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The Genetic Basis for Inflorescence Variation Between Foxtail and Green Millet (Poaceae)

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ABSTRACT

Grass species differ in many aspects of inflorescence architecture, but in most cases the genetic basis of the morphological difference is unknown. To investigate the genes underlying the morphology in one such instance, we undertook a developmental and QTL analysis of inflorescence differences between the cereal grain foxtail millet and its presumed progenitor green millet. Inflorescence differences between these two species are the result of changes in primary branch number and density, spikelet number, and bristle (sterile branchlet) number; these differences also account for inflorescence variation within the clade of 300+ species that share the presence of bristles in the inflorescence. Fourteen replicated QTL were detected for the four inflorescence traits, and these are suggested to represent genes that control differences between the species. Comparative mapping using common markers from rice and maize allowed a number of candidate genes from maize to be localized to QTL regions in the millet genome. Searches of regions of the sequenced rice genome orthologous to QTL regions on foxtail millet identified a number of transcription factors and hormone pathway genes that may be involved in control of inflorescence branching.

GRASSES (Poaceae) are important economically and ecologically and feed the world either directly via grain crops or indirectly as the primary fodder of most livestock. Inflorescence architecture is one of the most useful and conspicuous characteristics for distinguishing among the ~10,000 species, and variation in inflorescence architecture is important in determining yield of cereal grains. Even among closely related species the structure of the inflorescence may vary in the number of branches, the number of orders of branching, and the extent of elongation of various axes (Kellogg 2000; Doust and Kellogg 2002). To understand the evolution of these phenotypes, we need to characterize them carefully and identify the genes that underlie them.

The most complete source of information on genes controlling inflorescence development in grasses comes from studies on the model system, maize. Many genes have been found to affect inflorescence development in maize and these are all possible candidates for the control of inflorescence morphology in other grass species. However, several aspects of the morphology of maize are highly unusual within the grasses, and thus its developmental evolution may not be representative of other grass species. In contrast, other cereal domestication events have involved a greater number of inflorescence characteristics that vary among related wild species. We were interested to investigate morphological changes in a wild-domesticate pair in which the domesticate is morphologically similar to multiple wild species. Study of domestication in such a pair of species might shed light not only on the process of domestication, but also on morphological diversification in general.

We have examined the domesticated crop foxtail millet (*Setaria italica*) and its close relative and presumed wild progenitor, green millet (*S. viridis*), which are grasses in the subfamily Panicoideae, tribe Paniceae. They are in the same subfamily as maize, sugar cane, and sorghum, but in a different tribe, having diverged from the maize lineage roughly 28 million years ago (Gaut and Doebley 1997). Foxtail millet is an important crop in China, and green millet is one of the world's worst weeds of arable land (Harlan 1992). Domestication of foxtail millet led to the production of much larger inflorescences with a more complex branching pattern, as well as a reduction in basal (tillering) and axillary vegetative branching (Harlan 1992; Doust and Kellogg 2002; Doust *et al.* 2004).

Some of the same changes during domestication are also seen in the domestication of maize from its wild progenitor teosinte, and quantitative trait locus (QTL)

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studies of crosses between maize and teosinte have revealed five major regions of the genome controlling differences between the two species (Doebley and Stec 1991, 1993). Later analyses of mutants and gene expression identified *teosinte branched1* as causing differences between teosinte and maize (Doebley *et al.* 1997), with noncoding regions upstream of the gene being under selection (Clark *et al.* 2004). Increased expression of *tb1* in maize relative to its wild progenitor influences inflorescence architecture and sex expression and represses lateral vegetative branch elongation (Hubbard *et al.* 2002). However, in a previous study, we found that *tb1* plays only a minor role in controlling vegetative branching in foxtail millet (Doust *et al.* 2004).

We have previously characterized the development of both foxtail and green millet (Doust and Kellogg 2002). Both species produce sterile inflorescence branches, commonly called bristles. These generally terminate the various inflorescence axes and also appear to be approximately paired with spikelets (Figures 1–3; Doust and Kellogg 2002).

The differences between inflorescences of green and foxtail millet can be ascribed to variation in the number and density of primary branches, the number of orders of branching, and the extent of spikelet suppression (Doust and Kellogg 2002). These differences appear at varying stages during inflorescence development. Variation in the number of primary branches occurs early in development, reflecting the number of lateral primordia produced by the inflorescence meristem (greater numbers in foxtail than in green millet; Figures 1, A–C and 2A). Variation in how many orders of branches are initiated occurs at intermediate stages of development, with approximately five orders of branching in green millet (Figure 1, C and D) and up to eight orders of branching in foxtail millet (Figure 2, B–D). Each branch initiates meristems that will differentiate into spikelets (containing two florets) or elongated sterile branchlets (bristles), as well as higher-order branch axes (Figure 1, C–E). The last branch to be initiated terminates in either a spikelet or a sterile branchlet. Suppression of some initiated spikelets occurs later in development (Figure 1G). Variation in density of primary branches is the result of differential elongation of the main axis late in development, which continues to occur as the inflorescence emerges from the sheath (Figures 1, E and F and 2, E and F).

When first initiated, spikelets and bristles appear to be paired (Figure 3) and, if all spikelets grew to maturity, the numbers of spikelets and bristles would be approximately equal. However, a number of spikelets cease development and persist as tiny rudiments (Figure 1G). This failure of development may happen to spikelets at various stages and, in some cases, cessation of growth of the spikelets is so early that it is difficult to recognize the structures as spikelets. The result is that there are often many more bristles than spikelets, especially in green millet.

To determine the number—and ultimately the identity—of genes controlling the developmental characters, we have used QTL analysis of differences in inflorescence structure between foxtail and green millet. We have then exploited the colinearity of grass genomes to identify a likely subset of candidate genes for these differences (Gale and Devos 1998; Hake and Rochefort 2004). To localize genes of interest we have used common markers among foxtail millet, maize, and rice genomes. Cross-species comparisons are feasible because the millet genome is broadly colinear with rice and maize (Devos *et al.* 1998; Wang *et al.* 1998).

Our previous work has identified several QTL of major effect that control vegetative branching (Doust *et al.* 2004); for each of these QTL we have hypotheses of candidate genes. Here we describe QTL that control inflorescence branching, compare their location to that of vegetative QTL, and suggest possible candidate genes.

MATERIALS AND METHODS

Mapping: A genetic map of a cross between foxtail millet (*S. italica* acc. B100) and green millet (*S. viridis* acc. A10) using RFLP markers was previously constructed at the John Innes Center (Norwich, United Kingdom; Wang *et al.* 1998). The original molecular map used 160 RFLP probes, consisting of anonymous foxtail millet, pearl millet, and wheat genomic clones and two known-function clones identifying the *waxy* and *carboxypeptidase* loci (Wang *et al.* 1998). Additional rice probes were added to investigate the synteny of foxtail millet with rice, giving a map containing 257 loci and spanning 1050 cM (Devos *et al.* 1998). For the QTL analysis, 119 of these markers were chosen to cover the genome at ~10-cM intervals. F_3 offspring selfed from 120 of the original 127 F_2 plants were used to evaluate the number and location of QTL controlling the morphological characters distinguishing the two parents.

Additional RFLP markers from maize were added to the original map using 23 probes from University of Missouri-Columbia, a maize *tb1* cDNA clone kindly provided by J. Doebley, and probes amplified from foxtail millet using primers for *terminal ear1* (*te1*; White and Doebley 1999), *phytochrome B* (Mathews *et al.* 2000), *knotted1* (*kn1*; unpublished primers provided by Anthony Verboom, University of Missouri-St. Louis), *barren stalk1* (*ba1*; unpublished primers provided by Andrea Gallavotti, University of California at San Diego), and *branched silkless1* (*bd1*; Chuck *et al.* 2002). Southern hybridizations were performed against restricted F_2 DNA of the original *S. italica* acc. B100 × *S. viridis* acc. A10 population (Devos *et al.* 1998; Wang *et al.* 1998). Marker data were scored by two people separately and then results were cross-checked. Position of these markers on the genetic map was established using the two- and three-point linkage routines in Mapmaker version 3 (Lander *et al.* 1987).

