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HETEROSIS IN MAIZE (*Zea mays*, L.): CHARACTERIZATION OF HETEROTIC QUANTITATIVE TRAIT LOCI (QTL) FOR AGRONOMIC TRAITS IN NEAR ISOGENIC LINES (NILs) AND THEIR TESTCROSSES

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Esame finale anno 2011

Ad Anita, Amanda
e Carlo Alberto

Oh, don't be silly.
Everyone wants this.
Everyone wants to be us.

Miranda Priestly
from "The devil wears Prada"

SUMMARY

ABSTRACT

In a previous study on maize (*Zea mays*, L.) several quantitative trait loci (QTL) showing high dominance-additive ratio for agronomic traits were identified in a population of recombinant inbred lines derived from B73 × H99. For four of these mapped QTL, namely 3.05, 4.10, 7.03 and 10.03 according to their chromosome and bin position, families of near-isogenic lines (NILs) were developed, *i.e.*, couples of homozygous lines nearly identical except for the QTL region that is homozygote either for the allele provided by B73 or by H99. For two of these QTL (3.05 and 4.10) the NILs families were produced in two different genetic backgrounds. The present research was conducted in order to: (i) characterize these QTL by estimating additive and dominance effects; (ii) investigate if these effects can be affected by genetic background, inbreeding level and environmental growing conditions (low *vs.* high plant density). The six NILs' families were tested across three years and in three Experiments at different inbreeding levels as NILs *per se* and their reciprocal crosses (Experiment 1), NILs crossed to related inbreds B73 and H99 (Experiment 2) and NILs crossed to four unrelated inbreds (Experiment 3). Experiment 2 was conducted at two plant densities (4.5 and 9.0 plants m^{-2}). Results of Experiments 1 and 2 confirmed previous findings as to QTL effects, with dominance-additive ratio superior to 1 for several traits, especially for grain yield per plant and its component traits; as a tendency, dominance effects were more pronounced in Experiment 1. The QTL effects were also confirmed in Experiment 3. The interactions involving QTL effects, families and plant density were generally negligible, suggesting a certain stability of the QTL. Results emphasize the importance of dominance effects for these QTL, suggesting that they might deserve further studies, using NILs' families and their crosses as base materials.

ABBREVIATIONS

a additive effect

α average effect of the QTL allele substitution (Experiment 3)

ASI anthesis-silking interval

BPH best parent heterosis

d dominance effect

δ interaction (SSS *vs*. LAN) × (BB *vs*. HH) (Experiment 3)

d/a dominance ratio

EP number of ears per plant

F inbreeding coefficient

FAM NILs' family

GYP grain yield per plant

HG heterotic group

HPD high plant density(9.0 plants m^{-2})

JV juvenile vigor

KE number of kernels per ear

KM kernel moisture

KP number of kernels per plant

KW average kernel weight

LAN Lancaster Sure Crop heterotic group

LPD low plant density (4.5 plants m^{-2})

MP parental mean

MPH mid parent heterosis

NIL near isogenic line

PD plant density

PH plant height

PS days to pollen shedding

QTL quantitative trait locus

RC reciprocal crosses

RIL recombinant inbred line

SD largest stalk diameter

SSS Iowa Stiff Stalk Synthetic heterotic group

TS tester parental lines B73 and H99

***Keywords**: heterosis, maize, QTL validation, near-isogenic lines, plant density.*

INTRODUCTION

1. HETEROSIS: GENERAL ASPECTS

1.1 GENERAL DESCRIPTION OF THE PHENOMENON AND HISTORY

Nature tells us, in the most emphatic manner,
that she abhors perpetual self-fertilization.
Charles Darwin

Heterosis, or hybrid vigor, is a term coined by Shull in 1908 to define the ability of hybrids to outperform their inbred parents in respect to characteristics like growth, stature, biomass, fertility and yield (Semel *et al.*, 2006). Animal behavioral studies and human cultural taboos suggest that most species have evolved mechanisms to avoid crosses to related mates that would lead to the opposite phenomenon of heterosis (Goff, 2010), *i.e.* inbreeding depression. Inbreeding depression indicates the progressive decline in performance, health and fitness that can be measured when individuals of allogamous plants and animals (including humans) are crossed to related mates. In plants, the extreme level of inbreeding is self pollination, that can lead to dramatic situations like in alfalfa (*Medicago sativa,* L.) which produces non vital seeds after a few generations of selfing (Li and Brummer, 2009). Since heterosis and inbreeding depression are opposite phenomena, the vigor lost during inbreeding is recovered by out-crossing (Semel *et al.*, 2006).

All the traits showing the phenomenon of heterosis are quantitative traits. Quantitative traits are characteristics of the individuals that can be measured in the phenotype; from the genetic point of view, these traits are controlled by a high number of genes spread in the

genome, each having on average small effects (Schön *et al.*, 2004; Hayes and Goddard, 2001; Visscher, 2008). On these traits, heterosis can be calculated as the difference between the performance of the hybrid and either the performance of the best parent (Best Parent Heterosis, BPH) or either the average performance of the two parents (Mid Parent Heterosis, MPH). While MPH is scientifically interesting, it has relatively little economic importance; BPH is particularly interesting on the applied point of view. Often, the parents of heterotic offspring are inbred; in this case, the quantification of heterosis reflects both hybrid vigor and recovery from inbreeding depression (Springer and Stupar, 2007a).

Historically, hybrid vigor has been widely exploited by men ever since in many species, like for example mule, the interspecific hybrid obtained by the cross of a female horse and a male donkey. Greeks and Romans already knew that, in comparison with their parents, mules have bigger size, higher resistance in work and longer work life, adaptability to stressful conditions and poorer nutrition; anyway, these examples can be ascribed to intuition only (Troyer, 2006). The first scientific description of the phenomenon had to wait for Charles Darwin, who conducted experiments on 57 plant species in order to explain why reproduction by outcrossing is prevalent in nature, although requiring complex biological mechanisms leading to the prevention from self fertilization (Charlesworth and Willis, 2009). Based on these experiments, Darwin (1877) stated that "cross-fertilization is generally beneficial and self fertilization injurious", causing loss in vigor and fertility in most of the species he studied. In the same years, other scientists like Beal (1876 – 1882), Sanborn (1890), McClure (1892), Morrow and Gardner (1893) tested and compared the performance of a series of maize crosses and the correspondent parental lines and noted that the best combinations yielded up to 50% more than their parental means (Smith *et al.*, 2004). However, these works conducted on maize before the rediscovery of Mendel's laws

contributed to the description but not to the understanding of the phenomenon of heterosis (Crow, 2000).

The approach changed drastically with the works of Shull (1908, 1909) and East (1908, 1909). These two scientists conducted experiments concerning self and cross fertilized maize plants and obtained very similar results, underlying the outstanding performances of the hybrids over the inbred maize lines. However, Shull and East disagreed on the practical use of hybrids for cultivation, since the inbred lines available at that time had so poor performances, particularly in seed production, that East thought their use would have been impossible and non convenient for mass commercial production (Crow, 2000). The solution of this problem was proposed by D.F. Jones (1918, 1922), who suggested the use of double crosses, obtained by the cross of two unrelated single cross hybrids. With this procedure, commercial hybrid seeds were produced on hybrid plants, which were more vigorous and fertile and had higher seed production than inbred lines, thus overcoming the problem of insufficient seed yield (Fig.1).

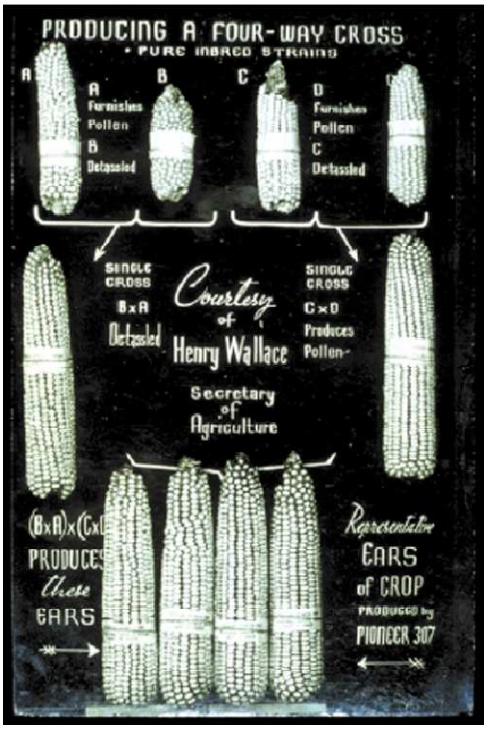

Fig.1. Scheme for the production of double crosses maize hybrids (Duvick, 2001).

Double cross hybrids were much more stable, uniform and productive than open pollinated varieties, even if double cross hybrids showed a little loss in uniformity and performance as compared to single crosses. So, while the market accepted this innovation, maize breeders worked on the improvement of inbred lines, and could then develop high yielding inbreds, which allowed the production of single cross hybrids at a competitive price and granting even higher yielding materials. These achievements have to be related to the historical contest in which these improvement happened in the United States. The beginning of 20th century was a moment of strong agricultural development in cultivated areas, in cultivation techniques, in products for fertilization, control of weeds and parasites, all contributing to significant increase in yields (Cardwell, 1982; Castelberry *et al.*, 1984; Russel, 1991; Duvick, 1992). Anyway, all the progresses achieved with these agronomic innovations could not satisfy the demand in terms of quantity and quality of

products; it is to be recalled that the 1930s was decade of 'The Great Depression', and public interest was to have huge and affordable supplies of food (Duvick, 2001). In this contest, the contribution of plant breeding, boosted by the rediscovery of Mendel's laws, became fundamental, and now we observe that the development of hybrid maize seeds is considered one of the greatest, if not the greatest, economic contribution of genetics (Dobzhansky, 1950; Bourlaug, 2000; Crow, 2008) (Fig.2).

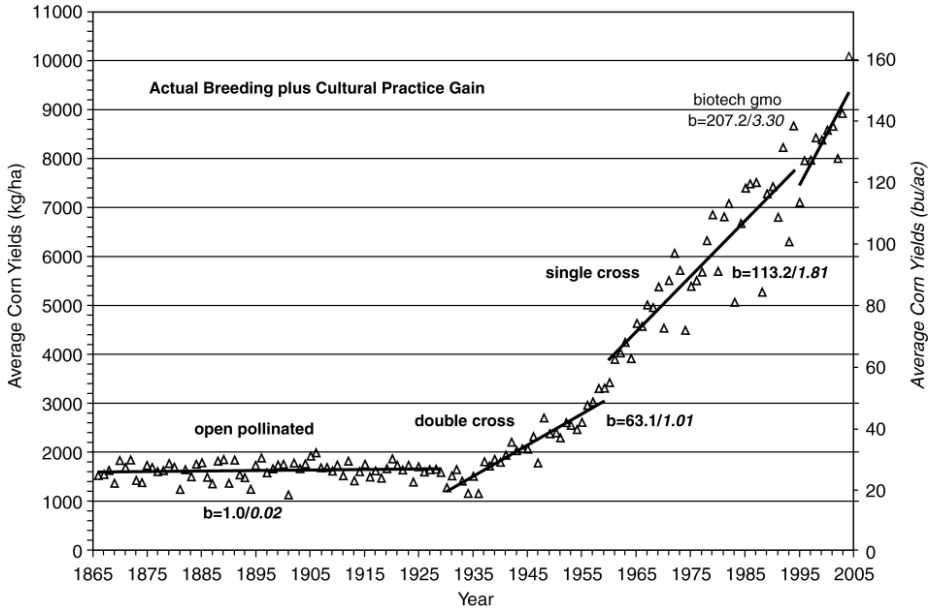

Fig.2. Average U.S. corn yields and kinds of corn from Civil War to 2004. The b values indicate production gain per unit area per year (USDA-NASS, 2005) (Troyer, 2006).

