepresented allele and increased genetic variance (Zhang et al. 2010). Two QTL were detected at positions, where B73 allele frequency was below 30 % for WEI for BUR11LN at 1003.5 cM on chromosome 9 (27.2 % B73 allele frequency) and for GY for MAR10LN at 764.6 cM on chromosome 8 (29.6 % B73 allele frequency). Five QTL on chromosome 4 including DEN and DIA QTL between positions 1348.2 and 1559.0 cM, two QTL on chromosome 7 (DEN and LEN at 854.3 and 1181.1 cM for BUR11LN and MAR10NN, respectively), and one QTL on chromosome 9 (246.9 cM; GY MAR10NN) showed B73 allele frequencies greater 70 %. However, CWTs were close to the according genome averages for any trial at those positions indicating no increased risk for false positive QTL detection. Therefore, the use of CWT based on 1000 permutations for each testing site enables testing for QTL at positions where segregation is distorted and QTL located in regions with segregation distortion can be considered trustworthy.

## QTL consistency within Syn10 and between Syn4 and Syn10

QTL showed low consistency within IBM10 and might be trial specific, although the power might have been too low to detect QTL with small effects. Nitrogen management did not affect QTL detection and inconsistent QTL across

nitrogen management levels were not more frequent than within. Also, similar numbers of QTL were detected for both treatments. To overcome inconsistency and implement estimation of epistasis, genotype-by-environment interaction and possible response curves of QTL at varying nitrogen supply, more complex models and more test environments, genotypes, and years are needed (Cooper and Delacy 1994).

QTL with low heritability (small effects) are expected to show larger confidence intervals (Hyne et al. 1995). Differences in heritability do explain low consistency in our study. Broad sense heritability of traits derived from repeatabilities was moderate to high and highest for DEN, but there were not more consistent QTL detected for DEN than for any other trait. About 10 % of our QTL were found in more than one trial. The number of co-locations across trials might have been reduced by considering QTL within 10 cM distance only. For example, three larger clusters on chromosomes 1, 4, and 10 showed multiple related QTL within larger intervals of 25–41 cM (Fig. 3). However, the number of co-locating QTL remains low (19 %) when allowing for larger 25 cM distances. Therefore, most likely, the majority of QTL had small effects with low power of being detected in different trials and environments.

Few QTL were consistent with findings in IBMSyn4 (Jansen et al. 2013). Possible reasons for low consistency between the two studies can be extended to the use of different lines, map resolution, marker sets, and environments (Austin et al. 2000). However, two out of the three DEN QTL and one DIA QTL, that were consistent with findings in IBMSyn4, were co-located with known genes involved in the branching pathway. This supports that for most cob traits no other major QTL segregate within IBM populations and most QTL effects are small.

## Candidate genes

Known genes with positions at QTL can help to understand trait architecture, if their function can be related to the associated trait. In a region on chromosome 7, where DEN QTL of IBMSyn10 and IBMSyn4 overlap, the *ramosal* gene is located between 110,331.5 and 110,332.3 kb (*ral*; Locus Lookup tool; Andorf et al. 2010). *ral* codes for a transcription factor with dramatic but complex effect on branching length in the maize inflorescence (Vollbrecht et al. 2005). On chromosome 2 *ba2* (barren stalk2) is located coding for another transcription factor that affects branching in maize inflorescences (Vollbrecht et al. 2005). It maps between 31,889.8 and 33,429.8 kb between two density QTL (at 624.35 and 643.9 cM or 30,550.0 and 33,650.0 kb, respectively, Locus Lookup tool; Andorf et al. 2010). Interpreting the cob as condensed lateral branches (Murdy 1960; Galinat 1975) could explain this consistent co-location of

density QTL and *ral* and the co-location of *ba2* with density QTL on chromosome 2.

#### Use of IBMSyn10 for fine mapping

With ten generations of intermating, a high resolution covered with 8575 markers, and an average distance of 1.8 cM between neighboring markers the IBM10DH population provides the potential of fine mapping QTL (Hussain et al. 2007). As compared to IBMSyn4-RILs (Lee et al. 2002) six additional generations of intermating and intensive genotyping yield increased genetic resolution. Overall, marker coverage and recombination was superior in IBMSyn10 with respective physical and genetic distances of 250 kb and 1.9 cM between markers, as opposed to 1550 kb and 4.7 cM for the map used for IBMSyn4 (Jansen et al. 2013). The number of obtained QTL in single trials was similar in high resolution IBMSyn10 and IBMSyn4 (about 4). Identification of two candidate genes would have been successful in both studies for *ral* and possibly for *ba2*.

The theoretical advantage and risk of smaller QTL confidence intervals due to higher recombination rate for DHLs can be illustrated using example *ral* on chromosome 7. The marker (umc1393) associated with cob tissue density in IBMSyn4 maps about 11 cM (i.e., 27 cM, 7700 kb in IBMSyn10 map) away from *ral*. In IBMSyn10 the position of the QTL peak is 6 cM (about 1500 kb) away from the *ral* position. QTL mapped with IBMSyn10 intermated for six additional generations (as compared to RILs of IBMSyn4) showed smaller physical and genetic distances. As from these results and prior publications LOD drop-off-based confidence intervals might not be optimal (Van Oijen 1992, Bennewitz et al. 2002). Alternative methods to establish empirical confidence intervals using CWTs have been proposed but require substantial calculation resources (Crossett et al. 2010). In general, to fully realize the advantages of the high recombinant DHL population, adequate marker density must also be backed up with a sufficient number of lines and test environments to estimate QTL effects and interactions at higher accuracy and power (Knapp and Bridges 1990). For physically isolating QTL, a possible advantage using the high resolution IBMSyn10 remains dependent on how confidence intervals are estimated and interpreted for fine mapping.

#### Implementation for breeding

If QTL effects are small and possibly environment specific, introducing single QTL using backcrossing or even transgenic approaches is not effective to improve general trait performance QTL with larger effects might be found within genetic resources with extreme cob architecture (Jansen and Lübberstedt 2012; Loesch et al. 1976). Based on

findings in the IBM population, selection of multiple QTL for grain yield and cob yield for example using phenotypic or genomic selection (Meuwissen et al. 2001) for specific target environments are proposed.

**Author contribution statement** Constantin Jansen and Yongzhong Zhang were responsible for finishing experiment and writing the paper. Hongjun Liu supplied to the mapping data. Constantin Jansen, Yongzhong Zhang and Gonzalez-Portilla analyzed the data. Nick Lauter, Bharath Kumar, Ignacio Trucillo-Silva, Juan Pablo San Martin, Michael Lee, Kevin Simcox and Jeff Schussler helped Yongzhong Zhang and Constantin Jansen to carry out the field trial. Kevin Simcox, Jeff Schussler, Kanwarpal Dhugga and Thomas Lübberstedt made the final revision.

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**Conflict of interest** The authors declare that no conflict of interest exists.

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