QTL trials: Multiple replicates of the 120 $F_{2:3}$ families were grown in four separate trials in a climate-controlled greenhouse at UM-St. Louis. Trials 1 and 2 were at a high density of 5 plants of each family per pot while trials 3 and 4 had 15 plants per family at a lower density of 1 plant per pot. The position of the pots was randomized to minimize the effect of differences in light intensity and other environmental variables within the greenhouse. Trials 1 and 3 were grown in

May–June (early summer) of 2000 and 2001, respectively, and trials 2 and 4 were grown in July–August (late summer) of 2000 and 2002, respectively. Soil, fertilizer, and water conditions were standardized, and a 16-hr day length was maintained by artificial lighting when necessary. Trials 2 and 4 had both a higher natural light intensity and higher average temperatures than trials 1 and 3, owing to the later growing time, although temperatures in both trials were kept between 25° and 35°.

Measurement of phenotypic traits: Plants were harvested after the seeds had ripened, and measurements were taken of the inflorescence that terminated the primary culm. All plants in trials 1 and 2 and five randomly chosen plants for each family in trials 3 and 4 were measured. Primary branch density was estimated by counting the number of primary inflorescence branches in a 1-cm region in the middle of the inflorescence axis. Multiplication of this number by the length of the inflorescence (measured from the inflorescence tip to the most basal branch that produces fertile spikelets) gives an estimate of the number of primary branches per inflorescence. We verified the reliability of this approach by establishing a significant correlation ($P < 0.01$; $R^2 = 0.34$) between the estimated number of primary branches and the actual number; the actual number was determined by counting all primary branches for 184 randomly selected inflorescences of various sizes. The numbers of spikelets and bristles were measured for one primary branch from each inflorescence (taken from the middle of the inflorescence). Because approximately one bristle and one spikelet per order of branching are produced (Figure 3; Doust and Kellogg 2002), the number of bristles can be used as a surrogate for the number of orders of branching of the primary branch (Figures 1D, 2D, and 3).

Data management: Means were calculated for each trait for each of the families in each trial. To construct mean high- and low-density trait values, means per family were calculated for combined high-density (trials 1 and 2) and combined low-density (3 and 4) trials. The means of both the individual and the two density trial averages were tested for normality (Kolmogorov-Smirnov test, $P < 0.05$; SPSS 2001) and transformed where necessary.

QTL detection: We investigated QTL (1) from each trial individually (referred to here as individual QTL), (2) with the trials combined by density (high-density QTL *vs.* low-density QTL), and (3) with all four individual trials analyzed together (joint QTL). Most results presented here reflect analyses of the high- *vs.* low-density trials, but other analyses are presented where appropriate. QTL were detected using composite interval mapping (CIM), as implemented in QTLCartographer (Basten *et al.* 2002). CIM tests the hypothesis that a QTL is present in an interval between two adjacent markers, while at the same time controlling for the effects of segregating QTL elsewhere in the genome (Rieseberg *et al.* 2003a). Background markers were selected at $P = 0.05$, and five background parameters were included as cofactors in each CIM model. Tests were made at 2.0-cM intervals and a window size of 10 cM was used to exclude flanking markers from the search for new QTL. Data were also analyzed by multiple interval mapping, using the recommended information criterion 1 with a threshold of 0.0 for acceptance of new QTL (Basten *et al.* 2002). This gave qualitatively similar results, so only the CIM results are presented here. Individual and high- and low-density averages were analyzed for CIM using the program module Zmapqtl (Basten *et al.* 2002). Where multiple QTL were detected in close proximity, they were accepted only when the LOD score dip between the QTL peaks was >1 LOD interval (Basten *et al.* 2002). QTL in high- and low-density trials were considered to be identical if their 1-LOD support intervals overlapped and the sign of their additive effects was the same. In addition, a joint analysis of each trait for all four trials taken together was analyzed using the module JZmapqtl (Basten *et al.* 2002). The joint analysis allows an estimate of the amount of genetic by environmental ($G \times E$) interaction between the trial values for each trait and provides a measure of both the main and the interaction effects of detected QTL.

Significance thresholds for QTL for individual and joint CIM analyses were calculated by 1000 permutations of the original data, using the same parameter settings as for the original analysis (Churchill and Doerge 1994; Doerge and Churchill 1996). To strike a balance between detection of true QTL and a high rate of acceptance of false positive results, chromosome-wide significance levels at $P < 0.05$ were used to declare the presence of QTL. The chromosomes differed in the number and density of markers, and thus the critical LOD score values for $P < 0.05$ differed among them. Permutation tests gave critical LOD score values per chromosome similar to those of a Bonferroni correction based on the total number of independent comparisons (after correcting for linkage between markers on a particular chromosome; Cheverud 2001). A more stringent genome-wide significance level of $P < 0.05$ was also calculated. The identification of QTL based on multiple chromosome-wide significance levels will increase type I error compared to the genome-wide level because nine different tests are being performed (one for each chromosome), but will also increase the probability of identifying more true QTL (Cheverud 2001).

In this study, we focused primarily on QTL for a particular phenotypic trait that were found in both high- and low-density trials. This is because our interest is in QTL that may represent genes explaining species differences, which we expect to produce the same phenotype under multiple environmental conditions. By this approach we are ignoring QTL and, by extension, genes that are important under certain environmental conditions (as represented by the different densities and growing times).

It was not possible to directly measure transgressive segregation (the extent to which the range of values of the hybrid populations exceeded that of the two parents) in the high- and low-density trials, because the parents of the cross were grown in only trials 2 and 4. However, the parental and hybrid ranges were examined for evidence of transgressive segregation in those individual trials.

Additive and dominance effects: The additive effect of each QTL changes the phenotypic value away from the overall mean of the parental values (the midpoint of the distance between the mean for foxtail millet and that for green millet) and toward the mean of one parent or the other. Dominance effects are detected by examining the phenotypes of individuals heterozygous for markers at a particular QTL. Heterozygotes are expected to have phenotypic values corresponding to the mean of the parental values; deviation from the mean indicates a dominant effect at or near that marker. The sign of the effect indicates the direction of change in the phenotype; in this study, a positive sign indicates an increase and a negative sign a decrease in the trait value.

Additive effects are presented for each of the replicated QTL in the high- and low-density trials. It was not possible to measure the extent to which the additive effects for the replicated QTL explained the parental differences, because parents were grown in only trials 2 and 4. The range of values of the $F_{2:3}$ population means in the high- and low-density trials was compared with the sum of the absolute values of the additive effects ($\Sigma|A|$) of the replicated QTL. If combinations of all positive or all negative additive effects are sufficient to explain the observed phenotypic extremes, then the range of values of the hybrids should be equal to twice $\Sigma|A|$. Transgressive segregation for a trait can be inferred when the range of the hybrid values exceeds twice $\Sigma|A|$.