1.2 HETEROSIS IN/AND CORN

I know [my corn plants] intimately, and I find it
a great pleasure to know them.
Barbara McClintock

Because of the historical factors mentioned above, the economic importance and the particular morphological and genetic characteristics, maize (*Zea mays*, L.) is still considered a model species for the study of the phenomenon of heterosis. This species is diploid (n = 10), allogamous, monoecious, with separated male and female inflorescences in the same plant, facilitating both crossing and selfing. Moreover, maize can bear inbreeding depression, thus allowing the obtaining of vital and fertile plants even after many cycles of self pollination. In addition, vigor can be restored when two inbred lines are crossed (Fig.3).

Fig.3. Inbreeding depression and heterosis in maize. P1 and P2 represent inbred lines, that produce heterotic high-performing F1. Plants of F2 – F8 generations show the loss in vigor occurring with subsequent cycles of self pollination.

The work of maize breeders during time was mainly focused on obtaining both high yielding inbred lines for hybrid seed production and high performing hybrids for cultivation. These two aspects involve MPH: increasing parental inbred yield decreases heterosis values when hybrid yield is held constant. The goal of maize breeders was to increase both the terms of the difference determining heterosis, to assure a global improvement of the system.

In maize breeding history, a very important aspect has been, and still is, the allocation of inbred lines to a specific heterotic group. An heterotic group includes related or unrelated genetic materials sharing similar combining ability and heterotic response when crossed with individuals belonging to different heterotic groups (Melchinger and Gumber, 1998). The distinction in heterotic groups is very important because heterosis increases with the increase of parental genetic distance. However, heterosis' increase has been reported to reach an optimum and then, as parental genetic distance increases again, a decline is observed (Moll *et al.*, 1965); this latter observation is probably due to the different levels of adaptation of the parental lines involved in the cross (Link *et al.*, 1996). The information concerning heterotic groups is particularly interesting, since it can give a preliminary information about the performance that can be exhibited by the cross between inbred lines belonging to different groups.

A wide range of natural genetic diversity has been captured in the current maize germplasm (Flint-Garcia *et al.*, 2005; Troyer, 2006). Results obtained in several studies (Kauffmann *et al.*, 1982; Mungoma and Pollak, 1988) indicate that inter-population crosses outyielded intra-population crosses by over 20% on average. Many heterotic groups have been identified for maize in time and in different geographic conditions. For example, the most successful case in the U.S. Corn Belt involves crosses between inbred lines originated by Reid Yellow Dent germplasm (especially Iowa Stiff Stalk Synthetics, SSS) originally

evolved in Illinois, and Lancaster Sure Crop (LAN), evolved in Lancaster County, Pennsylvania (Hallauer, 1990). The two original populations were adapted to different geographic conditions and had different genetic backgrounds, and their crosses showed very interesting results (Hallauer *et al.*, 1988). In Europe, after the Second World War, the economy improved and there was an increased in demand for feed grains, including maize. American maize hybrids were not adapted to the European climate, except in the south, so European breeders developed inbreds from early European flint varieties, that, when crossed to U.S inbreds, gave rise to high yielding hybrids, with adaptation to the cool growing season of northern Europe (Duvick, 2001). Hybrid maize is now an important crop in all Europe. From all these facts, it is evident that breeders work can move in different directions. Inbred lines can be selected from breeding pools inside each heterotic group, developing the available and selected materials to give rise to better combinations. Moreover, elite materials can also be crossed to materials from anywhere in the world, to explore new combinations and select new inbred lines for almost an uncountable number of new traits, as they are needed (Duvick, 2001). As the new hybrids replace older ones, new genetic variability is available for farmers. Actually, as Duvick (2001) states, it 'seems fair to say that, in a given season, individual farmers work with less diversity but, over the years, they have access to more diversity than in pre-hybrid days'.

Another important aspect is that, at the beginning of the last century, the cultivated open-pollinated varieties had a high level of susceptibility to environmental stresses, thus leading to low yield levels (Madden and Partenheimer, 1972). The utilizations of hybrids has enormously reduced this problem, since tolerance to abiotic stresses is a trait subjected to strong selection; moreover, this trait shows high levels of heterosis. In time, yields of new hybrids have significantly increased under stress conditions, such as the ones determined by population density, competition with weeds, low and high water stress and nutrient

deficiency (Duvick, 1984; Castelberry *et al.*, 1984; Tollenaar, 1992; Nissanka *et al.*, 1996). This increase seems to be closely related to morphological and physiological changes occurred progressively in plant during selection programs. Giving some examples, as compared with old materials, new hybrids tend to have more upright leaves, this leading to a better interception of light in dense populations (Duvick, 1984); moreover, tassel size and branches have been progressively reduced (Duvick, 1984); S and N uptake prove to be more efficient (Cacco *et al.*, 1983); in water stress conditions, respiration rate during silking, is lower (Nissanka *et al.*, 1996); grain filling involves a longer period (Tomes, 1998). This general lower susceptibility of modern hybrids to environmental negative factors is with no doubt a key point in hybrid advantage, also for reducing 'yield uncertainty' (Duvick, 2001).

1.3 HETEROSIS IN/AND OTHER CROPS

Come forth into the light of things, let nature be your teacher.
William Wordsworth

Autogamous and allogamous plants show very different levels of manifestation of heterosis, which is more evident in allogamous than autogamous species. As an example, the proportion of increase in yield is on average 10% in wheat hybrids and 200% in maize hybrids, as compared to the corresponding parental lines performance (Gallais, 1988). This evidence could be accounted for considering that, in autogamous species, individuals homozygous for deleterious alleles are progressively eliminated in a population through selection, thus reducing the genetic load of the population (Gallais, 1988) and consequently the inbreeding depression in these species.

It is important to note that the exploitation of hybrids in different crops depends on multiple factors, and the degree of heterosis is just one of them. Other factors are also to be

considered, and in particular the cost of hybrid seeds production, that could be excessive to justify their use. A low cost and efficient method to obtain F_1 hybrids is essential for hybrid commercialization, and there are no doubts that biological, genetic and morphological factors are in many cases a strong limitation (Duvick, 2001). However, in some cases hybrid cultivation could be advisable even for species with low heterosis degree or with seeds sold at high price, especially because of traits related to pests resistance, uniformity of production, yield and post-harvesting characteristics like shelf-life of the products.

Many allogamous species could be mentioned as examples for exploitation of heterosis.

Oil sunflower (*Helianthus annuus*, L.) has been grown as hybrids starting in the U.S.A., but now its hybrids are planted in all parts of the world where sunflower is grown commercially as an oil crop. Sunflower hybrids yield about 50% more than the open pollinated varieties (Miller, 1987). Performance improvement in hybrid sunflower are primarily due to better stability of performance, for example as for resistance to pests, diseases and lodging. Moreover, high oil percentage is an important trait in this oil crop. Parents as well as hybrids have acquired these improvements because of breeding efforts, so actually the increased yield in oil sunflower gradually depends less on heterosis *per se* and more on non-heterotic traits, for gains in yield and yield stability (Duvick, 1999).

Among autogamous specie, at least four main crops can be mentioned. Considering wheat (both *Triticum durum* and *Triticum aestivum*, L.), the increase of crop yield is an important objective in many breeding programs, and the major emphasis for this crop is on the development of improved inbred varieties. Nevertheless, important efforts have been made to find the economically feasible systems for the production of valuable F_1 hybrids (Rasul *et al.*, 2002). Krishna and Ahmed (1992) noted in their work that the highest levels of heterosis were obtained for grain yield (12.52%) and kernel weight (14.60%). Further studies of Morgan (1998) showed that heterosis for grain yield was less when the parents were high

yielding and suggested that probably the elite parental lines already had many of the genes beneficial for yield fixed in the homozygous state, and so the F_1 was unable to show much heterosis. Moreover, Yagdi and Karan (2000) observed heterosis for spike length, number of spikelets per spike, number of grains per spike, grain weight and grain yield per plant. However, the limiting factor for the exploitation of heterosis in wheat is still the cost of hybrid seed as compared to the increase realized.

Tomato (*Solanum lycopersicum*, L.) is a an autogamous plant whose hybrids are cultivated both for fresh market and for industrial use. Most hybrids, in this species, are produced through manual emasculation and pollination of flowers, because only a few male sterile materials are available. F_1 hybrids offer the advantage of higher shelf-life, quality of the product, yield and yield stability; moreover, many cases of complementation for disease resistance are reported, conferring the F_1 higher levels of resistance against pests (Melchinger and Gumber, 1998). The processing-tomato industry seeks varieties with both high total fruit yield and high sugar content (Brix value), but total yield is the trait primarily sought (Gur and Zamir, 2004). Works conducted to evaluate heterosis in tomato and to enter the details of the genetic basis of heterosis in these species evidenced that tomato heterosis is driven predominantly by genomic regions that control reproductive traits (*i.e.*, yield) through multiplicative effects of component traits (total number of flowers per plant and fruit weight) (Semel *et al.*, 2006; Krieger *et al.*, 2010).

Rice (*Oryza sativa*, L.) is a very important autogamous crop especially in the developing countries, and a possible exploitation of heterosis is of extreme interest. New hybrid rice can reach an increase of 30 to 45% in yield as compared to conventional rice cultivars (Yuan, 1992). High yielding hybrids have been obtained from interspecific crosses of *Oryza indica* and *Oryza japonica* (Xiao *et al.*, 1995), even if these hybrids tend to show a variable degree of sterility. Actually, the majority on cultivated hybrids are represented by *indica* x

indica crosses, and they reach also 70% heterosis as compared to their parents (Melchinger and Gumber, 1998). It is to be noted that RFLPs analyses indicated that the average genetic distance between *indica* lines were three to four times higher than between *japonica* lines, thus indicating that a higher level of heterosis is expected in crosses between *indica* than in crosses between *japonica* lines (Melchinger and Gumber, 1998).

Grain sorghum (*Sorghum bicolor*, L.) has been cultivated as hybrid starting in the U.S.A. (Doggett, 1988). Most of the advantage, in comparison with parents, that the hybrid showed was under severe drought stress, reaching up to 40% increase in grain yield.

Heterosis is evident not only in artificially selected populations, but it can also be observed in natural populations (Mitton, 1998; Hansson and Westerberg, 2002). Considering a random sample of coniferous trees, allelic frequencies were in agreement with Hardy-Weinberg law; however, an excess of heterozygotes frequency was observed when only the mature, oldest, or largest trees were sampled (Mitton and Jeffers, 1989). Moreover, a study of Pinyon pines suggested that heterozygotes are more resistant to herbivore pressure (Mopper *et al.*, 1991). The mechanism that gives a better performance of the heterozygotes in these tree species has not been determined, but it is possible to hypothesize that heterosis is an important factor in fitness for many organisms (Springer and Stupar, 2007a).

2. **THEORIES AND APPROACHES**

2.1 CLASSICAL GENETIC HYPOTHESES ON HETEROSIS

Science... never solves a problem without creating ten more.
George Bernard Shaw

To approach the details of the hypotheses concerning heterosis, general considerations have to be presented. Considering one locus with two alleles Q and *q* (being Q the allele that increases trait phenotypic value), the genetic effects that can be observed are reported in Fig.4. In the example, parental lines P_1 and P_2 are homozygous for Q and *q* allele respectively; F_1, resulting from their cross, is *Qq*; MP is parental mean.

