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Heterogeneous inbred family (HIF) analysis: a method for developing near-isogenic lines that differ at quantitative trait loci

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Abstract Analysis of near-isogenic lines (NILs) that differ at quantitative trait loci (QTL) can be an effective approach for the detailed mapping and characterization of individual loci. Although NILs are useful for genetic and physiological studies, the time and effort required to develop these lines have limited their use. Here we describe a procedure to identify NILs for any region of the genome that can be analyzed with molecular or other genetic markers. The procedure utilizes molecular markers to identify heterogeneous inbred families (HIFs) that segregate for a genomic region of interest. Each HIF is isogenic at the majority of loci in the genome, but NILs differing for markers linked to QTL of interest can be extracted from segregating families. The application of this procedure is described for two QTL associated with seed weight in sorghum. A population of 98 HIFs was screened with two RAPD markers from different linkage groups that were associated with seed weight. Three segregating families were identified for each marker. The progeny of these HIFs were characterized for the segregation of seed weight and other yield components and for markers flanking each QTL. NILs derived from each HIF had significantly different seed weights confirming the presence of at least two loci that influence seed weight in sorghum.

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Introduction

Quantitative genetics has enjoyed a renaissance in the past decade (Tanksley 1993). The ability to map and manipulate genetic loci that condition the expression of a quantitative trait has blurred the distinction between the fields of qualitative and quantitative genetics. Analysis of quantitative traits using molecular markers has great potential both for improving the efficiency of plant breeding and for identifying and characterizing the physiological and biochemical mechanisms associated with complex biological processes (Dorweiler et al. 1993, Paterson et al. 1990).

The mapping of quantitative trait loci (QTL) in segregating populations has limited resolution (Paterson et al. 1988). Loci associated with the expression of a quantitative trait can be mapped with a precision of about 10–20 cM, but additional experiments are required to obtain more precise map information. One method to resolve the map position of a QTL is by analyzing a series of near-isogenic lines (NILs) that differ for markers flanking the QTL (Paterson et al. 1990; Kaeppler et al. 1993). This approach can identify a small region of the genome associated consistently with a quantitative trait, thereby defining more precisely the map position for that QTL.

NILs differing for QTL have also been used to study the phenotype associated with a specific locus (Dorweiler et al. 1993). Discerning the specific phenotype of a QTL in a segregating population can be difficult, but this can be overcome by examining the expression of a locus in NILs. By offering a fixed genetic background

for direct comparisons, NILs can be used to determine the phenotype associated with a specific locus.

Although NILs are useful in the evaluation of QTL, this area of research has been limited by the cost, time, and effort associated with developing the appropriate genetic materials. Backcross introgression has been the most commonly used method for developing NILs for QTL studies (Dorweiler et al. 1993; Muehlbauer et al. 1991; Paterson et al. 1990; Young et al. 1988). The backcross procedure is used to introgress a small region of a donor genome containing a specific allele for a QTL into a recipient genome with a different allele. Although conceptually straightforward, several generations of marker-assisted selection are required. When many different loci are being investigated, the cost and labor associated with this procedure can become prohibitive. This is especially true for self-pollinated crops where the development of large backcross populations can be labor intensive. The evaluation of QTL in a single genetic background can also be complicated by problems with penetrance or epistasis. Expression of a QTL may be masked by lack of penetrance, or confounded by epistatic interactions in certain genetic backgrounds.

An alternative procedure for developing NILs utilizes a selfing and selection scheme (Allard 1960; Fehr 1987; Haley et al. 1994). With this approach, NILs are selected from an inbred line that is not entirely homozygous. Progeny of this line will segregate for those loci not yet fixed and will represent a heterogeneous inbred family (HIF) of nearly-isogenic individuals. NILs differing at loci with large effects have been selected from HIFs based on the segregation of qualitative phenotypes (Haley et al. 1994). This type of phenotypic discrimination between NILs with different alleles of a QTL is not normally possible. As discussed in this paper, NILs that differ for specific QTL can be identified in HIFs by screening for differences in genotype at specific marker loci. We describe a procedure in which molecular markers were used to evaluate a population of HIFs to identify NILs differing at two QTL associated with seed weight. Three HIFs segregating for each marker locus were identified, and the genetic and phenotypic characterization of NILs selected from these HIFs is described. We believe this procedure will be a useful approach for developing NILs that contrast at specific QTL, particularly in self-pollinated crops.