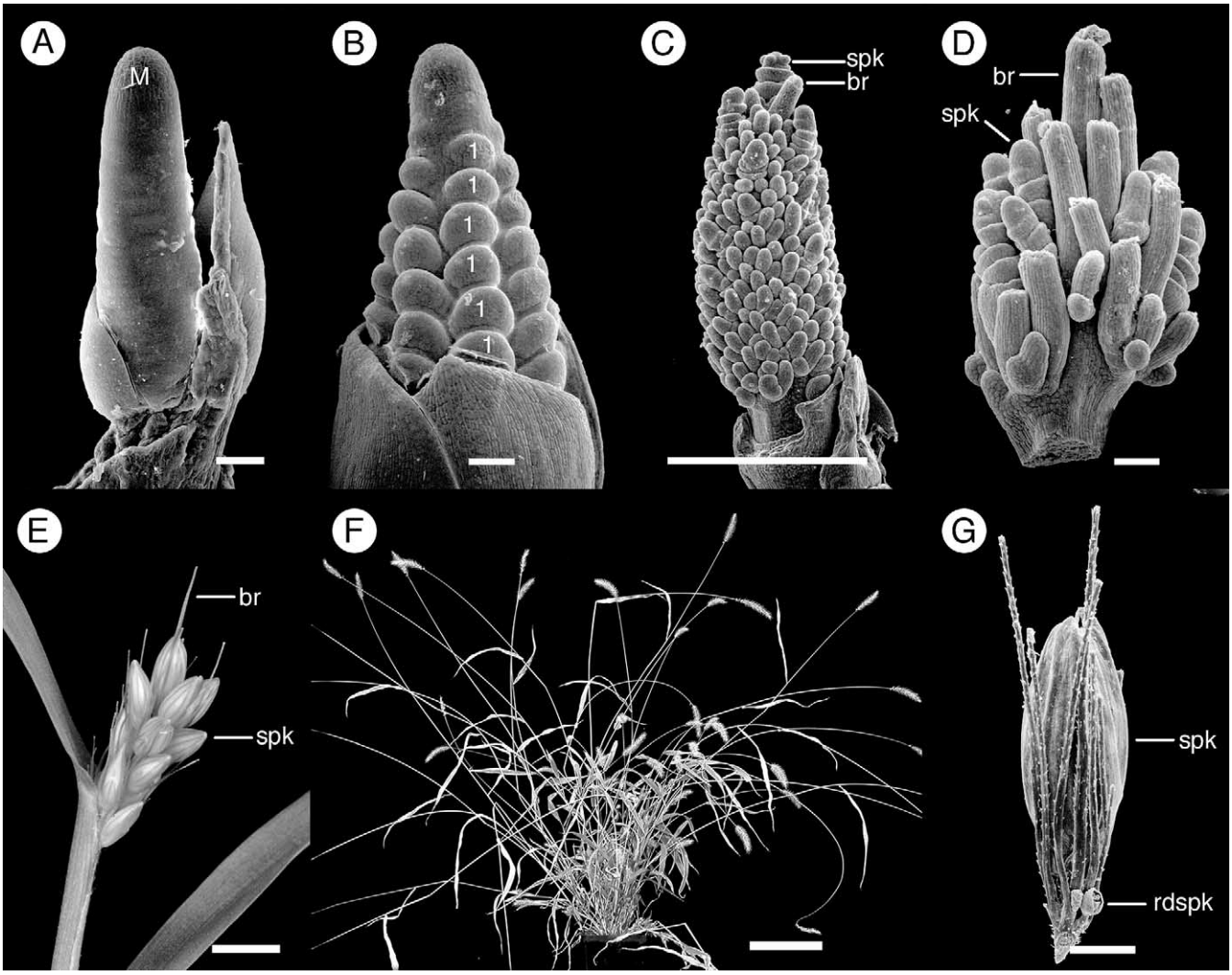

Figure 1.—Developmental stages and mature morphology of the inflorescence in green millet (*S. viridis*). (A) Inflorescence meristem, with sheathing leaf base. (B) Inflorescence meristem initiating primary branch primordia. (C) Inflorescence with many primary branches, each of which has produced up to four more orders of branching. The higher-order branch primordia at the distal end of the inflorescence are differentiating into spikelets and bristles. (D) A piece of an inflorescence, showing a number of primary branches with differentiating spikelets and bristles. (E) A mature inflorescence showing spikelets and bristles. (F) A mature plant of green millet, with multiple tillers and axillary branches, each tipped with an inflorescence. (G) A primary branch at maturity showing a mature spikelet with several bristles and two rudimentary spikelets. M, meristem; 1, primary branch; spk, spikelet; br, bristle; rdspk, rudimentary spikelet. Bars, 50 μm (A and B), 500 μm (C), 50 μm (D), 5 mm (E), 7 cm (F), and 500 μm (G).

Epistasis: The program Epistacy (Holland 1998) was used to identify digenic epistatic interactions by examining each pair of markers for each trait and testing for a significant interaction term. Because of the large number of comparisons that must be made for each trait, a Bonferroni correction to the $P < 0.05$ significance level was applied so that overall significant interactions were reported at $P < 1.0 \times 10^{-5}$ (Rieseberg *et al.* 2003a).

Comparative mapping: To identify possible candidate genes from maize, we used markers mapped on both maize and foxtail millet to define intervals on the maize map that correspond to QTL regions in foxtail millet. Where regions between common markers were larger than the 1-LOD confidence intervals of the QTL, the entire region on the maize map was searched for candidate genes. MaizeGDB (Lawrence *et al.* 2004) and GRAMENE (Ware *et al.* 2002a,b) were used to identify genes that had mutant phenotypes in which inflorescence branching was affected. Several of these genes, including *ba1*, *bd1*, *zea floricaula leafy1* (*zfl1*), and *luminidependens* (a marker closely linked to *tassel seed4*, *ts4*) were screened for RFLP polymorphisms between the parents and, in the case of *ba1* and *bd1*, were mapped.

Identification of candidate genes from the rice genome: Common markers mapped on both the foxtail millet and rice genomes (Devos *et al.* 1998) were used to delimit regions that contained the identified QTL. These regions were in most cases covered by a number of unassembled contigs. Each of these contigs was scanned using FgeneSH (Salamov and Solovyev 2000), and open reading frames (ORFs) were translated and BLASTed against ORFs from other contigs from the