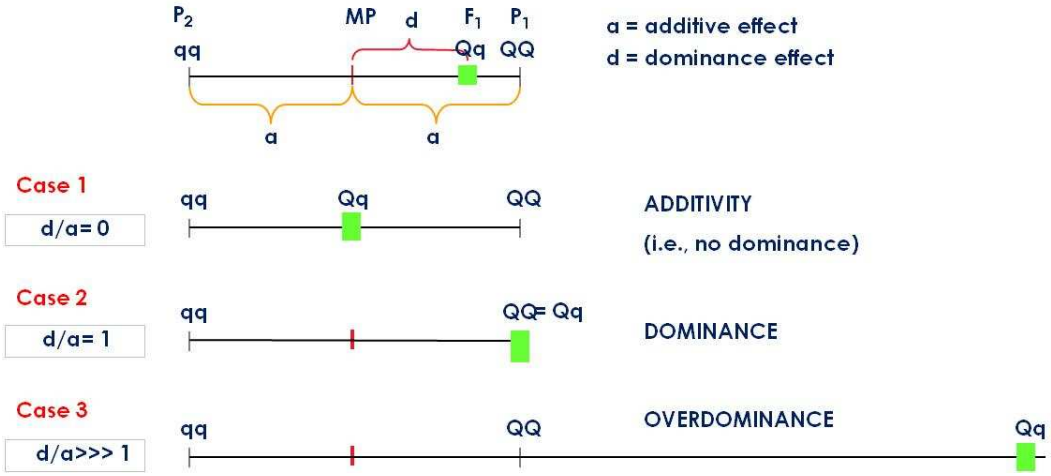

Fig.4: scheme of the possible genotypes and effects at one locus with two alleles, Q and q. P_1 (QQ) and P_2 (qq) are the two parental lines, F_1 (Qq) is the generation derived from their cross; MP is the parental mean. a and d are additive and dominance effect at the locus, respectively.

Additive effect (*a*) is by definition the average effect of allele substitution, and it corresponds to the difference between MP and P_1 or P_2 value. Dominance effect (*d*) is the difference between F_1 value and MP. In case 1 of the example, F_1 value coincides with MP, thus leading to a value of *d* equal to 0. This is the case of additivity model, where only additive effects are present; the dominance ratio *d*/*a* is equal to 0. In case 2, F_1 and MP have different values, with F_1 being equal to P_1 (higher performing parent). In this case, *d* is equal to *a*, so the Q allele shows complete dominance, with *d*/*a* equal to 1. Intermediate cases are possible, being MP < *d* < *a*; in these cases, 0 < *d*/*a* < 1, and this condition is indicated as partial dominance. In case 3, F_1 has a value much higher than the best performing parent; *d* is much higher than *a*, *d*/*a* is higher than 1; this is the case of overdominance.

Starting from these models, considering a single locus at a time, several hypotheses were proposed, involving the number of loci that influence the expression of quantitative traits exhibiting heterosis. The formulation of hypotheses concerning heterosis is one of the controversies that characterized the scientific community in the 20th century (Crow, 2008). Actually, the definition given by Shull was essentially a description of the phenomenon, and the knowledge of its genetic basis appeared immediately fundamental for a rational approach to its exploitation. From the earliest days, two main hypotheses were developed for its explanation (Birchler *et al.*, 2003). The dominance hypothesis states the superiority of the hybrid derives from the capacity of dominant alleles (given by one parent) to mask detrimental recessive alleles (given by the other parent). In this case, the level of heterosis depends on the kind of mutations involving the recessive alleles: large-effect mutated alleles, like those involving a loss of function, show a noteworthy higher performance of the hybrid, whose dominant allele restores the loss of function. Mildly deleterious mutations are often only partially recessive, so heterozygote performance could be only slightly superior

than parental lines (Crow and Simmons, 1983). An early criticism moved against this theory was that, if it was true, it should be possible to select an inbred line homozygous for all the superior (*plus*) alleles, so equal to hybrid in performance; however, this line has never been found. The response to this argument was that, considered the high number of loci involved, it is very difficult to pile all the favorable alleles into one genotype because of linkage that could keep deleterious and superior alleles linked together in repulsion.

The other classical relevant hypothesis proposed for heterosis is overdominance. This hypothesis states that the peculiar allelic interaction itself occurring in the hybrid allows the heterozygote class to perform better than each homozygote. In this latter case, it appears clear that, being the interaction of two different alleles at one locus that would give rise to heterosis, it wouldn't be possible to obtain an homozygote performing as the heterozygote, neither on the theoretical point of view.

Other two classical hypotheses were proposed in time. The pseudo-overdominance (or associative overdominance) hypothesis occurs in case of two-locus linkage in repulsive phase that exhibit partial to complete dominance (Jones, 1917). In this case, the hybrid has only an apparent overdominant phenotype, because dominant alleles mask the deleterious effect of the recessive alleles at both loci, and thus the hybrid performance outstands both parents.

Another classical hypothesis for heterosis invokes epistasis, thus heterosis would be the result of the effect in the hybrid of the interaction of favorable alleles at different loci, themselves showing additive, dominant and/or overdominant effect (Stuber *et al.*, 1992; Li *et al.*, 2001; Luo *et al.* ,2001).

Among all these alternatives, the main hypotheses overwhelmed each other in time, according to the subsequent researches and results that were obtained by breeders. For example, in the earliest days, overdominance prevailed until D.F. Jones (1917) pointed out

that multiple linked loci could account for heterosis without taking into account overdominance. Later during the 40s, Hull, Comstock and Robinson and Dickerson re-evaluated the overdominance hypothesis, considering different species of plants and animals (for example, maize and swine) (Crow, 2008). Although all those inconsistent findings, it has become clear that heterosis is the result of the cumulative action of a large number of favorable dominant alleles (Hallauer and Miranda, 1981). And after a century the debate is still open.

2.2 MOLECULAR HYPOTHESES ON HETEROSIS

All our science, measured against reality, is primitive and childlike –
and yet it is the most precious thing we have.
Albert Einstein

Considering the molecular level, different models can be proposed to try to give an explanation to heterosis (Birchler *et al.*, 2003), some evoking the 'classical' hypothesis but entering in more accurate details of the underlying mechanisms. One of the first molecular hypothesis advanced to explain heterosis states that, when the hybrid is produced, all the different slightly deleterious alleles at multiple loci in the two parental inbred lines are complemented, thus generating a progeny that exceeds each of the two parents. Considering a single locus at a time, complementation in hybrids would explain the hybrid being equivalent to the better of the two parents for the effect of any individual gene (Birchler *et al.*, 2003); heterosis would result only if complementation at each gene involved was cumulative in the phenotype. Actually, several observations suggest that the basic principle of heterosis is something more than simple complementation (Birchler *et al.*, 2003). The strongest evidence is that, although breeders work has progressively produced better

inbred lines, the magnitude of heterosis has not decreased, as it would have been expected with complementation, but has rather been maintained or even slightly increased (East, 1936; Duvick, 1999). Another indication of the insufficient explanation of complementation is that the characteristics of the two parental inbred lines don't necessarily predict the level of heterosis in their hybrid, which must still be measured with a cross. Actually, the slight increase in hybrid vigor over time might have occurred through selection of the best combinations of alleles in the set of loci showing heterosis, rather than through substitution of alleles that regulate the efficiency of physiological processes. Considered that quantitative traits are often regulated by dosage-dependent loci, heterosis could result from different alleles present at loci contributing to the plant regulatory hierarchies. In this context, the study of gene expression is a frequently utilized application of molecular markers, especially in species like maize and rice, considering the differences in expression levels of hybrids in comparison of their inbred parents (Kollipara *et al.*,2002; Guo *et al.*, 2003, 2004, 2006; Auger *et al.*, 2005; Bao *et al.*, 2005; Swanson-Wagner *et al.*, 2006; Meyer *et al.*, 2007; Song *et al.*, 2007; Springer and Stupar, 2007b; Uzarowska *et al.*, 2007; Hoecker *et al.*, 2008; Stupar *et al.*, 2008; Zhang *et al.*, 2008; Wei *et al.*, 2009; Frisch *et al.*, 2010; Jahnke *et al.*, 2010; Riddle *et al.*, 2010). Studies conducted on maize identified an interesting number of genes with altered expression levels in the comparison between the hybrid and the parental lines. It is noteworthy that, in many cases, only small differences were observed in gene expression; however, it is not clear if these differences are actually a cause or a consequence of heterosis. Anyway, the results reported in these experiments are quite different, with some studies reporting a high percentage of additive gene expression changes (Li *et al.*, 2009), others reporting a high percentage of non-additive changes (Stupar *et al.*, 2007, 2008), or even both (Swanson-Wagner *et al.*, 2006). The causes of all these different results are still unknown (Goff, 2010), but might depend on

relevant significant differences in genotypes, plant material, experimental designs and statistical procedures applied in the various studies. However, it might also be an indication that, in different tissues or developmental stages, different global expression patterns might prevail, which might be related to heterosis (Hochholdinger and Hoecker, 2007).

Epigenetic mechanisms, such as DNA methylation, are genome-wide general regulatory mechanisms that affect gene regulation (Kovacevic, 2005). Nuclear DNA is organized in a complex structure involving euchromatin, transcriprionally active, and heterochromatin, inert; both forms of chromatin affect gene activity and gene silencing. DNA methylation level is one of the major determinants of chromatin state, thus implying that the extent and the distribution of methylation on the genome is correlated to the rate of expression of many genes (Matzke *et al.*, 1989; Bird, 2002). Results from several studies indicated that hybrids have in general a lower level of methylation in comparison with their parents. Moreover, hybrids showing different levels of heterosis have different levels of DNA methylation: highly heterotic hybrid show lower methylation levels than less-heterotic hybrids. In addition, Tani *et al.* (2005) showed that inbred lines display a higher percentage of methylation changes as compared to their hybrids in different growing conditions of low or high plant density. Thus, the involvement of methylation in manifestation of hybrid vigor should be an object of study as an indicator of the presence of vigor due to heterosis.

Several studies include genome organization for the dissection of heterosis. Again, the case of maize is particularly interesting. Considering the concept of colinearity (*i.e.*, the fact that genomes of individuals in a given species have the same gene content), recent studies evidenced significant deviations from colinearity on the micro level between different inbred lines of maize. For example, among 72 genes identified in various position of the genome of inbred lines B73 and Mo17, 27 genes were absent in one of the inbred lines (Brunner, 2005). Since in maize many genes are members of small gene families, then

deletions in inbred lines might have only minor quantitative effects on plant performance, because these genes might often be functionally compensated by duplicate copies in other positions in the genome (Fu and Dooner, 2002). However, complementation in hemizygous of many genes with minor quantitative effects might determine a significant increase in hybrids' performance and would be consistent with the dominance hypothesis (Fu and Dooner, 2002). This theory could also explain inbreeding depression as the loss of functional genes in subsequent generations when hemizygous genes get lost after several rounds of self pollination of the hybrids (Hochholdinger and Hoecker, 2007). The high degree of non-colinearity in the genomes of different inbred lines of maize might explain, at least partially, the exceptionally high degree of heterosis in this species. However, it is probable that other molecular mechanisms are involved in heterosis, because it is unlikely that all species contain a degree of non-colinearity in their genome as high as that of maize, hence explaining the different levels of heterosis (Hochholdinger and Hoecker, 2007).

In addition to the molecular explanations, the researches conducted for the dissection of heterosis are involving also biochemical and physiological approaches. Actually, the results coming from gene expression studies seem not to be associated with any specific biochemical pathway, but appear to be randomly dispersed among pathways and functions. Thus, there seems not to be a specific biochemical pathway responsible for hybrid vigor (Goff, 2010). Recent works, aimed at dissecting heterosis on the biochemical point of view, involve protein metabolism. Data reported by Hawkins *et al.*(1986) indicate that inbred organisms are less metabolically efficient because of an increased energy-expansive rate of protein turnover as compared to non-inbred counterparts, which consequently have more energy available for synthesis of additional biomass (Ginn, 2010). This metabolic efficiency hypothesis (Ginn, 2010) is an interesting objective of study which is

entering the debate held among population geneticists over the cause and meaning of correlations between multi-locus heterozygosity and fitness related to traits such as growth rate, viability, and fecundity (Mitton, 1978; Koehn and Hilbish, 1987; Borrell *et al.*,2004).
However, despite all the molecular data available, there is still no clear indication of a correlation between one of the genetic hypotheses and the molecular events leading to heterosis (Hochholdinger and Hoecker, 2007).