Materials and methods

Identification of QTL associated with seed weight in sorghum

An $F_{5:7}$ sorghum mapping population was genotyped using 170 random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) markers as previously described (Tuinstra et al. 1996). In summary, DNA was isolated from several plants in each line using a slightly modified CTAB (Hexadecyltrimethylammonium bromide) DNA isolation method (Bernatzky and Tanksley 1986). RAPD and RFLP markers were scored for segregation according to standard procedures (Williams et al. 1992; Saghai-Maroof et al. 1984). RFLP loci were named for maize clones used as probes on Southern blots. RAPD marker names indicated the parental line in which the marker was amplified (t = TX7078 and b = B35), the RAPD primer name used to amplify the marker (Operon Technologies, Alameda, Calif. or UBC RAPD Primer Synthesis Project, University of British Columbia, Vancouver, B.C., Canada), and the size of the polymorphic band ($\times 10$ base pairs). Thus, *t329/132* indicates a RAPD marker amplified from TX7078 with UBC primer 327 giving a product of 1320 bp, and *bB20/205* is a 2050-bp RAPD product amplified from B35 with Operon primer B20. These loci were ordered into a linkage map using a Macintosh version of the MAPMAKER program (Lander et al. 1987). Genetic maps were developed at a LOD significance threshold of 6.0, and genetic distances were estimated based on the recombination frequencies between markers. This genotypic information was used to identify QTL for seed weight. Seed weights were measured as described below, and QTL associated with seed weight were identified by single factor analysis using Proc GLM procedure in Statistical Analysis Systems (SAS Institute 1988). QTL were declared significant on a per marker basis at $P \leq 0.01$.

Developing a population of heterogeneous inbred families (HIFs)

A population of HIFs was derived from the $F_{5:8}$ sorghum mapping population. The population was developed from a cross between two inbred sorghum lines, TX7078 and B35. Single-seed descent was used to advance 98 F_2 individuals to the F_5 generation. Heterogeneous lines were developed by bulking the selfed seed in each F_5 line. A head row representing each F_5 plant was grown, 8–10 plants from each line were selfed, and the seed was bulked to advance to the next generation. This process of selfing and bulking seed from several plants within each line was repeated until the F_8 generation. A progeny sample of 8 individuals was selected from each $F_{5:8}$ line so that the population consisted of 784 (98×8) progeny lines. The seed from each line was maintained separately, and the 8 progeny from each line represented a heterogeneous inbred family of near-isogenic lines.

Identification and characterization of HIFs segregating for QTL associated with seed weight

HIFs segregating for markers tightly linked to two QTL for seed weight, *t329/132* and *tH19/50*, were identified by testing for marker segregation in the 8 progeny from each HIF. DNA was isolated from each of the 784 progeny lines as described for the mapping population. Three HIFs segregating for each RAPD marker were identified in the population.

A set of NILs was selected from each of the segregating HIFs. Each set consisted of 4 lines with each parental allele represented by 2 individuals. These lines were characterized for 13 other RAPD markers from across the genome to determine the average heterogeneity of each HIF. The segregation of other markers flanking the target QTL was evaluated to estimate the size of the heterogeneous region differentiating the lines in each HIF.

Phenotypic characterization

Field studies to characterize differences in seed weight in the mapping population were conducted near San Juan de Abajo, Nayarit, Mexico, during the spring of 1992 and 1994. The population was grown in 14- foot rows in a randomized complete block with four

replications. A seed sample was taken from each plot, and the seed weight determined by weighing 100 random seeds per plot. The 2-year seed weight average for each line was used to identify QTL for seed weight.

The 8 progeny lines from each HIF were evaluated for differences in seed weight at the Purdue University Agronomy Research Center, near West Lafayette, Ind., during the summer of 1994. The progeny lines were grown in single-row plots, and six heads were selfed in each plot. The 100 seed weight of each selfed head was measured and used to determine the mean seed weight of each progeny line. The association between the segregating marker and seed weight in each HIF was evaluated using the Proc GLM procedure in SAS. Significant differences between lines with contrasting marker alleles were determined using the least significant difference (LSD).