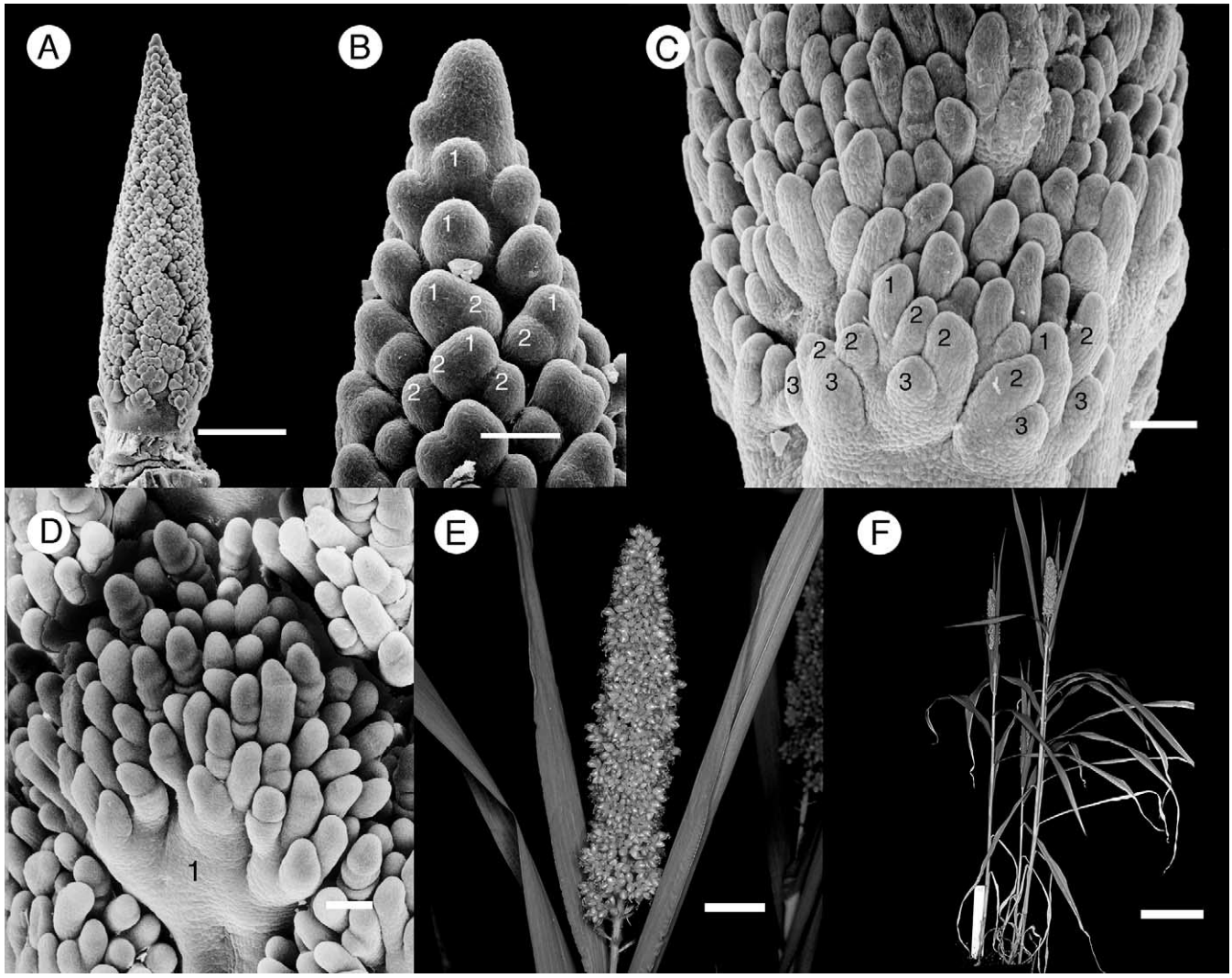

FIGURE 2.—Developmental stages and mature morphology of the inflorescence in foxtail millet (*S. italica*). (A) Inflorescence of foxtail millet with many primary branches, each of which has initiated further orders of branching. (B) Distal end of inflorescence, showing initiation of primary and secondary branches. (C) Basal end of inflorescence showing initiation of primary, secondary, and tertiary branches. (D) Primary branch late in development showing many orders of branching (>8). Spikelets and bristles are just starting to differentiate. (E) Mature inflorescence of foxtail millet with very tightly packed spikelets and short bristles. (F) A plant of foxtail millet, with three tillers but no axillary branches. 1, 2, and 3, primary, secondary, and tertiary branches. Bars, 500 μm (A), 50 μm (B–D), 2 cm (E), and 15 cm (F).

same QTL region to reduce redundancies (ALTSCHUL *et al.* 1997). The final data set of translated proteins for each region was BLASTed against the NCBI database (ALTSCHUL *et al.* 1997). Hits with eV $< 10^{-7}$ were evaluated, and possible candidate genes were identified. Where annotated chromosomes for the rice genome were available, these were searched directly for candidate genes, using the intervals defined by the common markers between foxtail millet and rice.

A dual approach was also used, identifying the location of orthologs of developmentally important maize genes on the rice genome and then using common markers between the rice and millet genomes to identify whether the presumed orthologous regions on the millet genome would contain the orthologs. This enabled greater precision in placement of genes relative to QTL because there is greater coverage of the millet genome with rice markers than with maize markers.

RESULTS

Phenotypic traits: The distribution of phenotypic values for each trait in each trial was, in general, positively skewed, so that the tail of the distribution to the right of the mean (increasing in value) was greater than that to the left (Figure 4). Natural log transformation of trait means significantly improved the normality of the distributions, with all traits assessed as normal in a one-sample Kolmogorov-Smirnov test (SPSS 2001).

The parents of the cross were grown as part of the experimental design in trials 2 and 4 (high density and low density, respectively). Differences in mean trait values between the two parents were consistent in sign

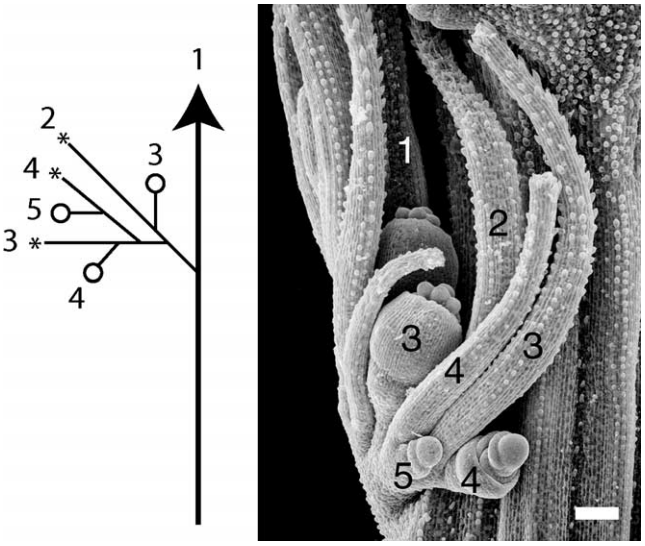

FIGURE 3.—Electron micrograph and line-drawing interpretation of the orders of branching in a primary branch of Setaria. In early development, each order of branch axes has approximately one spikelet and one bristle; 1, 2, 3, 4, and 5 denote primary, secondary, tertiary, quaternary, and quinternary branch axes, respectively; circles represent spikelets; asterisks represent bristles. Bar, 50 μm.

between high- and low-density trials; primary branch number, spikelet number, and bristle number were greater in foxtail millet as opposed to green millet, while the reverse was true for primary branch density (Figure 5). The difference in spikelet and bristle number between foxtail and green millet was greater at low density than at high density, while the reverse was true for primary branch number and density. Primary branch number and density were not significantly different for either species at low density, but were at high density ($P < 0.05$). Spikelet and bristle number were significantly different at both densities for both species. These observations suggest that, in general, both species respond in a similar manner to changes in planting density, although there is variation in the magnitude of the differences in trait values.

Transgressive segregation was seen in almost all traits, with the hybrid range exceeding the parental range in all cases except for spikelet number in trial 4. The extent to which the hybrid range exceeded the parental range varied greatly, both among traits and among trials (Figure 6).

The overall means per trial of the $F_{2:3}$ populations were significantly greater for low planting density than for high planting density for all four traits (univariate ANOVA, $P < 0.001$).

QTL detected: The CIM approach, using a chromosome significance level of $P < 0.05$, found 33 QTL in the high-density and 38 QTL in the low-density trials. Of these, 5 in the high density and 12 in the low density

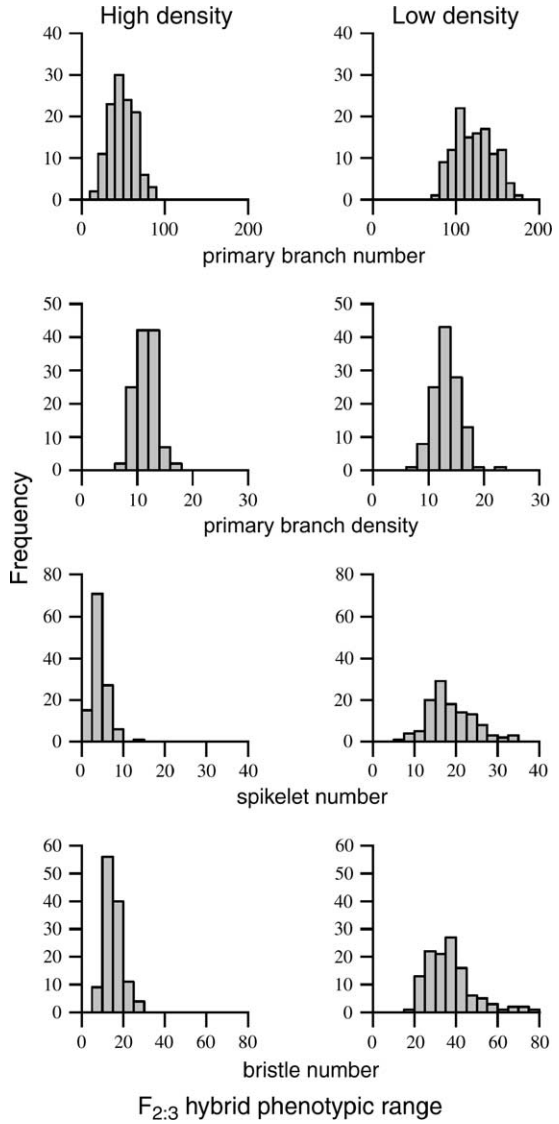

FIGURE 4.—Histograms showing distribution of $F_{2:3}$ means (untransformed) for the four phenotypic traits.