2.3 MOLECULAR MARKERS AND QTL STUDIES

Doubt is not a pleasant condition, but certainty is absurd.
Voltaire

The study of quantitative traits involving the use of genetic markers has become a key approach in plant genetics for the dissection of the genetic basis of these traits and to help breeders designing novel plant improvement programs (Stuber, 1992). Genetic markers are sequences of DNA which have a specific location in the genome and that are linked to loci involved in the control of a particular quantitative trait. Pioneer studies like those of Sax (1923), Rasmusson (1933), Everson and Schaller (1955) started focusing on natural morphological mutations detectable in the phenotype; this approach soon revealed its strong limitations, since phenotypic markers are limited in number, often difficult to follow in any given cross and sometimes even affecting plant traits, thus producing confounding phenotypic effects (Stuber *et al.*, 1992). The introduction of molecular markers offered a new unique tool, since a huge number of DNA polymorphisms can be detected, having no effect on the phenotype of quantitative traits under investigation. The greatest challenge given by these tools in the search for the molecular basis of heterosis is establishing a causative link between heterotic phenotypes and the molecular events that underlie them

(Lippman and Zamir, 2007). So, the advent of molecular markers opened new scenarios and widened the possibilities to study heterosis.

Molecular markers have been extensively utilized for the choice of the best parental combination for the production of hybrids to increase the efficiency of breeding programs, since field evaluations are time and resources consuming (Frish *et al.*, 2010). The prediction of hybrid performance has been evaluated through various molecular-based measures (Schrag *et al.*, 2010). First, the genetic distance between the parental lines has been investigated, especially with molecular marker systems such as AFLPs, SSRs, and SNPs (Liu *et al.*, 2002; Barbosa *et al.*, 2003); other parameters have been taken into account, like hybrid value (Dudley *et al.*, 1991), best linear unbiased prediction (Bernardo, 1994), predicted specific combining ability (Charcosset *et al.*, 1998), vector machine regression (Maenhout *et al.*, 2010) and parental gene expression profiles (Frisch *et al.*, 2010). However, all these studies have not reported a unique parameter that can explain or predict the performance with a certain precision, and the selection of the lines for highly-performing hybrid production is still based on an empirical evaluation of the performance of the hybrid progeny.

A very important application of molecular markers has been the creation of dense genetic maps available for mapping Quantitative Trait Loci (QTL). QTL are regions of the genome involved directly in the control of complex traits, and so useful for dissecting the genetic architecture of quantitative traits (Mackay, 2001). Mapping QTL involves measuring the trait under investigation in specific mapping populations and utilizing the genotypic information coming from molecular marker analyses; if a QTL is linked to a marker locus, there will be a difference in the mean value of the quantitative trait among individuals with different genotypes at that marker locus. As much as the QTL and the marker are close, the difference between mean values of different marker class genotypes will be evident; if the

QTL and the marker locus are unlinked, each marker genotype will have the same mean value. So, QTL mapping consists on testing the differences for trait means between marker genotypes for each marker. The marker class exhibiting the greatest difference in the mean value of the trait is the one closest to the QTL of interest (Mackay 2001).

Most conventional QTL studies usually focus on mapping loci for a defined phenotype, and those which evolve in QTL cloning generally involve loci with large effects and high heritabilities (Salvi and Tuberosa, 2005). Studies for the dissection of heterosis, by contrast, have small similarity to previous QTL mapping, because such a dissection is based on complex interactions, altogether named multiplicative heterosis, which occur throughout plant development. Each trait contributing to this multiplicative heterosis has its own inheritance and is subjected to a certain environmental influence (Schnell and Cockerham, 1992). So, mapping heterotic QTL is actually equivalent to mapping multiple traits simultaneously (Lippman and Zamir, 2007).

The most powerful and widely used design for the classical genetic analysis of heterosis is Design III devised by Comstock and Robinson (1948, 1952) (Schön *et al.*, 2010). Design III utilizes the F_2 population obtained by selfing the single cross of two inbred lines; random individuals of this F_2 are backcrossed to both parental lines, and the quantitative trait of interest is measured in these two populations. The analysis of variance of the progenies gives an estimates of additive and dominance effects of the QTL, that can be used to infer the genetic bases of the quantitative trait and to study heterosis (Garcia *et al.*, 2008). This analysis of Design III has been extended by Cockerham and Zeng (1996), that developed a statistical theory that allows the estimate of the QTL effects on both backcross populations simultaneously and the evaluation of the presence of epistasis. The role of epistasis might be actually relevant, since with single marker analysis the estimate of additive and dominance effects of a QTL can be confounded with different types of

epistatic effects (Schön *et al.*, 2010). The study of Melchinger *et al.* (2007b) stresses the importance of epistasis in the manifestation of heterosis in Design III populations.

Several works conducted in maize to evaluate the phenomenon of heterosis took advantage of the application of Design III. A critic example is the work of Stuber *et al.* (1992). In this study, QTL for heterosis in maize were mapped, especially focusing on yield and its component traits, to identify the regions involved in heterosis and to widen the view on its bases. The work of Stuber *et al.* (1992) considered two elite inbred parental lines, Mo17 and B73, that were crossed to produce the F_1, and then self fertilized to obtain the F_2 population. 264 F_2 individuals were self fertilized to obtain F_3 families, each backcrossed to the two parental lines. Stuber *et al.* (1992) determined the genotypic constitution of the parents for several marker loci and performed QTL analysis. All but one QTL identified for grain yield exhibited overdominance, and plant yield correlated significantly with the proportion of heterozygous markers in the genome. A recent re-analysis of the data (Garcia *et al.*, 2008), performed with more sophisticated statistical methods like multiple interval mapping, obtained analogous results, identifying a higher number of QTL but again with a strong evidence for overdominance. However, the possibility that such overdominance underlines pseudo-overdominance is relevant. Considering again the work of Stuber *et al.* (1992), the fine mapping of the genomic region including the QTL with the largest effect on yield showed that the overdominant QTL first identified consisted actually in two QTL linked in repulsion (Graham *et al.*, 1997). Lu *et al.* (2003) conducted another study in maize, focusing again on the importance of dominance, overdominance and pseudo-overdominance for heterosis at the molecular marker level. As for Stuber *et al.* (1992), Lu *et al.*(2003) utilized materials from Iowa Stiff Stalk Synthetics and Lancaster Sure Crop heterotic groups; the F_2 population of LH200 and LH216 inbred lines was random-mated for three generations. This cycles of random mating were performed trying to have

a balance between the risk of breaking linkages between markers and QTL, necessary for QTL detection, and the aim of breaking linkages between QTL, hence reducing pseudo-overdominance. This study again evidenced the presence of overdominance at the molecular marker level, and identified structures that comprise either one QTL that exhibits overdominance, or more than one QTL each exhibiting only partial or complete dominance, but tightly linked so that they function as one inherited unit (Lu *et al.*, 2003). So, despite these cited works and many others, results have not been conclusive yet (Schön *et al.*, 2010).

2.4 PREVIOUS WORK CONDUCTED AD DiSTA

One should always play fairly when one has the winning cards.
Oscar Wilde

The present work has its bases in a previous work of Frascaroli *et al.* (2007) conducted at DiSTA, University of Bologna, in cooperation with other Research Units. In this previous work, a study was conducted starting from B73 and H99 inbred lines. B73 belongs to Iowa Stiff Stalk Synthetic (SSS) heterotic group, and it has been historically a very important inbred, considered the best for crosses for a long time, since it is a high performing line, good in cross-combinations; H99 was developed from Illinois Synthetic 60C and belongs to Lancaster Sure Crop (LAN) heterotic group (Melchinger *et al.*, 1991) and it differs dramatically from B73 for phenotypic characteristics, like plant height, cycle development, though showing a good productive level, and for molecular characteristics (Livini *et al.*, 1992; Lu and Bernardo, 2001). B73 and H99 were used to produce a population of 142 Recombinant Inbred Lines (RILs) by a single seed descent procedure conducted for 12 selfing generations starting from the F_2 population. The 142 RILs were field tested as lines *per*

se and in testcrosses populations obtained by crossing them as female parents with B73 [TC(B)], H99 [TC(H)], and their F_1 [TC(F)]. These three populations were analyzed according to Triple Testcross design (TTC) as described by Kearsey and Jinks (1968) and Kearsey *et al.* (2003). The materials were field tested in three environments, and evaluated according to a randomized complete block design for basic generations, whereas it was a modified split-plot design for the four populations (Lu *et al.*, 2003): the four populations corresponded to the main plots and the 142 RILs (either *per se* or combined with a tester) corresponded to the subplots. Data were collected for many traits concerning early life of plant (percentage of seedling emergence, seedling dry weight), life cycle and maturity traits (days to pollen shedding, anthesis-silking interval, kernel moisture), morphological traits (plant height) and production and its component traits (grain yield, kernel weight and number of kernels).

The population of RILs was the reference mapping population; it had been previously genotyped and used for the production of a genetic linkage map (Sari-Gorla *et al.*, 1997; Frova *et al.*, 1999). QTL were identified with Composite Interval Mapping method (CIM) (Zeng, 1994) using PlabQTL software (Utz and Melchinger, 1996). For QTL analysis, several data sets were considered, and in particular, RIL population, TC(F), two independent data sets obtained by summation (SUM) and subtraction (DIFF) of TC(H) and TC(B) values, and finally, midparental heterosis (Hmp) of each TC hybrid, calculated considering each RIL and the tester inbred line (*i.e.*, B73 or H99). In this analysis, in case of no epistasis, additive (*a*) effects are evidenced in QTL analysis of RILs, TC(F), and SUM data set; the analysis of TC(H) Hmp, TC(B) Hmp, and DIFF data sets identified QTL on the basis of their dominance effects (*d*). A mixed linear model was used to map digenic epistatic QTL also in the SUM and DIFF data sets. QTLMapper (Wang *et al.*, 1999) was used, a software that performs simultaneous interval mapping of both main-effect and digenic epistatic QTL in a data set

with two possible genotypes at each marker locus. The analysis was first conducted without including epistasis, to confirm the QTL detected with the PlabQTL; then, the analysis was conducted including epistasis in the model.

A number of QTL were mapped for all the mentioned traits. Each QTL was identified by the name of the flanking markers, specifically referring to the mapping population, and by the indication of the bin, a conventional subdivision of each chromosome of maize genome in smaller portions (http://www.maizegdb.org). For all the mapped QTL, the degree of dominance was calculated (the ratio between dominance and additive effects $|d/a|$), identifying as partially dominant those QTL with $|d/a|$ between 0.2 and 0.8, as dominant those QTL with $|d/a|$ between 0.8 and 1.2, as overdominant those QTL with $|d/a|$ higher than 1.2 (Stuber *et al.*, 1987). Considering the QTL mapped for grain yield, 21 QTL were detected and 16 of them showed a marked effect on the expression of heterosis, with $|d/a|$ being superior to 1. Moreover, most of these QTL overlapped with heterotic QTL detected in the same experiment for other agronomic traits (Fig.5), thus suggesting that, besides linkage effects, the underlying genes might have pleiotropic effects on the overall plant vigor by means of a sequence of causally related events, that start from the very beginning of the plant life and culminate in grain yield (Frascaroli *et al.*, 2007).