The core set of lines from segregating HIFs were characterized for differences in seed weight, grain yield, and seed number per plant in experiments conducted near San Juan de Abajo, Nayarit, Mexico, during the spring of 1995. Lines were grown in 14-foot rows arranged in a split-split block design. Whole plots represented the genetic loci and subplots represented each segregating HIF. The 4 lines in each HIF were randomized within each subplot. The experiment contained eight replications. Phenotypic differences between NILs with contrasting marker alleles were evaluated using the Proc GLM procedure in SAS. Significant differences were determined using the least significant difference (LSD).

Results and discussion

Identification of QTL for seed weight

Single factor QTL analysis in a sorghum mapping population identified two regions of the genome that were associated with variation in seed weight (Fig. 1; Table 1). On one linkage group, *umc84* was the marker most tightly associated with seed weight, and the allele derived from the B35 parent was associated with higher seed weight. The additive effect of this QTL was estimated to be 0.21 g/100 seed (Table 1). On the other linkage group, *tH19/50* was the marker most tightly linked to the QTL for seed weight. At this locus, the allele derived from TX7078 was associated with higher seed weight. The additive effect of this locus was estimated to be 0.15 g/100 seed (Table 1). Near-isogenic lines differing at these QTL were developed to further characterize these loci.

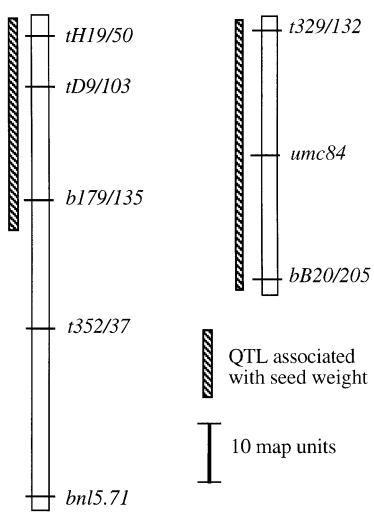

Fig. 1 Linkage maps of RAPD and RFLP markers on two sorghum linkage groups. QTL associated with seed weight were identified by single factor analysis of variance ($P < 0.01$). The genomic regions associated with seed weight are indicated by the *shaded bar* adjacent to each linkage group

Table 1 Two QTL for seed weight identified by the association between RAPD and RFLP markers and seed weight in a recombinant inbred (RI) population. The significance of the association was determined by single factor analysis of variance

Marker	F value	Probability ($P > F$)	Additive effect (g/100 seed)
t329/132	16.00	< 0.0001	0.14
umc84	36.17	< 0.0001	0.21
bB20/205	11.23	0.0011	0.12
tH19/50	20.87	< 0.0001	0.15
tD9/103	15.86	< 0.0001	0.13
b179/135	7.83	0.0062	0.10
t352/37	6.17	0.0147	0.09

Development and characterization of near-isogenic lines

NILs were developed by screening heterogeneous inbred families (HIFs) to identify families that were heterogeneous for the RAPD marker most tightly associated with each QTL (Fig. 2). Progeny of $F_{5:8}$ HIFs were screened with RAPD markers *tH19/50* and *t329/132*. RAPD markers were used in the primary screening of HIFs because the initial genotypic evaluation of the mapping population contained only RAPD markers. *umc84* was mapped to this region after the HIFs were screened for segregation of RAPD markers. HIFs 13, 47, and 48 were shown to be segregating for marker *t329/132* (Fig. 3), and HIFs 36, 66, and 96 were segregating for marker *tH19/50*.

The seed weights of the 8 progeny lines derived from each HIF were measured to determine the association between seed weight and the segregating marker (Table 2). In HIFs segregating for *t329/132*, lines carrying the allele derived from B35 had a significantly higher seed weight than lines with the allele derived from TX7078. The analysis of HIFs segregating for *tH19/50* indicated that lines carrying the allele derived from the TX7078 parental line had a significantly higher seed weight than lines carrying the allele derived from B35. For both loci, the association between the RAPD marker and seed weight was consistent with observations in the original mapping population.