were also significant at the more stringent genome level of $P < 0.05$. The 71 QTL found at the chromosome significance level of $P < 0.05$ were compared between high- and low-density trials to identify those that were overlapping and could represent the same gene or genes. We found 14 replicated QTL, 3 each for primary branch number and primary branch density, 6 for spikelet number, and 2 for bristle number (Table 1). Because we were looking for QTL that accounted for differences between the two species, rather than differences between environments, we focused on these replicated QTL and did not consider further the QTL found in only one trial. The QTL that were replicated in position and direction of additive effect at high and low densities can be interpreted as identifying genomic regions responsible for differences between the parents independent of environment. Some of the QTL for different

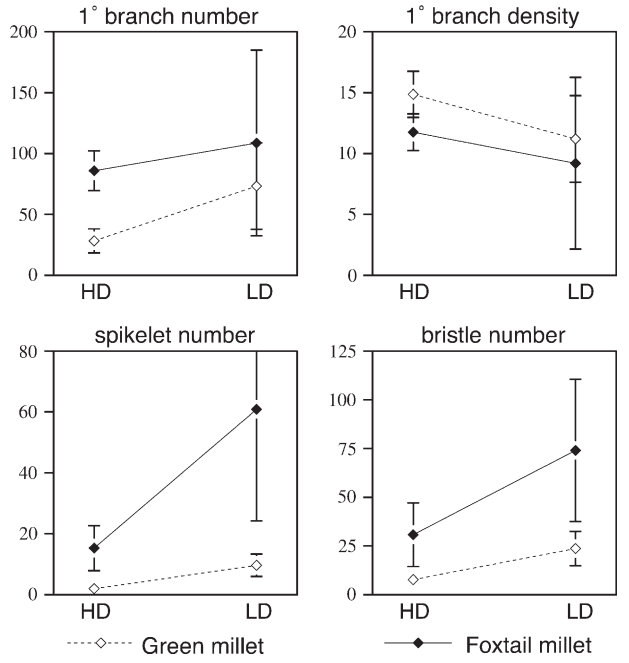

FIGURE 5.—Differences in phenotypic means (untransformed) for green and foxtail millet parents at high and low planting density.

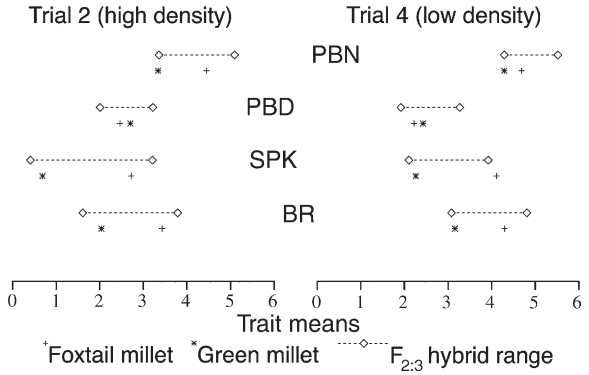

FIGURE 6.—Transgressive segregation in trials 2 (high density) and 4 (low density) as evidenced by the greater range of hybrid population means *vs.* the parental means (data natural log transformed). PBN, primary branch number; PBD, primary branch density; SPK, spikelet number per primary branch; BR, bristle number per primary branch.

traits overlap, suggesting that individual QTL may have effects on more than one trait (Table 1; Figure 7).

Joint analyses for each trait across the four individual trials found six significant QTL at the genome significance level of $P < 0.05$ (Table 2). Four of these had significant main effects but nonsignificant $G \times E$ interactions and were in the same position as the replicated QTL for high- and low-density trials. The other two joint QTL had significant $G \times E$ interactions and did not correspond in position to any replicated or nonreplicated QTL from the high- and low-density trials or to QTL identified in either of the individual high-density trials (trials 1 and 2). However, one of the joint QTL with a significant $G \times E$ interaction, that for primary branch number on chromosome IV, is in the same position as QTL in both individual low-density trials (trials 3 and 4). The other joint QTL, that for bristle number near the centromere on chromosome V, is in the same position as a single QTL in one of the individual low-density trials (trial 3).

Additive and dominance effects: The additive effects of QTL for primary branch density and bristle number were all of the same sign (Table 1), corresponding to lower density of branches and higher numbers of bristles in foxtail millet relative to green millet. Primary branch number and spikelet number were also higher in foxtail millet relative to green millet, but the QTL had a mixture of both positive and negative additive effects (Table 1). QTL with additive effects of differing sign for a particular trait indicate that each parent contains a mixture of alleles for that trait, some acting to change the phenotype toward that of one of the parents while others act to change the phenotype toward the other parent.

The hybrid range was compared to double the sum of the absolute values of the additive effects ($\Sigma|A|$) (the sum of the absolute values of the additive effects is doubled because the range spans both sides of the overall mean). $\Sigma|A|$ explains only between 38 and 81% of the hybrid range in the high-density trial and between 50 and 88% in the low-density trial (Table 1). This suggests that, as in trials 2 and 4, significant transgressive segregation occurs in the high- and low-density trials.

Dominance effects were generally much less than additive effects, and the differences observed are in several cases below the power of this experimental design to unambiguously detect (LANDER and BOTSTEIN 1989). Larger dominance effects were detected for several of the spikelet QTL at both planting densities. The sign of dominance effects was not always consistent between high- and low-density trials.

Epistasis: An analysis of digenic epistasis revealed 17 significant epistatic interactions between markers, occurring exclusively in the high-density trials (Table 3). In 9 cases one of the markers in the interaction was associated with a QTL; in 2 of these the QTL were found in both the high- and low-density trials, and in the other 7 cases the QTL were found in only one or the other of the density trials. In the remaining 7 cases neither marker in the interaction was associated with a detected QTL. In no case was there a significant interaction where both markers were associated with QTL. It is noteworthy that the interactions were identified in only the high-density trial, where additive effects explained less phenotypic variation than in the low-density trial.

Candidate genes: We confirmed colinearity between the millet and maize genomes, predicted from rice and

TABLE 1

Replicated QTL detected at the chromosome-wide level of $P < 0.05$ in both high- and low-density trials

Trait	High-density chromosome position	A	D	R^2 (%)	Low-density chromosome position	A	D	R^2 (%)
PBN	III-8	+0.11	−0.04	6.3	III-6	+0.08	+0.01	7.6
	VII-5	−0.14	+0.02	8.1	VII-6	−0.06	+0.06	5.7
	IX-6	+0.12	+0.02	5.0	IX-5*	+0.11	−0.04	14.9
	PBN % $F_{2:3}$ range explained = 48.1%				% $F_{2:3}$ range explained = 60.2%			
PBD	IV-5	−0.07	−0.02	8.2	IV-5*	−0.11	+0.01	15.3
	VII-7	−0.07	+0.07	10.5	VII-7	−0.07	−0.02	6.7
	IX-16	−0.04	+0.04	4.8	IX-16*	−0.14	−0.02	23.1
	PBD % $F_{2:3}$ range explained = 47.4%				% $F_{2:3}$ range explained = 50.0%			
SPK	III-8	+0.11	−0.13	7.0	III-8	+0.08	−0.01	3.0
	III-11	+0.17	+0.10	6.4	III-11*	+0.16	+0.06	14.3
	V-14	+0.12	+0.02	4.1	V-14	+0.07	−0.08	5.1
	VIII-2	−0.10	−0.13	6.5	VIII-4	−0.05	+0.09	3.2
	IX-4	+0.11	+0.14	5.1	IX-4	+0.07	+0.01	2.5
	IX-14*	+0.26	+0.25	23.5	IX-16*	+0.25	+0.07	31.3
	SPK % $F_{2:3}$ range explained = 80.9%				% $F_{2:3}$ range explained = 88.3%			
BR	III-8*	+0.17	−0.03	19.1	III-8*	+0.17	−0.01	19.1
	V-14	+0.09	+0.03	6.3	V-14*	+0.20	−0.08	28.4
	BR % $F_{2:3}$ range explained = 37.7%				% $F_{2:3}$ range explained = 52.5%			