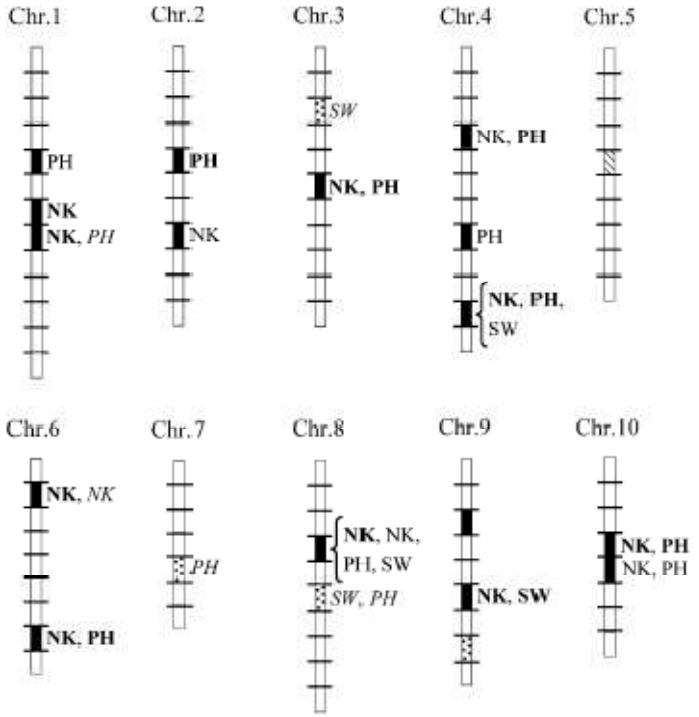

Fig.5. Representation of QTL detected for grain yield in a population of 142 RILs derived from B73 x H99. Chromosomes' segments represent the bins. Colored segments correspond to overdominant QTL, striped segments to dominant QTL, spotted segments to partial-dominant or additive QTL. Overlaps with QTL identified for other important traits like seedling dry weight (SW), plant height (PH) and number of kernels per plant (NK). The overlaps are boldface if the colocating QTL is overdominant, roman type if dominant and italic if partial-dominant or additive (Frascaroli et al., 2007).

Considering that the work of Frascaroli *et al.* (2007) shared B73 with both the works of Stuber *et al.* (1992) and Cockerham and Zeng (1996), a comparison is possible. Again, considering the QTL mapped for grain yield, 16 overdominant QTL were identified; five of them colocated with QTL showing high levels of $|d/a|$ in the work of Stuber *et al.* (1992), and other eight overdominant QTL were mapped in adjacent bins to those mapped by Cockerham and Zeng (1996). These cases of colocation are particularly meaningful, since they could imply a practical perspective for breeding research. Actually, the identification

and better characterization of these regions controlling hybrid vigor could lead to the selection of particularly favorable heterotic allelic combinations, even in other maize genotypes.

2.5 PREPARATION OF NEAR ISOGENIC LINES (NILS)

Scientists are explorers. Philosophers are tourists.
Richard Feynman

Starting from all the reported evidences of several congruent genomic regions both in different traits and genetic materials, the logical following step is the development of Near Isogenic Lines (NILs), *i.e.*, pair of inbred lines identical for all the genome except for the portion including the QTL of interest. Traditionally, NILs are produced via recurrent backcrosses assisted by markers flanking the QTL of interest, but these kinds of procedures are very long and time consuming. An alternative scheme involves cycles of selfing and subsequent selection applied on heterogeneous individuals, such as Heterogeneous Inbred Families (HIFs), or partially heterozygous materials (Allard, 1960; Fehr, 1987; Haley *et al.*, 1994). A more rapid procedure consists on the selection of advanced self fertilized materials, namely Residual Heterozygous Lines (RHLs), which are still heterozygous for the regions of interest. These kind of materials, identified, in the present case, in an advanced phase of RILs' production procedure, made it possible to obtain NILs for the QTL of interest in a background which was a mosaic of the two original parental inbred lines B73 and H99 (Pea *et al.*, 2009). This approach allowed to obtain NILs with a more rapid procedure; moreover, several pairs of NILs for the same QTL could be produced in different backgrounds, thus allowing the evaluation of epistatic effects, *i.e.*, the interaction of the QTL with its genetic background.

The specific procedure followed for NILs production is reported in Fig.6 (Pea *et al.*, 2009). The production of the NILs started from 71 RIL-$F_{4:5}$ families grown in the field and genotyped according to a 10 plants leaf sample in 2003. All plants were genotyped before flowering according to two markers flanking the QTL region, m_1 and m_2, who could show H or B alleles, corresponding to H99 or B73 alleles, respectively. The individuals identified as heterozygote (preferably carrying on the same chromosome both markers alleles provided by the same parent, to reduce the possibility of recombinations events at QTL region) were selected and crossed to the corresponding RIL-$F_{12:13}$, obtaining the generation pseudo-backcross one (ΨBC_1). Then, double heterozygous individuals were advanced to the following generation, which consisted in a backcross to the corresponding RIL-$F_{12:13}$, in 2004, a marker assisted selection on single individuals and a generation of self fertilization, giving rise to BC_1-S_1 generation, in 2005. Single BC_1-S_1 plants having both the flanking markers homozygous for B73 or H99 alleles (*i.e.*, m_1B/m_1B - m_2B/m_2B or m_1H/m_1H - m_2H/m_2H) were then selected within each population and self fertilized, obtaining the generation BC_1-S_2 in 2006 which represented the pairs of recombinant NILs (*i.e.*, pairs of BC_1-S_2 lines with either genotype m_1B/m_1B - m_2B/m_2B or m_1H/m_1H - m_2H/m_2H, respectively) for each chosen QTL (Pea *et al.*, 2009). Thus, taking into account the considered QTL and assuming no crossing over within the m_1 - m_2 segment, we assume that NIL BB has the genotype m_1B/m_1B - qB/qB - m_2B/m_2B, and the NIL HH has the genotype m_1H/m_1H - qH/qH - m_2H/m_2H (Fig.6).

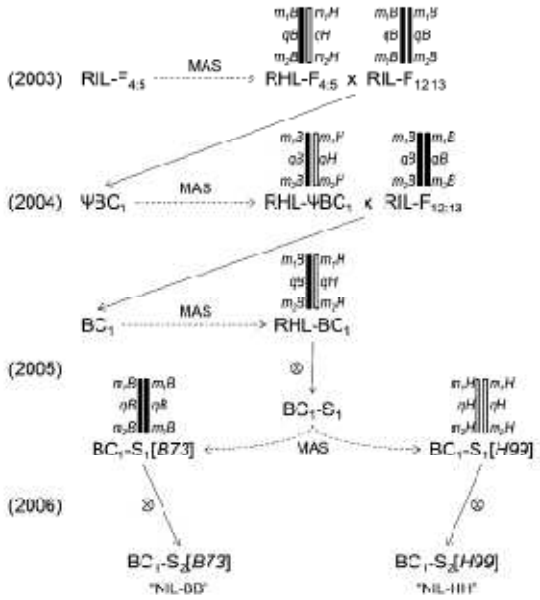

Fig.6. Scheme of NILs' production (Pea et al., 2009).

Starting from the findings of our previous work (Frascaroli *et al.*, 2007), six QTL, named according to their bin position 3.05, 4.10, 7.03, 8.03, 8.05, and 10.03, were chosen for the development of NILs as previously described. The length of the introgressed chromosome segments ranged from 13 cM (QTL 4.10) to 33 cM (QTL 7.03) (Pea *et al.*, 2009).

PROJECT OVERVIEW

Research is to see what everybody else has seen,
and to think what nobody else has thought.
Albert Szent-Györgi

Given the availability of NILs' families for the heterotic QTL described, six NILs' families were chosen to undertake the present study. In particular, four QTL were chosen because they showed overdominance for relevant agronomic traits, such as plant height and kernel weight (QTL 7.03), or grain yield and number of kernel per plant (QTL 3.05 and 4.10, both with two NILs' families each, and 10.03). These six NILs' families were evaluated *per se* and in combination with the related inbred lines B73 and H99 and with four unrelated testers in order to:

(i) characterize QTLs for complex traits and their components by estimating additive and dominance effects;

(ii) investigate whether these effects can be affected by:

   a. genetic background,

   b. inbreeding level

   c. environmental growing conditions, namely the competition among plants as determined by low *vs.* high plant density.

MATERIALS & METHODS

Science is the captain, practice the soldiers.
Leonardo da Vinci

3.1 PREPARATION OF *AD HOC* PLANT MATERIALS

The starting material consisted on six of pairs of the NILs previously described, identified according the bin of the QTL of interest (3.05, 4.10, 7.03, 10.03) and the RILs from which the material was derived (RIL 08 and 40 for QTL 3.05, RIL 40 and 55 for QTL 4.10, RIL 35 for QTL 7.03 and RIL 63 for QTL 10.03), in both versions BB and HH according to the parental allele carried at the QTL. So, for all NILs pairs (3.05_R08, 3.05_R40, 4.10_R40, 4.10_R55, 7.03_R35, and 10.03_R63), selfing and crosses were made in Cadriano (Bologna, Italy; 44°33' N lat., 11°24' E long) to prepare the materials for field investigation. Three different Experiments were conducted, distinguished as 1, 2 and 3 on the basis of materials' inbreeding coefficient (*F*). Starting from the NILs, crosses were made with different materials, as described hereafter.

1. Experiment 1. The preparation of the material for this Experiment consisted on crossing the NILs, differing only for the allele at the QTL of interest. The materials obtained were highly homozygous ($F \approx 1$), except for the target QTL in both reciprocal crosses (RC, Fig.7). These crosses together with the lines *per se* were evaluated in Experiment 1.

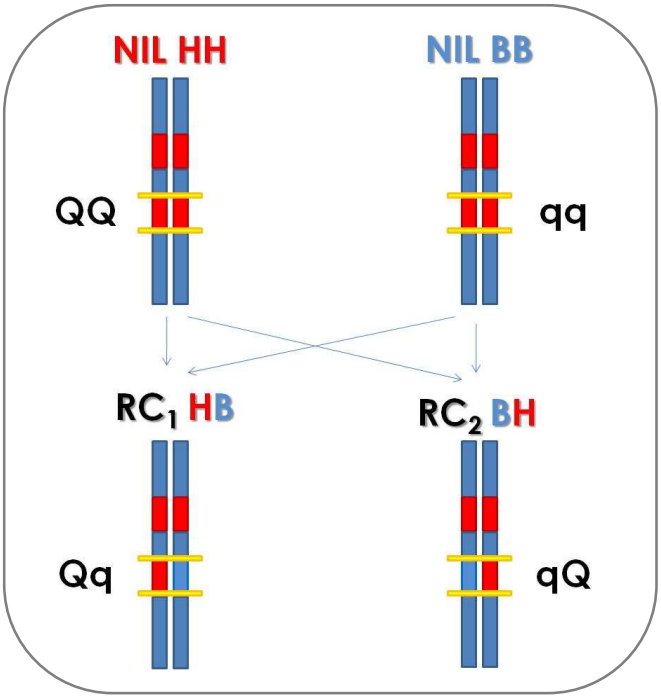

Fig.7. Scheme concerning the materials tested in Experiment 1. Red bars represent the genome of H99 and blue bars represent the genome of B73. NILs' chromosomes is as a mosaic of different segments of the two parental lines. The yellow lines represent the flanking markers of the QTL of interest. The letters represent the genotype of the different materials at the QTL of interest, being Q the allele of H99 parental line and q the allele of B73 parental line. It should be noted that BB and HH NIL are homozygous for the parental alleles, while the two reciprocal crosses (RC) are heterozygous. All the rest of the genome is assumed to be identical and homozygote.

2. Experiment 2. The six NILs pairs were crossed to both parental inbred lines B73 and H99. Since the NILs were actually mosaics of the original parents' genome, the materials obtained after the crosses with one parent are homozygous in some genomic portions, and heterozygous in the other genomic portions; the reverse is true when considering the crosses with the other parent (Fig.8). Thus, the average *F* value of these crosses is expected to be 0.5. These crosses were evaluated in Experiment 2.