These QTL for seed weight were further characterized by analyzing a set of 4 NILs selected from each

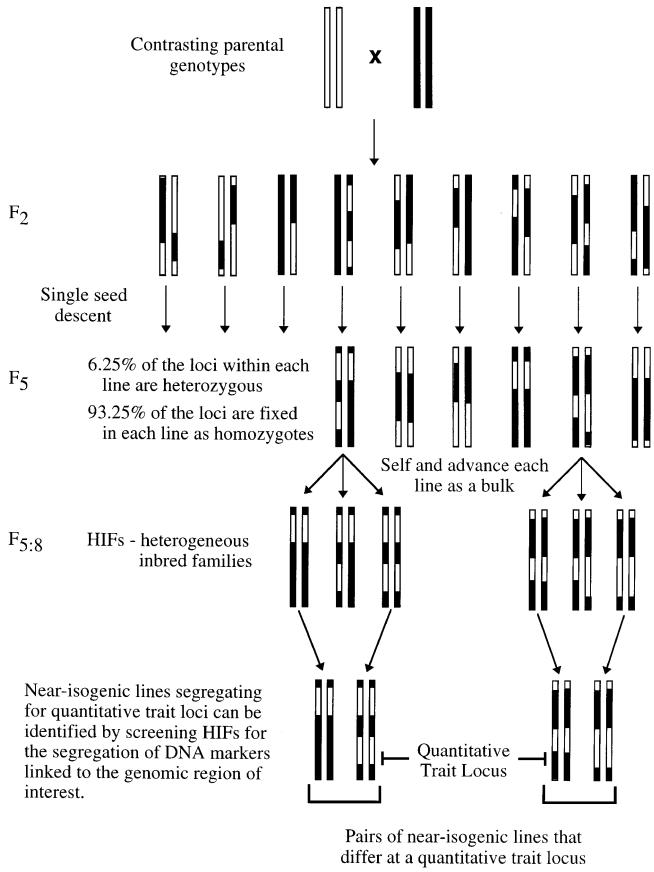

Fig. 2 Production of heterogeneous inbred families from a non-fixed recombinant inbred population. Families of near-isogenic individuals are maintained by advancing lines from the bulked seed of several plants within each recombinant inbred line. Pairs of near-isogenic lines differing at marker loci associated with quantitative trait loci can be selected from heterogeneous families.

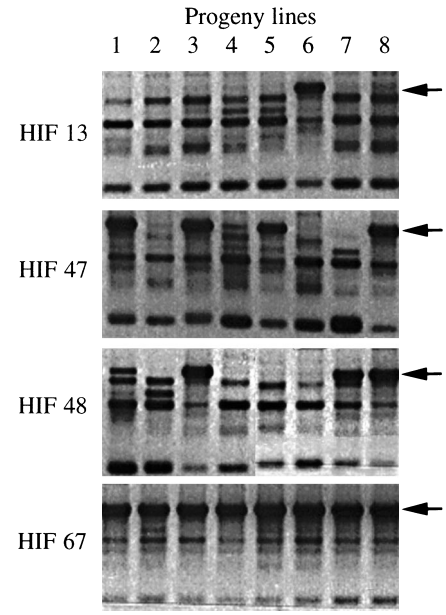

Fig. 3 Screening of progeny lines derived from heterogeneous inbred families using the RAPD marker *t329/132* (indicated by *arrow*). Progeny in HIFs 13, 47, and 48 are segregating for the marker, whereas HIF 67 is an example of a non-segregating family

Table 2 Mean seed weight of lines with contrasting marker genotypes in HIFs segregating for RAPD marker *t329/132* or *tH19/50*. Mean seed weights were averaged across three HIFs segregating for each marker

RAPD marker	Source of marker allele	Seed weight (g/100 seed)
t329/132	TX7078	3.07*
	B35	3.45
tH19/50	TX7078	3.28*
	B35	3.01

* Indicates differences significant at $P < 0.05$

HIF. These lines were characterized for differences at 13 unlinked RAPD marker loci to determine the average percentage of heterogeneity. On average, 6.41% of the markers were heterogeneous in each set of NILs thereby confirming the genetic similarity of these lines. This was consistent with an expected 6.25% heterozygosity in the F_5 progenitor lines.