An asterisk next to a QTL position denotes that it was significant at the more stringent genome level of $P < 0.05$. Positive additive effects denote an increase in the value of the phenotypic trait; negative effects denote a decrease. PBN, primary branch number; PBD, primary branch density; SPK, spikelet number per primary branch; BR, bristle number per primary branch; chromosome position, chromosome and marker position closest to a QTL LOD score peak; A, additive effect; D, dominant effect; R^2, percentage of the phenotypic variation explained; % $F_{2:3}$ range explained, percentage of the $F_{2:3}$ hybrid range that is explained by $\Sigma\ |A| \times 2$ (see MATERIALS AND METHODS).

other cereal alignments, by placing 23 new RFLP markers and four genes from maize on the millet genome map. All markers fell into regions that would be predicted on the basis of synteny with other markers. In particular, we verified that millet chromosomes V and IX, which contain many of the QTL affecting inflorescence architecture, are indeed colinear with regions on maize chromosomes III and I, respectively, that also contain QTL for inflorescence architecture (DOEBLEY and STEC 1991; LUKENS and DOEBLEY 1999). These genomic regions have been labeled "domestication regions" (DOEBLEY and STEC 1991).

We hybridized clones of two potential candidate genes for inflorescence architecture that were polymorphic between the two parents. A clone of *bd1* was placed at the base of linkage group II, between markers *rgr1789* and *psf360*, and a clone of *ba1* was placed on the long arm of linkage group V, between markers *psm768* and *rgc385*, both as predicted by synteny with the maize and rice genomes (Figure 7). The clone of *luminidependens* (closely linked to *ts4* in maize) did not identify polymorphisms between the parents and so could not be mapped. The same was true for *zfl1*, but a closely linked marker identified through MAIZEGDB, *umc44*, was mapped to the long arm of linkage group VII. The position of this marker in rice was checked via Gramene, where it was found to be closely linked to the rice ortholog of *zfl1*.

The position of a number of other candidate genes for inflorescence variation was inferred using synteny between the millet, maize, and rice genomes. Of these, good matches between QTL and potential gene position were obtained for *barren inflorescence2* (*bif2*) and *ts4* (Figure 5).

We also examined regions of the sequenced rice genome corresponding to the QTL regions on foxtail millet, using common markers mapped on both the rice and the foxtail millet genomes to delimit appropriate rice genomic regions. We identified several hormone biosynthesis pathway genes and many transcription factors in the putatively colinear regions on rice. The rice genome was also used to map the position of candidate genes from maize more precisely onto the millet genome.

DISCUSSION

Phenotypic traits: The mean values for primary branch number, spikelet number, and bristle number were higher for the foxtail millet parent than for the green millet parent, while those for primary branch density were lower. However, at low densities, primary

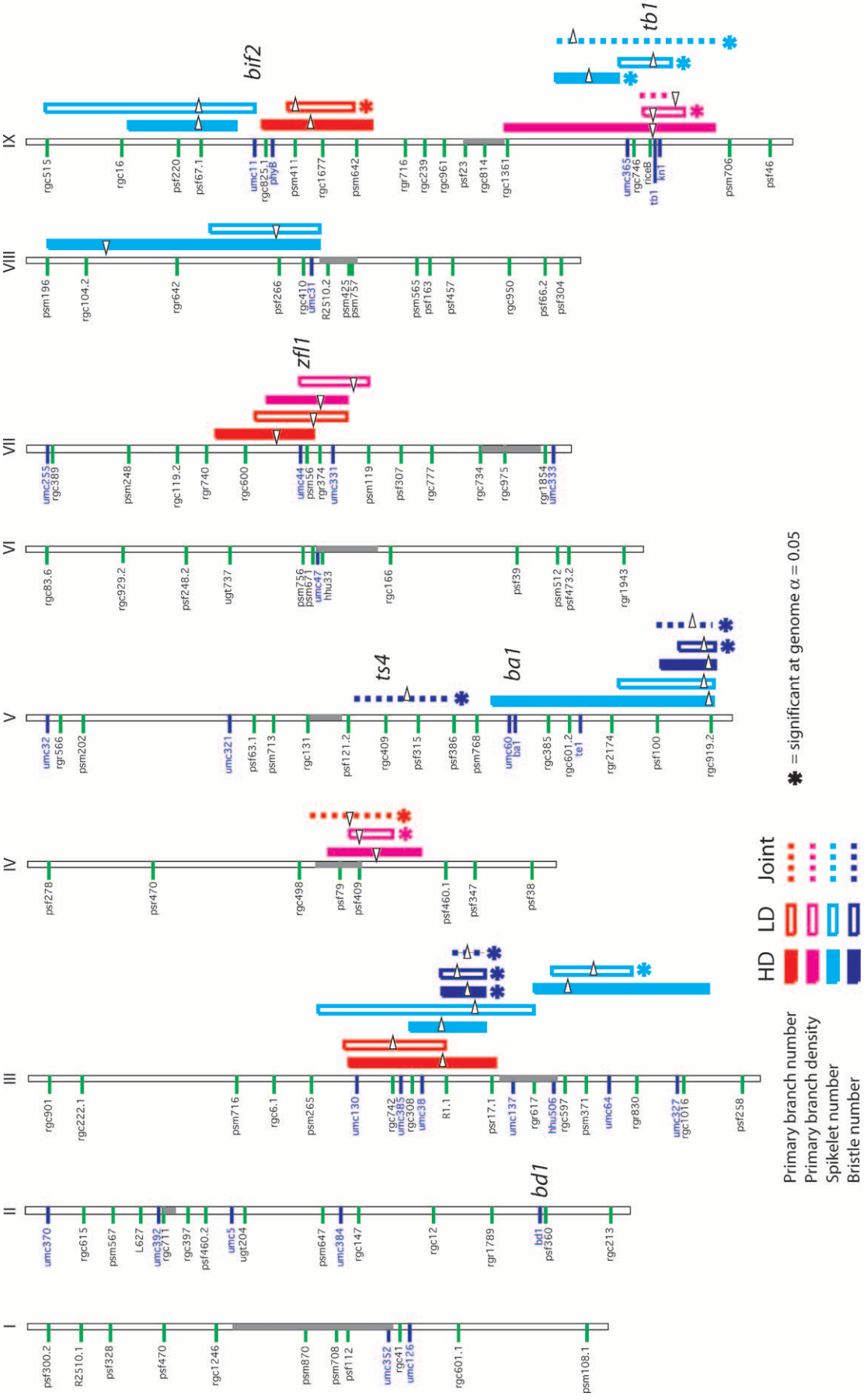

FIGURE 7.—Millet genome map, showing all replicated high- and low-density QTL at the chromosome significance level of $P < 0.05$. Replicated QTL that are also significant at the genome level of $P < 0.05$ are indicated by an asterisk. High-density trials are indicated by solid bars and low-density trials by open bars. Length of the bar denotes the 1-LOD confidence interval. Joint QTL, estimated from the individual trials and significant at the genome level $P < 0.05$, are indicated by dashed lines. Arrows within the confidence interval indicate the position of the highest LOD score; direction of arrow indicates direction of effect, with left indicating a decrease in the phenotypic trait value and right an increase. Markers that were added to enable comparative mapping with the maize genome are indicated in blue; original markers used in the QTL analysis are in black. Approximate gene positions are indicated by *bd1, branched silkless1; ts4, tasselseed4; ba1, barren stalk1; zfl1, zea leafy1; bif2, barren inflorescence2; tb1, teosinte branched 1.*