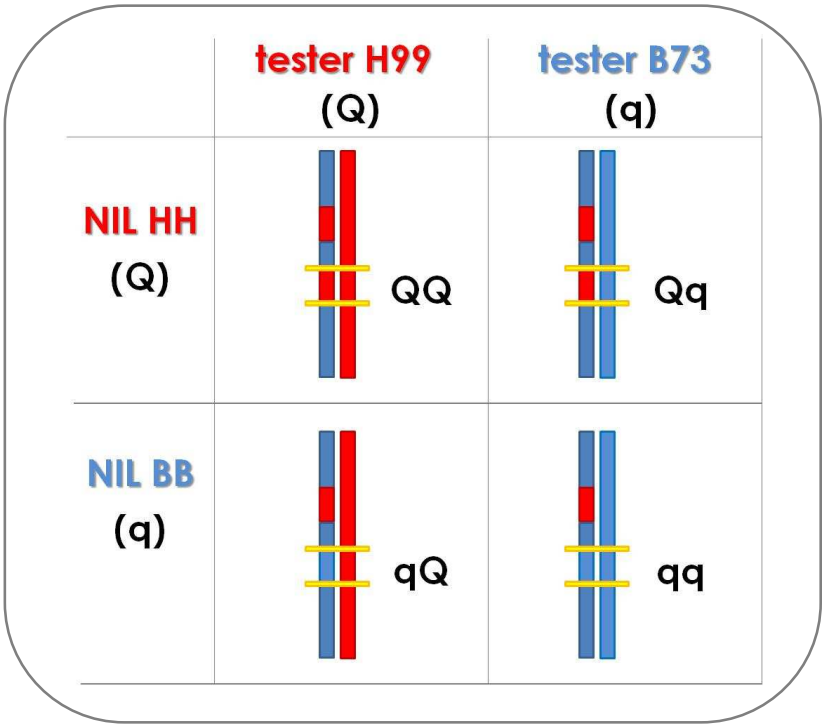

Fig.8. Scheme concerning the materials tested in Experiment 2. Red bars represent the genome of H99 and blue bars represent the genome of B73. In crosses, the parentals' bars are the one color segments, while NILs' chromosome is as a mosaic of different segments of the two parental lines. The yellow lines represent the markers flanking the QTL of interest. As to the QTL of interest, Q is the allele provided by H99, q by B73. In brackets, the gametes produced by the lines involved in the crosses are reported. In the progeny, the genotype QQ is homozygous for the allele provided by H99, the genotype qq is homozygous for the allele provided by B73, while qQ and Qq are the two heterozygotes.

3. Experiment 3. The six NIL pairs were crossed to four unrelated testers, A632 and Lo1016 belonging to Stiff Stalk Synthetic (SSS) heterotic group (the same of B73), and Mo17 and Va26 belonging to Lancaster (LAN) heterotic group (the same of H99) (Fig.9). These inbreds were chosen because they were well-adapted to our environments and because they differed from each other and from the two parental inbred lines both for molecular aspects and for agronomic characteristics (Livini *et al.*, 1992; Pejic *et al.*,

1998). The *F* value of such crosses is expected to be very close to 0. These testcrosses were evaluated in Experiment 3.

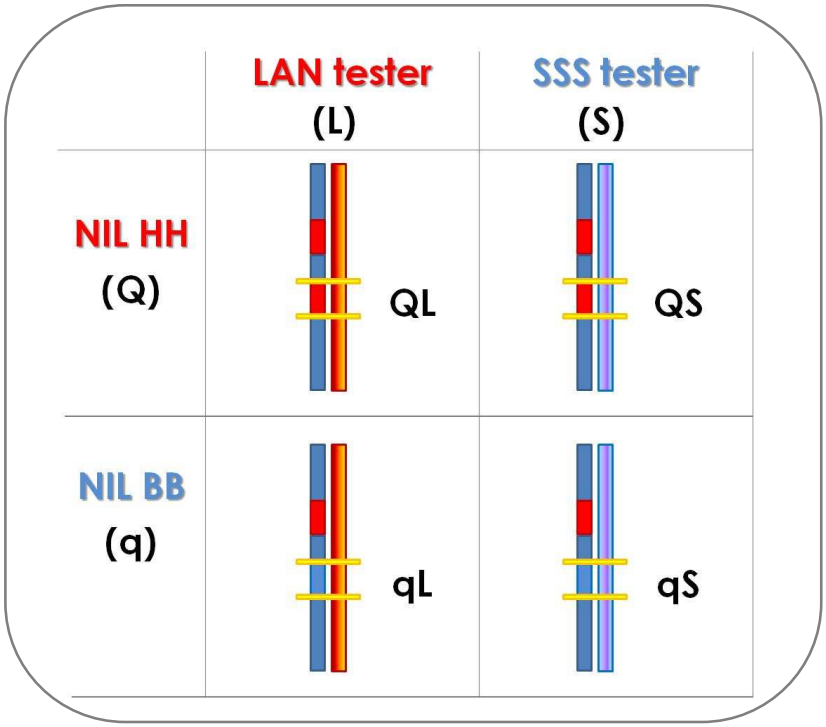

Fig.9. Scheme concerning the materials tested in Experiment 3. Red bars represent the genome of H99 and blue bars represent the genome of B73. In crosses, SSS or LAN tester chromosome bars are the one color segments; note that LAN tester color resembles H99 color (since H99 belongs to LAN heterotic group), and that SSS tester color resembles B73 color (since B73 belongs to SSS heterotic group). NILs' chromosome is as a mosaic of different segments of the two parental lines. The yellow lines represent the markers flanking the QTL of interest. As to the QTL of interest, the alleles provided by H99 and B73 are indicated with Q and q, respectively; the alleles provided by LAN or SSS tester lines are indicated as L and S, respectively. In brackets, the gametes produced by the lines involved in the crosses are reported.

A summary of the tested genotypes and the expected mean values according to QTL effects are reported in Table 1.

Table 1. Genotypes tested for each NILs' family and expected mean value for the QTL of interest of the genotypes tested in the three different Experiments.

Experiment 1		Experiment 2		Experiment 3	
Genotype	Expected QTL value	Genotype	Expected QTL value	Genotype	Expected QTL value
BB [a]	- a [b]	BB x B73 [c]	- a [b]	BB x A632 [d]	$BS_{(A632)}$ [e]
HH	a	BB x H99	d	BB x Lo1016	$BS_{(Lo1016)}$
BB x HH	d	HH x B73	d	BB x Mo17	$BL_{(Mo17)}$
HH x BB	d	HH x H99	a	BB x Va26	$BL_{(Va26)}$
				HH x A632	$HL_{(A632)}$
				HH x Lo1016	$HL_{(Lo1016)}$
				BB x Mo17	$HS_{(Mo17)}$
				BB x Va26	$HS_{(Va26)}$

[a] *BB and HH: homozygous NIL for B73 and H99 allele at the QTL of interest, respectively.*

[b] *a represent additive effect, d dominance effect at the QTL of interest.*

[c] *B73 and H99 correspond to the tester inbred lines.*

[d] *tester inbred line A632 and Lo1016 belong to Stiff Stalk Synthetic (SSS) heterotic group; tester inbred line Mo17 and Va26 belong to Lancaster (LAN) heterotic group.*

[e] *specific contribution of testers. B or H indicate whether the cross involves BB or HH NILs; S or L indicate the heterotic group of the tester line (SSS or LAN, respectively). In brackets, the specific contribution of each tester is reported.*

3.2 Description of Experiments

3.2.1 Experiment 1 (materials with F ≈ 1)

For each NILs' family, the two NILs *per se* and their two RC were tested, except the cases of NILs 3.05_R8 and 10.03_R63 because of seed shortage.

The field design of each trial was a randomized complete block with two replications. Plots consisted on single rows spaced 0.85 m, including after thinning 19 plants with a density of 6.0 plants m^{-2}.

The sources of variation concerning genotypes of Experiment 1 are reported in Table 2.

Table 2. Components of the source of variation concerning genotypes and corresponding degrees of freedom (df) of Experiment 1 for each trial and for the two families of NILs per QTL. Sources concerning families and their interaction were excluded for QTL with only one NILs' family.

Components	Df	Description
Families (FAM)	1	Variation between NILs' families
BB *vs.* HH	1	Variation due to twice additive effect (*a*) at the QTL
Reciprocal crosses (RC)	1	Variation between reciprocal crosses, estimating maternal and/or cytoplasmic effect at the QTL
(BB and HH) *vs.* RC	1	Variation due to dominance effect (*d*) at the QTL
FAM x (BB *vs.* HH)	1	Variation due to interaction between families and *a*
FAM x RC	1	Variation due to interaction between families and maternal and/or cytoplasmic effect
FAM x [(BB *vs.* HH) *vs.* RC]	1	Variation due to interaction between families and *d*

The sources of variation include the differences between families, among genotypes and the corresponding interactions. This scheme is complete only for QTL 4.10 in this Experiment, since only this QTL was evaluated in two different NILs families; thus, only for QTL 4.10 it was possible to consider this source of variation, which was ruled out, with all its interactions, for the other three QTL.

Considering each NILs' family, the variation between BB and HH estimates was due to twice the QTL additive effect (*a*); the variation between RC estimates the variation due to reciprocal effects, while the variation between the mean value of BB and HH NILs

and the mean value of RC estimates the variation due to dominance effect (*d*) of the QTL. For each family, *a* was calculated as half the difference between HH and BB, while *d* was calculated as the difference between the mean value of the two RC and the mean value of the two NILs.

3.2.2 EXPERIMENT 2 (MATERIALS WITH F ≈ 0,5)

Crosses among the NILs and the original parental lines B73 and H99 were tested at two plant densities (PD), *i.e.*, 4.5 (low plant density, LPD) and 9.0 plants m^{-2} (high plant density, HPD). The experimental design was a split-split-plot with three replications; main plots were the two PD, while the two testers were considered sub-plots, because B73 is much taller than H99 and large differences were expected and measured in the crosses with these two inbred lines. Finally, the sub-sub-plots were represented by the two NILs of each family. Two border rows were used to separate the two main plots as well as the two sub-plots. Sub-sub-plots were single rows spaced 0.85 m between rows, including after thinning 15 plants for the LPD and 27 plants for HPD.

The sources of variation concerning genotypes of Experiment 2 are reported in Table 3.

Table 3. Components of the source of variation concerning genotypes and corresponding degrees of freedom (df) of Experiment 2 for each trial and for the two families of NILs per QTL. Sources concerning families and their interaction were excluded for QTL with only one NIL family.

Components	Df	Description
Families (FAM)	1	Variation between NILs' families
Testers (TS)	1	Variation due to the differences between tester B73 and H99
BB *vs*. HH	1	Variation due to additive effect (*a*) at the QTL
TS x (BB *vs*. HH)	1	Variation due to dominance effect (*d*) at the QTL
FAM x TS	1	Variation due to the interaction between families and testers
FAM x (BB *vs*. HH)	1	Variation due to interaction between families and *a*
FAM x TS x (BB *vs*. HH)	1	Variation due to interaction between families and *d*

For the two NILs' families of QTL 3.05 and 4.10, the variation concerning families and all the corresponding interactions were considered, while the families source of variation and all the interaction were excluded for single family QTL 7.03 and 10.03. All the other sources of variation were common to all QTL, and included the variation between tester inbred lines B73 and H99 (TS) across the two NILs of each QTL, the variation between mean values of the two NILs (BB *vs*. HH) across the two testers (giving an estimate of the variation due to the average effect of the QTL allele substitution) and the variation of the interaction TS × (BB *vs*. HH), (giving an estimate of the variation due to *d*). The average effect of the QTL allele substitution was calculated as the difference between the mean value of the crosses of HH NIL and the mean value of the crosses of BB NIL [*i.e.*, (HH × B73 + HH × H99)/2 - (BB × B73 + BB × H99)/2]. Being *p* and *q* the average allelic frequencies over the two related testers, the average effect is equal to: $a + d\,(q - p)$ (Falconer and McKay, 1996). Actually, *p* and *q* in this crosses are both on average

equal to 0.5, since *p* is equal to 1 for one tester and equal to 0 for the other. So, the average effect of the QTL allele substitution is equal to the additive effect (*a*). The *d* effect was calculated as the difference between the mean value of crosses of BB NILs with H99 and HH NILs with B73 (being the heterozygous material at the QTL of interest) minus the mean value of BB NILs crossed to B73 and HH NILs crossed to H99 (being the homozygote material at the QTL of interest).