These lines were characterized for differences in seed weight, grain yield, and seed number per plant. The evaluation of NILs contrasting at *t329/132* indicated significant differences in average seed weight associated with the marker in lines derived from HIFs 13 and 48 (Table 3). A similar pattern of expression was observed in NILs derived from HIF 47, but the difference in seed weight was not significant. Large differences in average grain yield and seed number per panicle were also noted (Table 3). Surprisingly, the pattern of expression for grain yield and seed number was not consistent across all NILs. In NILs derived from HIFs 13 and 47, the B35 allele was associated with higher grain yield and seed number, but in NILs derived from HIF 48 the TX7078 allele was associated with higher yield and seed number. Despite the contrasting expression of grain yield and seed number in different sets of NILs, the expression of seed weight was consistently associated with *t329/132* confirming the effects of a QTL for seed weight near this marker (Table 3).

The analysis of markers flanking *t329/132* indicated considerable heterogeneity within and between sets of NILs (Fig. 4). This heterogeneity provided useful information for fine mapping of QTL. Differences in seed weight of individual lines were compared with recombination events near *t329/132* (Table 3; Fig. 4). Seed weight appeared to co-segregate with *t329/132* in HIF 13, with *umc84* in HIF 47, and with the interval between *t329/132* and *umc84* in HIF 48. These

Table 3 Differences in seed weight, grain yield, and seed number per plant in NILs derived from HIFs segregating for *t329/132*

Source of NILs	Line	Source of marker allele	Seed weight (g/100 seed)	Grain yield (g/plant)	Seed number (no./plant)
			Phenotypic differences between NILs[a]		
HIF 13	1	TX7078	1.93 a	10.99 a b	570 a
	2	TX7078	1.89 a	9.58 b	501 a
	3	B35	2.10 a	11.67 a	552 a
	4	B35	2.09 a	12.19 a	598 a
HIF 47	1	TX7078	1.82 a	9.53 a	526 a
	2	TX7078	2.25 b c	10.19 a	489 a
	3	B35	2.01 a b	12.54 b	661 c
	4	B35	2.45 c	12.87 b	532 a
HIF 48	1	TX7078	2.31 a	10.73 a	464 a
	2	TX7078	2.19 a	9.91 a b	457 a
	3	B35	2.52 b	8.78 b c	349 b
	4	B35	2.36 a b	8.25 c	350 b
			Average marker effect[a]		
HIF 13		TX7078	1.91 a	10.29 a	535 a
		B35	2.09 b	11.93 b	575 a
HIF 47		TX7078	2.03 a	9.86 a	509 a
		B35	2.23 a	12.72 b	588 b
HIF 48		TX7078	2.25 a	10.32 a	461 a
		B35	2.44 b	8.52 b	350 b

[a] Values significantly different at $P < 0.05$ are indicated by different letters

observations indicated the QTL for seed weight lies in the interval between marker *t329/132* and *umc84*.

The genetic and phenotypic analyses of NILs contrasting for *tH19/50* also indicated a significant association between the segregation of the marker and seed weight (Table 4). The association between the marker and average seed weight in NILs derived from HIFs 36 and 66 was consistent with that observed in the original mapping population. In these lines, variation in seed weight appeared to be associated with differences in seed number (Table 4). This QTL may condition variability in seed number, and the association with variability in seed weight could be due to photosynthate repartitioning. The similarity in grain yield in these NILs, despite differences in seed weight and seed number, is consistent with this hypothesis. The phenotypic differences observed in NILs derived from HIF 96 were more difficult to explain. Although these lines differed significantly in seed weight, the pattern of expression was not consistent with observations of seed weight in lines derived from HIFs 36 and 66. There are several possible explanations for this inconsistency. The difference in seed weight in NILs derived from HIF 96 can be attributed primarily to the effects of line 3 (Table 4). Although sets of lines derived from HIFs are nearly isogenic, NILs still segregate for a small portion of the genome, and the higher seed weight of line 3 could be explained by the segregation of a second QTL. Another explanation for these results could be genotype interaction. The QTL might have positive effects in some genetic backgrounds and negative effects in other genetic backgrounds. This explanation is less likely because if genotype interaction were important, linkage between the marker and trait would have been difficult to detect in the original mapping population. Environmental interaction or inadequate sampling could also be important factors contributing to these results. Additional testing will be required to answer these questions. These results highlight the importance of evaluating QTL effects in more than one set of NILs.