TABLE 2

QTL detected at the genome level of $P < 0.05$ by joint analysis of the four individual trials

Trait	Chromosome position	$G \times E$	A	D
PBN	IV-4	*	−0.03	−0.01
PBD	IX-16	NS	−0.06	0.02
SPK	IX-14	NS	0.21	0.10
BR	III-8	NS	0.10	0.01
BR	V-6	*	0.04	0.01
BR	V-14	NS	0.12	−0.01

* Significant at the genome level of $P < 0.05$; NS, not significant; $G \times E$, genotype by environment interactions; other abbreviations are as for Table 1.

branch number and density were not significantly different between the two parents. Primary branch density was negatively correlated with spikelet and bristle number, and observation of inflorescence development suggested that greater number of orders of branching in each primary branch, with associated increases in spikelet and bristle number, necessitates more space for each individual primary branch. This would lead to a decrease in the density of branches along the inflorescence axis in foxtail compared to green millet. This suggests an interaction between inflorescence axis elongation and orders of branching within each primary branch, but further study is needed to establish whether the basis of this interaction is genetic or the result of physical stresses experienced during growth.

There was a significant effect due to planting density for all traits, with larger mean trait values at low density. Together with the QTL results, this suggests that, as expected, variation between foxtail and green millet is a combination of fixed species differences and variable environmental responses.

Declared QTL: This study identifies 14 QTL that control the major portion of variation in the four measured phenotypic traits. In several cases, replicated QTL for separate traits overlap, raising the possibility that they actually indicate a single locus with pleiotropic effects. Taking all instances where this could be the case reduces the set of QTL differentiating the species to ~11. Thus, it is possible that only a small number of QTL may underlie the differences in inflorescence architecture between foxtail and green millet.

The number of QTL controlling differences between the species is expected to be an underestimate of the number of genomic regions responsible for the control of all variation in inflorescence traits. This is because only QTL that were replicated between high- and low-density trials were considered, thus excluding the 43 QTL that were unique to one or the other trial. These unique QTL were excluded because they indicate gene effects that occur only in certain environmental situations, but their presence suggests that genotype by environment interactions are important in determining the absolute values of each trait. The results from the joint analysis of individual trials support this conclusion as two of the six joint QTL detected had significant $G \times E$ interactions and were in positions that differed from the replicated high- and low-density QTL.

Spikelet and bristle number show a mixture of overlapping and separate QTL. Some overlapping QTL were expected because both spikelet and bristle number reflect the number of orders of branching within each primary branch, and developmental analyses indicate that spikelets and bristles initiate in approximately equal numbers. However, a proportion of the spikelets fail to develop to maturity, with green millet having more undeveloped spikelets than foxtail millet. Cessation of spikelet growth occurs later in development than initiation of branching and differentiation of primordia into spikelets or bristles. This suggests that QTL for spikelet number that overlap with QTL for bristle number represent regions for genetic control of branching within each primary branch, whereas nonoverlapping QTL for spikelet number may control spikelet development or suppression.

It is possible that not all QTL affecting these traits have been detected, due to the relatively small population of F_2 plants on which the study is based (Beavis 1998). The effect of the small population size may also be responsible for the relatively broad 1-LOD intervals for several of the detected QTL. The small population size potentially decreases the power to detect small but significant changes in dominant effects (Lander and Botstein 1989). However, the presence of such effects in replicated trials may support our inference of small but real dominance effects for these QTL.

Additive effects: QTL for primary branch, spikelet, and bristle number in the high- and low-density trials had total additive effects consistent with higher values in foxtail millet, whereas primary branch density had additive effects consistent with lower values in foxtail relative to green millet. We did not expect that the declared additive effects should explain all of the observed variation in each of the trials, as QTL that occur in only a single trial were excluded. However, the large dominance effects observed for some of the identified QTL, particularly in the high-density trials, suggest that additive effects are unlikely to explain all of the variation observed.

Transgressive segregation was observed for all traits in the trials in which the parents were grown. Transgressive segregation is commonly found in QTL studies and has been interpreted as the result of combination of favorable alleles in the hybrids from both of the parents (Rieseberg *et al.* 2003b). Transgressive segregation was also indicated in the high- and low-density trials, and in many cases the hybrid range exceeded that obtainable by combination of favorable QTL alleles. The increase

TABLE 3

Digenic interactions calculated using Epistacy (Holland 1998)

Trait	Locus1	QTL	Locus2	QTL	R^2 (%)
PBNHD	PSF266 (VIII)	—	RGC746 (IX)	Y	26
PBNHD	RGC410 (VIII)	—	RGC746 (IX)	Y	17
PBNHD	RGR1789 (II)	—	RGR740 (VII)	Y	21
PBDHD	PSF46 (IX)	—	UGT737 (VI)	—	22
PBDHD	PSM425 (VIII)	—	RGC919.2 (V)	—	18
PBDHD	PSM757 (VIII)	—	RGC919.2 (V)	—	18
PBDHD	RGC119.2 (VII)	Y	RGC389 (VII)	—	18
PBDHD	RGC166 (VI)	—	RGR1943 (VI)	—	28
SPKHD	HHU33 (VI)	—	PSF121.2 (V)	—	16
SPKHD	PSF121.2 (V)	—	PSM671 (VI)	—	18
SPKHD	PSF278 (IV)	—	PSM108.1 (I)	—	17
SPKHD	PSF386 (V)	—	PSM706 (IX)	Y	22
SPKHD	PSM706 (IX)	Y	PSM768 (V)	—	20
SPKHD	PSM708 (I)	—	RGC597.1 (III)	Y R	22
SPKHD	PSM756 (VI)	—	RGC104.2 (VIII)	Y R	23
BRHD	PSF473.2 (VI)	Y	PSM56 (VII)	—	22
BRHD	PSM202 (V)	—	RGC41 (I)	—	29

Results are reported only if the overall probability of interaction is $P < 10^{-5}$. Locus1 and Locus2 are the two markers for which an interaction is being tested; the chromosome on which each locus is found is in parentheses following the locus name. QTL recognizes those loci that are associated with a QTL for that trait and trial combination; Y denotes presence of QTL; R denotes presence of replicated QTL. Trait abbreviations are as for Table 1; the suffix HD denotes the high-density trial. R^2, percentage of variation explained by the digenic interaction.

in hybrid extreme values may be due to the exclusion of QTL that were not replicated between the high- and low-density trials or to the epistatic and dominance effects that were detected.

Epistasis: All of the significant digenic epistatic effects between markers occurred at high density, where additive effects explained less of the phenotypic variation than at low planting density. It was striking that in no case were both markers associated with QTL and that in just under half of the cases neither marker was associated with a QTL. This suggests that epistatic control can be exerted by genes that do not by themselves have a detectable phenotypic effect, indicating that a more complex model of interacting gene networks must exist to mediate these responses (Lark *et al.* 1995).

Inflorescence *vs.* vegetative QTL: In a previous article we discussed the position and possible candidate genes underlying QTL for vegetative branching in the same $F_{2:3}$ cross (Doust *et al.* 2004). That article discussed QTL found in the two low-density trials and used a chromosome-wide significance level for declaring QTL of $P < 0.01$ (a more stringent cutoff criterion than the $P < 0.05$ level used in this article). Three QTL appear to be in similar positions in both the vegetative and the inflorescence analysis. Two QTL for vegetative branching are in positions similar to replicated QTL for inflorescence traits; one for tillering at the base of chromosome III matches the broad position of a replicated QTL for spikelet number, and one for axillary branching at the base of chromosome IX matches inflorescence QTL for primary branch density and spikelet number. Another QTL for axillary branching near the centromere on chromosome V is in the same general position as a QTL identified from the joint analysis. It is possible that these overlapping QTL represent the same genes, and that therefore the same mechanisms govern vegetative and inflorescence branching, but a rigorous test of this hypothesis awaits fine mapping studies.