3.2.3 EXPERIMENT 3 (MATERIALS WITH F ≈ 0)

In this Experiment, the crosses among the NILs and the four unrelated testers A632, Lo1016, Mo17 and Va26 were evaluated. The experimental design of each trial was a randomized complete block design with two replications. Plots were single rows spaced 0.85 m, including 19 plants after thinning at a plant density of 6.0 plants m^{-2}. For each NILs' family, the sources of variation concerning genotypes of Experiment 3 are reported in Table 4.

Table 4. Components of the source of variation concerning genotypes and corresponding degrees of freedom (df) of Experiment 3 for each trial and for the two families of NILs per QTL. Sources concerning families and their interaction were excluded for QTL with only one NIL family.

Components	Df	Description
Families (FAM)	1	Variation between NILs' families
Testers (TS)	3	Variation due to the differences between the four tester lines, belonging to SSS or LAN heterotic group
SSS *vs.* LAN [a]	1	Variation due to differences between heterotic groups
Within SSS, within LAN	2	Variation due to differences between lines within each heterotic groups
BB *vs.* HH	1	Variation due to the average effect (α) of QTL allele substitution
TS x (BB *vs.* HH)	3	Variation due to specific combining ability
(BB *vs.* HH) x (SS *vs.* LAN)	1	Variation due to the interaction between NILs and testers' heterotic group (δ)
(BB *vs.* HH) x (within SSS, within LAN)	2	Variation due to the interaction between NILs and different lines within heterotic group
FAM x TS [b]	3	Variation due to the interaction between NILs' family and tester lines
FAM x (BB *vs.* HH)	1	Variation due to the interaction between NILs' family and the average QTL effect of allele substitution
FAM x TS x (BB *vs.* HH) [b]	3	Variation due to the interaction between NILs' family and the specific combining ability

[a] *SSS and LAN correspond to the heterotic group of the tester inbred lines, belonging A632 and Lo1016 to Stiff Stalk Synthetic (SSS) and Mo17 and Va26 to Lancaster (LAN) heterotic group, respectively.*
[b] *the partitioning of df is not present being the same as that of TS.*

The sources of variation include variation among the four testers, resulting from their general combining ability (g.c.a.) effects, with 3 df; these 3 df can be partitioned into

the variation due to differences between heterotic groups (*i.e.*, SSS *vs*. LAN, 1 df), and within heterotic groups (*i.e.*, within SSS (A632 *vs*. Lo1016), within LAN (Mo17 *vs*. Va26), 2 df altogether). Moreover, variation due to the average effect (α) of the QTL allele substitution (BB *vs*. HH) and the interaction among testers and BB and HH lines, attributable to specific combining ability (s.c.a.) are considered. For QTL in bins 3.05 and 4.10, each with two different families, the variation between families and all the corresponding interactions were also considered.

The average effect (α) of the QTL allele substitution was again calculated as the difference between the mean value of the crosses of HH NIL and the mean value of the crosses of BB NIL. In this case, the QTL allelic frequencies over the four testers are unknown; moreover, since more lines are involved, more than the two parental alleles could be present. Hence, unless the QTL allelic frequencies across testers are equal to 0.5 (as for Experiment 2), and/or *d* is equal to zero, the average effect of the QTL allele substitution is not comparable to the *a* value estimated in the previous two Experiments. Particularly interesting are the interactions TS × (BB *vs*. HH), and especially the component (SSS *vs*. LAN) × (BB *vs*. HH), because this latter reflects the differences (δ) that can be observed comparing crosses of materials belonging to the same or to different heterotic groups. In the specific case, considering for example BB NIL, the crosses within heterotic group are the ones realized with the two inbred testers A632 and Lo1016, belonging to the SSS group as the parental line B73 donor of the QTL allele; in contrast, the crosses between heterotic groups are the ones involving again the BB NIL and the two inbred testers of the opposite heterotic group Mo17 and Va26. For HH NIL the reverse is true, as the crosses between heterotic groups are the ones with A632 and Lo1016, while crosses within heterotic group are the ones with Mo17 and Va26. The effect associated to this interaction was calculated as the difference between the

mean value of the four crosses between heterotic groups (*i.e.*, two crosses BB × LAN and two crosses HH × SSS) and the mean value of the four crosses within heterotic groups (two crosses BB × SSS and two crosses HH × LAN).

3.2.4 FIELD TECHNIQUES COMMON TO ALL TRIALS

The three Experiments were conducted at Cadriano (Bologna, Italy; 44°33' N lat., 11°24' E long.) for three years (2008-2010). In each year (environment), the trials of Experiments 1, 2 and 3 were adjacent in the same field. Trials were treated using the same standard techniques for maize cultivation in the region. Sowing was made at mid-end of April. Fertilizer rates were 45 kg ha^{-1} for P (all applied before sowing) and 200 kg ha^{-1} for N (half before sowing and half after thinning). Weed control was made mechanically and by hand when needed. To attain favorable growing conditions, on average four-five irrigations were made from the mid-end of stem elongation (one-two weeks before silking) to the mid-end of the milk stage (two-three weeks after silking), providing on the whole 60-80 mm of water in each trial. Trials were hand-harvested in the first half of September, by discarding the first and the last plant of each row in Experiments 1, 2 LPD and 3, or by discarding the first two and the last two plants in experiment 2 HPD.

3.3 DATA COLLECTION AND STATISTICAL ANALYSIS

Statistics: the only science that enables different experts using the same figures to draw different conclusions.
Evan Esar

In all trials, data were collected at the single plot level for the following traits:

1. juvenile vigor, (JV, cm), estimated at the developmental stage of 10th leaf, measuring the distance from the ground to the tip of the uppermost leaf;
2. days to pollen shedding (PS, d), as the interval between sowing date and PS date defined when 50% of plants had extruded anthers;
3. anthesis-silking interval (ASI, d), as the difference between silking date, assessed when 50% of plants had extruded silks, and PS date;
4. plant height (PH, cm), measured at the base of the tassel;
5. largest stalk diameter (SD, mm), measured on the second elongated internode;
6. kernel moisture (KM, %) at shelling;
7. number of ears per plant (EP, no.);
8. grain yield per plant (GYP, g);
9. average kernel weight (KW, mg);
10. number of kernels per plant (KP, no.), calculated as the ratio between GYP and KW;
11. number of kernels per ear (KE, no.), calculated as the ratio between GYP and the product between EP and KW.

JV, PH and SD were investigated on a sample of five competitive plants per plot, while all other traits were investigated at the entire plot level. KW was estimated as the mean of a sample of 200 kernels per plot. Both GYP and KW were adjusted to standard 15.5% KM. In Experiment 1, JV was investigated in only one environment, and SD was not investigated in Experiment 3. Since ears were kept in drier for a few days at 35 °C before shelling, KM values have no biological meaning, and, hence, they are not presented and discussed.

In each trial, the analysis of variance (ANOVA) was conducted separately for each family of NILs. For those two QTL represented by two families (*i.e.*, QTL 3.05 in Experiments

2 and 3, and QTL 4.10, in all the three Experiments), the ANOVA was combined across families. Then, for each Experiment, the ANOVA was combined across trials (environments, 2 df) and all the interactions involving environments and the other sources of variation were investigated. A mixed model of ANOVA was followed, having considered plant densities (only for Experiment 2) and genotypes as fixed, and environments as random factors. The analyses were conducted using SAS GLM procedures (SAS Institute, 1996), and least square means over locations are presented.

RESULTS

Newton, forgive me.
Albert Einstein

4.1 COMPARISON AMONG TRIALS WITHIN EXPERIMENT AND AMONG EXPERIMENTS

A summary of the ANOVA concerning the comparison among trials (environment) within Experiments is reported in Table 5a, 5b and 5c.

Table 5a. ANOVA: significance of the environmental source of variation for the measured traits within the Experiment 1.

NILs' family	JV	PS	ASI	PH	SD	GYP	KW	KP	EP	KE
3.05_R08	-	-	-	-	-	-	-	-	-	-
3.05_R40	-	*	ns	**	**	**	**	**	**	**
4.10_R40	-	ns	**	ns	**	**	**	**	**	**
4.10_R55	-	**	**	*	**	+	**	**	**	**
7.03_R35	-	ns	ns	ns	**	**	**	**	**	**
10.03_R63	-	-	-	-	-	-	-	-	-	-

*+, *, ** : effect significant at P ≤ 0.10, P ≤ 0.05 and P ≤ 0.01, respectively; -: trait not estimated.*

Table 5b. ANOVA: significance of the environmental source of variation for the measured traits within the Experiment 2.

NILs' family	JV	PS	ASI	PH	SD	GYP	KW	KP	EP	KE
3.05_R08	**	**	**	**	**	**	**	**	**	**
3.05_R40	**	**	**	**	**	**	**	**	**	**
4.10_R40	**	**	**	**	**	**	**	**	**	**
4.10_R55	**	**	**	**	**	**	**	**	**	**
7.03_R35	**	**	ns	**	**	**	**	**	**	**
10.03_R63	**	**	**	**	**	**	**	**	ns	**

*+, *, ** : effect significant at P ≤ 0.10, P ≤ 0.05 and P ≤ 0.01, respectively; -: trait not estimated.*

Table 5c. ANOVA: significance of the environmental source of variation for the measured traits within the Experiment 3.

NILs' family	JV	PS	ASI	PH	SD	GYP	KW	KP	EP	KE
3.05_R08	ns	**	ns	**	-	**	ns	**	**	**
3.05_R40	ns	**	**	**	-	**	ns	*	**	ns
4.10_R40	ns	**	**	**	-	ns	*	**	**	ns
4.10_R55	ns	**	ns	**	-	**	ns	**	**	ns
7.03_R35	ns	**	**	**	-	ns	ns	ns	**	ns
10.03_R63	ns	**	**	**	-	ns	*	ns	**	ns

*+, *, ** : effect significant at P ≤ 0.10, P ≤ 0.05 and P ≤ 0.01, respectively; -: trait not estimated.*

The analysis pointed out that the differences among trials (environments) within each experiment were significant for most traits. In Experiment 1 (Table 5a), the differences were significant in particular considering traits related to GYP and its components; in Experiment 2 (Table 5b), almost all traits and QTL showed significant differences among the three trials. In Experiment 3 (Table 5c) significant differences among trials were found always for PS, PH, and EP for all NILs' families, and never for JV. All these findings reflected that, even if the

field work was conducted in the same location, genotypes were actually tested in widely different environmental conditions.

The overall mean values by Experiment across the four NILs' families (3.05_R40, 4.10_R40, 4.10_R55, 7.03_R35) tested in all the three Experiments and across the environments are reported in Table 6 together with the Coefficient of Variation (CV) calculated for each trait.

Table 6. Mean values and coefficient of variations (CV) for traits measured in the three Experiments across environments and across the four families of NILs common to the three Experiments.