Heterogeneous inbred family (HIF) analysis

HIF analysis is a procedure for identifying near-isogenic lines (NILs) that differ for a selected marker or genomic region (Fig. 2). HIFs can be generated from populations of early or advanced generation inbreds. NILs will be more homogeneous if they are derived from populations that are more inbred. However, this advantage must be balanced by the need to screen larger populations to identify segregating families. Segregating families can be identified at a higher frequency in populations derived from early generation inbreds. However, NILs derived from these families will be less isogenic, and phenotypic variability resulting from segregation of other loci could mask the expression of QTL. Decisions for developing HIFs from early or late generation inbreds will depend on the number of families that must be screened to identify NILs for comparison of specific QTL and on the degree of genetic similarity that is needed to evaluate QTL effects. For most studies, NILs identified in HIFs derived from

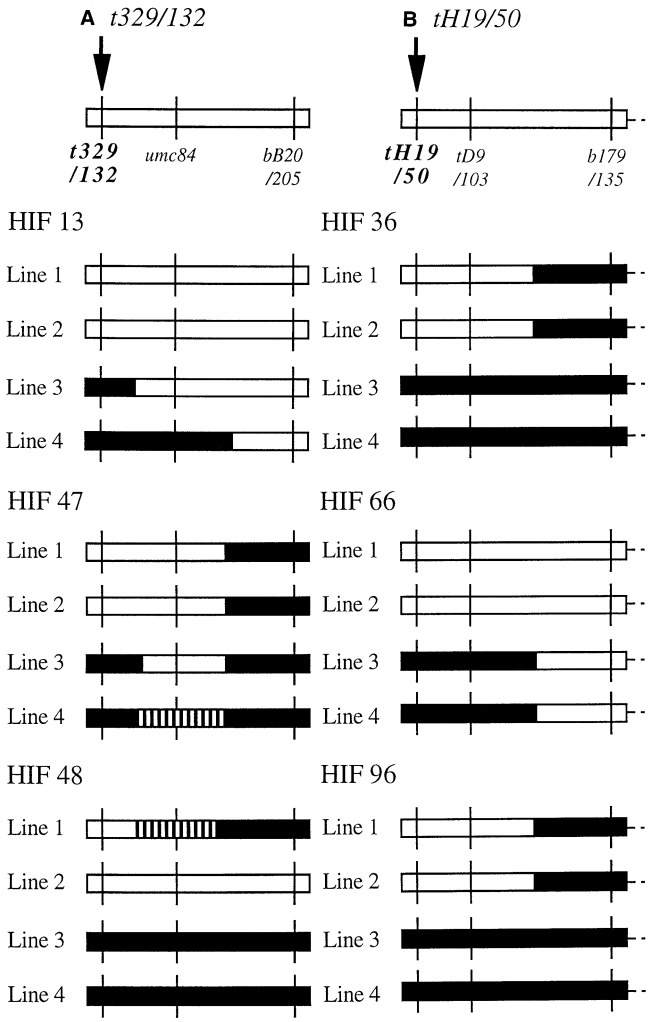

Fig. 4A, B The genotypes of NILs isolated from HIFs segregating for marker *t329/132* (**A**) and *tH19/50* (**B**). The genotypes of markers flanking each QTL are indicated for each line. Marker alleles derived from TX7078 are indicated as *unshaded regions* on the linkage group; alleles derived from B35 are *shaded*; and heterozygous regions are indicated by a *vertical pattern*

F_5 or F_6 generation inbred lines should provide a suitable compromise between the size of population to be screened and the genetic similarity of the resulting lines.

The initial genotypic analysis of the mapping population in this study included only RAPD markers. Progeny testing was required in the screening of HIFs because RAPDs are dominant markers. A more efficient strategy for HIF analysis would involve QTL mapping with co-dominant markers in heterogeneous inbred mapping populations. Once markers for the desired trait were identified by linkage analysis, a survey of the marker genotypes in the mapping population would indicate lines that were heterozygous or heterogeneous for the target genomic regions. NILs contrasting at these genomic regions could be easily identified by evaluation of the selfed progeny of these lines.