Candidate genes: For genes to be considered candidate genes in this study they had to have a mutant phenotype in a model organism that was similar to some aspect of the phenotypic differences between foxtail and green millet, and they had to be localized to a region of the genome (QTL) implicated in control of that phenotype. An example of such a gene is *zea floricaula leafy1* (*zfl1*), a gene that is duplicated in maize and affects variation in long branch number in the tassel inflorescence (Bomblies *et al.* 2003). In addition, *zfl1* has been linked to a QTL in maize that affects inflorescence phyllotaxy and may act to increase the number of primary branches that are initiated, presumably by increasing the size of the apical meristem (Bomblies *et al.* 2003). It also affects the transition to flowering and, in rice, may affect panicle branching (Kyozuka *et al.* 1998). It was not possible to map *zfl1* directly, because of the lack of polymorphisms between the parents. However, in maize and rice it is closely linked to the RFLP probe *umc44*, which was hybridized to the mapping filters and localized to foxtail millet chromosome VII. This places the gene in close proximity to QTL for primary

branch number and density on millet chromosome VII. This gene appears to be a good candidate for control of primary branch number, as it confers a similar phenotypic effect in maize and in foxtail millet and apparently maps to two of the replicated QTL detected in foxtail millet .

Tasselseed4 is another candidate gene for which the mutant phenotype of increased inflorescence branching (Irish 1997) is similar to phenotypic variation in foxtail and green millet and where the inferred position of the gene matches an identified QTL. It was not possible to map *ts4* to the millet genome directly because the gene has not yet been cloned, and a closely linked marker, *luminidependens*, was monomorphic between the parents of the cross. However, on the basis of comparative mapping, using the common markers *umc60* and *umc321*, *ts4* is near a joint QTL for bristle number near the centromere on chromosome V. In this case, the joint QTL was not matched by replicated high- and low-density QTL, presumably because of a significant genotype by environment interaction. *Ts4* affects determinacy of the spikelet pair meristem in maize, leading to proliferation of branches on which spikelet production is greatly delayed. We interpret the spikelet pair meristem as simply another order of branching and thus regard the *ts4* mutant as having increased numbers of orders of branching compared to wild type. Bristle number in foxtail millet is a measure of the number of orders of branching; thus the QTL effect and the phenotype of *ts4* match. More bristles (and thus more orders of branching) are in foxtail than in green millet.

Teosinte branched1 occurs in QTL for several traits in the comparison between maize and its wild relative, teosinte (Doebley and Stec 1991; Doebley *et al.* 1995; Lukens and Doebley 1999), and its promoter region has been shown to have been under selection (Wang *et al.* 1999; Clark *et al.* 2004). It is thus a good candidate gene for involvement in domestication of foxtail millet. *Tb1* was found to be a relatively minor player in the control of vegetative branching in foxtail millet (Doust *et al.* 2004), but on the basis of map position remains a candidate for control of inflorescence branching. However, the mutant phenotype of *tb1* in maize bears little resemblance to anything in foxtail or green millet. In maize, *tb1* affects sex expression in the male inflorescence (tassel), but is not involved in inflorescence branching or elongation or in spikelet differentiation or suppression (Hubbard *et al.* 2002). The two species of millet investigated here are, however, identical in sex expression, but differ in aspects of branching. Thus the position of *tb1* suggests it could underlie the QTL, but its mutant phenotype in maize does not support this hypothesis.

Other possible candidate genes from maize that may be involved in inflorescence variation include *branched silkless1* (*bd1*) and *barren stalk1* (*ba1*). *Bd1* and *ba1* were mapped directly onto the millet genome; the mutant phenotype of *bd1* in maize causes indeterminate branches to form instead of spikelets (Chuck *et al.* 2002), while that of *ba1* causes a reduction or elimination of branches and spikelets (Neuffer *et al.* 1997). Both genes thus affect branching and therefore are candidates for variation in branching in the millets. However, neither gene maps to one of the replicated or joint QTL. *Bd1* maps to a position on millet chromosome II that does not have QTL for any of the measured traits, while *ba1* maps to a position on chromosome V that is between the 1-LOD intervals for spikelet and bristle number. The lack of close correspondence between *bd1* or *ba1* and the replicated QTL suggests that, although these genes have mutant phenotypes that mimic the observed phenotypic variation, these genes are not likely candidates for differences in inflorescence branching between the parents.

Several replicated QTL are in regions where no obvious candidate gene from maize is apparent. These include QTL on chromosomes III and IV and the bottom of V. The failure to find candidate genes may be due to the inadequacy of the syntenic comparisons between millet and maize in those regions or may be due to the presence of genes underlying these QTL that are not characterized in maize floral architecture or are not present in the orthologous regions of the maize genome.

To attempt to identify other possible genes we examined sections of the rice genome that were presumed orthologous to regions of the millet genome covered by QTL. Many transcription factors were identified, but, as the phenotypic effect of these genes is unknown, they cannot as yet provide good candidate genes for this study. Rice genomic regions colinear to QTL regions on chromosomes V, VI, and VII also include a variety of auxin and gibberellin pathway mediators, including *semidwarf1*, the so-called "green revolution" gene in rice (Monna *et al.* 2002). Genes in the rice region colinear to the QTL region on millet chromosome IX include a cytochrome P450 gene with high similarity to the lateral meristem proliferating gene *SUPERSHOOT1*, from Arabidopsis, and genes with high similarity to the Arabidopsis *AXR1* gene, all of which are involved in auxin regulation (Stirnberg *et al.* 1999; Tantikanjana *et al.* 2001). This approach offers considerable promise in identifying novel candidate genes but would be significantly improved by better annotation of the rice genome. As this happens, we expect further candidate genes from rice and maize to be identified.

The changes in inflorescence morphology seen between green and foxtail millet are the product of domestication, and it is likely that the selective pressure was for increase in seed production (Harlan 1992). Although such selection must have been the focus of all domestications of cereal grains, the phenotypic traits affected and the genes thus acted upon are likely to differ in the

different domestication events. For example, comparisons of foxtail millet with the closely related crop pearl millet (*Pennisetum glaucum*; Doust and Kellogg 2002) show that seed production per primary branch in pearl millet increases only marginally over the presumed wild progenitor (*P. mollissimum*), but that the inflorescences are much longer, with a concomitant increase in primary branch number (Harlan 1992; Poncet *et al.* 1998, 2000). This underscores the importance of careful selection of genes as potential candidates for phenotypic variation and highlights the role that developmental and QTL analysis can have in selecting candidate genes for further analysis.

Conclusions: This study represents an important first step in understanding the control of inflorescence architecture diversification in millets, and the results are of value because they concentrate on repeatable QTL across high- and low-density trials. Our data show that the inflorescence characteristics that differentiate two millet species are each controlled by a small number of loci. Previous developmental analyses have shown that the characters distinguishing foxtail and green millet are similar to those distinguishing other species of Setaria, Pennisetum, and Cenchrus (Doust and Kellogg 2002). Among these species, much of the phenotypic variation involves inflorescence architecture, including the numbers of primary branches, density of primary branches, numbers of orders of branching, and numbers of spikelets that fail to develop. We hypothesize that the loci identified here are the same ones that natural selection has affected during the evolution of the 300+ species of the Setaria-Pennisetum clade. This can be tested by further investigations in mapping populations of closely related species such as pearl millet, as well as by studying the evolution and expression of those candidate genes suggested by QTL analysis and comparative mapping among wild species that exhibit variation in inflorescence morphology.

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