HIF analysis has several applications. First, marker linkage to a QTL can be confirmed by examining the phenotypes of NILs derived from segregating families. Linkage between markers and traits in a mapping population followed by confirmation of phenotype in NILs provides strong evidence supporting the position and effect of QTL. The ease with which NILs can be extracted from a large population of HIFs may also allow QTL mapped at low significance thresholds to be confirmed by subsequent examination of NILs. Second, NILs extracted from segregating HIFs are useful for the fine mapping of QTL. Each segregating HIF is independent and contains unique recombination events in genomic regions flanking the QTL. These recombinations can define the genetic interval known to contain the QTL (Paterson et al. 1990). Third, NILs that differ at QTL can be used to characterize the expression and function of a specific locus. NILs can be extracted from different recombinant lines so that the genetic background in which a QTL is best expressed can be used for phenotypic characterization of the locus. This should alleviate problems resulting from epistatic interactions or poor penetrance in the evaluation of QTL.

One of the limitations of HIF analysis for the evaluation of QTL is that the genetic background of NILs derived from HIFs are unique and cannot be easily replicated. NILs are not easily developed for evaluating the effects of more than one QTL in a single genetic background or for comparing the effects of QTL identified in different populations. Another important consideration in the analysis of QTL in NILs is statistical power. In this study, linkage analysis in the recombinant inbred (RI) mapping population indicated highly significant associations between markers and seed weight. Subsequent analysis of these QTL in NILs indicated a difference in phenotype, however the level of statistical significance was low. This can be explained by the difference in statistical power for comparing QTL in NILs versus RI lines. In an RI population, each marker allele is replicated many times in different genetic backgrounds. This internal replication of marker alleles provides the statistical power for testing differences in phenotype associated with a particular marker. Although the analysis of NILs reduces phenotypic variability resulting from the segregation of other loci, each marker allele is represented by only one or a few lines within a given set of NILs. As a result, the statistical power for testing phenotypic differences is reduced. The power for statistical tests can be increased by evaluating several sets of NILs contrasting at a single locus and by increasing the number of lines within each near-isogenic background. Increased replication of NILs can also be used to increase the power in statistical tests. This consideration of statistical power for testing phenotypic differences between NILs may limit the potential for fine mapping of QTL with small effects using this procedure.

Table 4 Differences in seed weight, grain yield, and seed number per plant in NILs derived from HIFs segregating for *tH19/50*

Source of NILs	Line	Source of marker allele	Seed weight (g/100 seed)	Grain yield (g/plant)	Seed number (no./plant)
			Phenotypic Differences between NILs[a]		
HIF 36	1	TX7078	2.74 a	12.73 a	464 a
	2	TX7078	3.25 b	10.40 b	317 b
	3	B35	2.18 c	9.92 b	464 a
	4	B35	2.56 a	12.22 a	474 a
HIF 66	1	TX7078	2.13 a	8.87 a	427 a
	2	TX7078	2.16 a	10.40 a	495 a b
	3	B35	1.72 b	9.96 a	589 b
	4	B35	1.87 b	9.11 a	471 a
HIF 96	1	TX7078	2.16 a	12.38 a	576 a b
	2	TX7078	2.38 a	14.98 b	660 a
	3	B35	2.80 c	17.18 c	627 a b
	4	B35	2.43 a	13.24 a b	545 b
			Average marker effect[a]		
HIF 36		TX7078	3.00 a	11.56 a	391 a
		B35	2.37 b	11.07 a	469 b
HIF 66		TX7078	2.15 a	9.63 a	461 a
		B35	1.79 b	9.54 a	534 a
HIF 96		TX7078	2.26 a	13.67 a	614 a
		B35	2.61 b	15.21 a	583 a

[a] Values significantly different at $P < 0.05$ are indicated by different letters

Conclusions

HIF analysis provides an efficient method for developing NILs, especially for self-pollinated crops. Several sets of NILs contrasting at a specific QTL can be identified in a single generation of marker-assisted selection. The examination of QTL for seed weight in sorghum indicated that HIF analysis could identify NILs that were useful for confirming linkage between markers and QTL, for fine mapping of QTL, and for evaluating the phenotype associated with specific QTL.

HIF analysis can be used to generate NILs in a range of recombinant genetic backgrounds. The analysis of a QTL in more than one background should be useful for identifying a genetic background in which the phenotype of a QTL is clearly expressed. This should facilitate detailed phenotypic characterization of individual QTL, particularly QTL that are strongly influenced by the environment and genetic background.

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