Trait	Mean			CV(%)		
	Experiment			Experiment		
	1	2	3	1	2	3
JV (cm)	111	129	110	5.2	5.4	4.9
PS (d)	59.5	61.3	60.2	2.1	1.8	2.2
ASI (d)	2.4	0.5	0.4	-	-	-
PH (cm)	138	196	242	4.6	2.6	4.1
SD (mm)	23.2	24.2	-	5.7	4.6	-
GYP (g)	64	135	157	17.9	8.5	10.2
KW (mg)	189	245	298	5.3	4.2	4.9
KP (no.)	344	495	531	17.0	8.6	10.8
EP (no.)	1.25	1.22	1.04	14.7	7.9	8.6
KE (no.)	276	412	509	14.8	9.2	8.2

The mean values of the three Experiments should be compared with extreme caution, because they were conducted in different though adjacent trials and because of the peculiar characteristics of the testers utilized in Experiments 2 and 3. Considering the mean values reported in Table 6, it is noteworthy that Experiment 1 exhibited the lowest values for all traits except ASI and EP. Mean values of Experiment 2 were intermediate between the

other two Experiments for every trait. The highest values were reported for Experiment 3, except for ASI and EP, which showed an opposite trend. All these evidences are consistent with the inbreeding level of the different materials tested in each Experiment, since materials of Experiment 1 have the highest level of homozygosity and those of Experiment 3 have the highest level of heterozygosity. The comparison among CV revealed the opposite trend, since the highest values of CV were calculated in Experiment 1, including the highest value of 17.0% calculated for GYP. These findings again underlined a relationship with the level of inbreeding since, as expected, the homozygous and less vigorous materials tested in Experiment 1 showed a higher reaction to the uncontrollable sources of variation of the environment.

4.2 COMPARISON AMONG GENOTYPES WITHIN EACH EXPERIMENT

4.2.1 EXPERIMENT 1

The results of the four NILs' families (3.05_R40, 4.10_R40, 4.10_R55, 7.03_R35) tested *per se* and as crosses in Experiment 1 are discussed hereafter. Considering the interactions of genotypic sources of variation with the environment (EN), for all NILs' families only few significant differences were evidenced, so the results for all QTL NILs' families are presented and discussed as means across the three environments.

4.2.1.1 NILs' family 3.05_R40

In Table 7a, the ANOVA concerning NILs 3.05_R40 genotypes and genotype by environment interaction is reported.

Table 7a. ANOVA: significance of genotype components source of variation and interactions with the environment (EN) in Experiment 1, NILs' family 3.05_R40.

Source of variation	JV	PS	ASI	PH	SD	GYP	KW	KP	EP	KE
BB *vs*. HH	ns	ns	ns	ns	ns	*	*	ns	ns	ns
RC	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
(BB, HH) *vs*. RC	ns	ns	ns	**	*	**	ns	**	ns	**
EN x (BB *vs*. HH)	-	ns	ns	ns	ns	ns	ns	ns	ns	ns
EN x RC	-	ns	ns	ns	ns	ns	ns	ns	*	ns
EN x [(BB, HH) *vs*. RC]	-	ns	ns	ns	ns	*	**	*	*	ns

**, ** : significant at P ≤ 0.05 and P ≤ 0.01, respectively ;ns: non significant; -: trait not estimated.*

Considering genotypes, significant differences were detected for the source BB *vs*. HH (estimating additive effect, *a*) for GYP and KW; significant difference were detected between NILs and their crosses (estimating dominance effect, *d*) for SD, and highly significant differences for PH, GYP, KP and KE. No significant differences were evidenced for reciprocal crosses, thus indicating the absence of maternal or cytoplasmic effects.

Mean values across the three environments for NILs' family 3.05_R40 are reported in Table 7b.

Table 7b. Mean values across three Environments of NILs' family 3.05_R40 and their crosses evaluated in Experiment 1.

Genotype	JV (cm)	PS (d)	ASI (d)	PH (cm)	SD (mm)	GYP (g)	KW (mg)	KP (no.)	EP (no.)	KE (no.)
BB	107	58.9	1.9	144	23.0	55	175	309	1.27	245
HH	105	59.5	2.0	139	24.8	74	202	363	1.38	259
BB × HH	104	58.5	1.8	145	24.4	81	195	410	1.42	281
HH × BB	112	58.5	1.8	150	25.4	74	193	379	1.32	281
Mean [a]	*107*	*58.8*	*1.9*	*144*	*24.4*	*71*	*191*	*365*	*1.35*	*267*

[a] *mean value of the four tested genotypes.*

Considering the results detected by the ANOVA, the significant differences for the comparison between BB and HH reflected a higher value of the HH NIL over the BB NIL for both GYP and KW. Moreover, the significant (SD) and highly significant (PH, GYP, KP and KE) differences between the crosses and NILs *per se* always resulted from a higher mean value for heterozygous genotypes at the QTL of interest as compared to homozygous genotypes.

4.2.1.2 NILs' family 4.10_R40

In Table 8a, the ANOVA concerning NILs 4.10_R40 genotypes and genotype by environment interaction is reported.

Table 8a. ANOVA: significance of genotype components source of variation and interactions with the environment (EN) in Experiment 1, NILs' family 4.10_R40.

Source of variation	JV	PS	ASI	PH	SD	GYP	KW	KP	EP	KE
BB *vs*. HH	ns	ns	ns	**	ns	ns	**	ns	ns	ns
RC	ns	ns	ns	*	ns	ns	ns	ns	ns	ns
(BB, HH) *vs*. RC	ns	ns	ns	**	**	**	ns	**	ns	**
EN x (BB *vs*. HH)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
EN x RC	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
EN x [(BB, HH) *vs*. RC]	ns	ns	ns	ns	ns	ns	ns	ns	*	ns

**, ** : significant at P ≤ 0.05 and P ≤ 0.01, respectively ;ns: non significant.*

Highly significant differences were found for BB *vs*. HH for PH and KW. Highly significant differences were detected for the comparison between NILs and their crosses for PH, SD, GYP, KP and KE. In only one case (PH) a significant difference was noted for RC.

Means values across the three EN for NILs 4.10_R40 are reported in Table 8b.

Table 8b. Mean values across three Environments of NILs' family 4.10_R40 and their crosses evaluated in Experiment 1.

Genotype	JV (cm)	PS (d)	ASI (d)	PH (cm)	SD (mm)	GYP (g)	KW (mg)	KP (no.)	EP (no.)	KE (no.)
BB	117	63.0	2.8	144	23.4	52	182	279	1.18	231
HH	109	59.3	1.3	124	22.7	57	200	287	1.09	264
BB × HH	120	60.5	2.0	146	24.0	72	179	397	1.12	358
HH × BB	117	61.3	2.3	138	25.1	78	189	405	1.19	344
Mean [a]	*116*	*61.0*	*2.1*	*138*	*23.8*	*65*	*188*	*342*	*1.15*	*299*

[a] *mean value of the four tested genotypes.*

For this QTL and family, PH showed a higher mean value for BB NIL rather than HH NIL, while KW showed a higher mean value of HH NIL. The highly significant differences in the comparison between the crosses and NILs *per se* for PH, SD, GYP, KP and KE again revealed higher values for the heterozygous materials at the QTL of interest as compared to homozygotes. The significant differences between RC for PH concerned a higher mean value of the cross realized utilizing BB NIL as the mother, so it could reflect a maternal or cytoplasmic effect in this particular case.

4.2.1.3 NILs' family 4.10_R55

In Table 9a, the ANOVA concerning NILs 4.10_R55 genotypes and genotype by environment interaction is reported.

Table 9a. ANOVA: significance of genotype components source of variation and interactions with the environment (EN) in Experiment 1, NILs' family 4.10_R55.

Source of variation	JV	PS	ASI	PH	SD	GYP	KW	KP	EP	KE
BB *vs*. HH	ns	ns	ns	*	ns	ns	ns	ns	ns	ns
RC	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
(BB, HH) *vs*. RC	ns	ns	ns	ns	+	**	ns	**	ns	**
EN x (BB *vs*. HH)	ns	ns	ns	ns	ns	*	ns	ns	ns	ns
EN x RC	ns	*	ns	ns	ns	ns	ns	ns	ns	ns
EN x [(BB, HH) *vs*. RC]	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

*+, *, ** : significant at P ≤ 0.10, P ≤ 0.05 and P ≤ 0.01, respectively ;ns: non significant.*

Most of the significant or highly significant differences among genotypes appeared for (BB, HH) *vs*. RC for SD, GYP, KP and KE; in only one case the source concerning the difference between NILs was significant (PH). No differences were noted for RC.

The results provided by NILs 4.10_R55, as means across the three EN, are presented in Table 9b.

Table 9b. Mean values across three Environments of NILs' family 4.10_R55 and their crosses evaluated in Experiment 1.

Genotype	JV (cm)	PS (d)	ASI (d)	PH (cm)	SD (mm)	GYP (g)	KW (mg)	KP (no.)	EP (no.)	KE (no.)
BB	105	57.3	0.5	136	19.4	58	149	392	1.35	296
HH	97	56.5	0.5	121	20.1	41	152	263	1.16	229
BB × HH	109	56.3	0.5	135	20.5	67	145	458	1.41	348
HH × BB	108	57.0	0.3	132	21.2	70	154	453	1.40	345
Mean [a]	*105*	*56.8*	*0.4*	*131*	*20.3*	*59*	*150*	*392*	*1.33*	*304*

[a] *mean value of the four tested genotypes.*

Considering NILs' performance, BB NIL showed higher mean values for PH than HH NIL. Again, for all the significant comparisons between the mean values of crosses and NILs *per se*, heterozygous materials at the QTL of interest exhibited the best performance.

4.2.1.4 NILs' family 7.03_R35

Table 10a shows the ANOVA concerning NILs 7.03_R35 genotypes and genotype by environment interaction.

Table 10a. ANOVA: significance of genotype components source of variation and interactions with the environment (EN) in Experiment 1, NILs' family 7.03_R35.

Source of variation	JV	PS	ASI	PH	SD	GYP	KW	KP	EP	KE
BB *vs*. HH	ns	ns	ns	*	ns	ns	ns	ns	ns	ns
RC	ns	ns	ns	ns	ns	ns	ns	ns	*	ns
(BB, HH) *vs*. RC	ns	ns	ns	**	ns	ns	ns	ns	+	ns
EN x (BB *vs*. HH)	ns	ns	ns	ns	ns	*	ns	ns	ns	ns
EN x RC	ns	*	ns	ns	ns	ns	ns	*	*	ns
EN x [(BB, HH) *vs*. RC]	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

*+, *, ** : effect significant at P ≤ 0.10, P ≤ 0.05 and P ≤ 0.01, respectively ;ns: non significant.*

Considering genotypes, the source revealing the difference between NILs was significant only for PH. Significant differences were found between crosses and NILs *per se* for PH and EP. Again, in only one case (EP) a significant difference emerged for RC.

Table 10b shows the mean values across the three EN for NILs 7.03_R35.

Table 10b. Mean values across three Environments of NILs' family 7.03_R35 and their crosses evaluated in Experiment 1.

Genotype	JV (cm)	PS (d)	ASI (d)	PH (cm)	SD (mm)	GYP (g)	KW (mg)	KP (no.)	EP (no.)	KE (no.)
BB	125	62.0	5.8	138	24.1	62	234	271	1.07	248
HH	109	60.8	6.0	123	24.9	56	215	257	1.18	216
BB × HH	121	62.5	4.8	147	24.0	68	234	296	1.15	253
HH × BB	118	61.0	5.3	146	23.7	64	231	279	1.35	214
Mean [a]	*118*	*61.6*	*5.4*	*138*	*24.2*	*63*	*229*	*276*	*1.19*	*233*

[a] *mean value of the four tested genotypes.*

The comparison between NILs showed a significantly higher value of BB NIL over HH NIL for PH. The comparison between mean values of crosses and NILs *per se* indicated again higher mean values of heterozygous material at the QTL of interest for PH and EP. The significant differences between RC for EP concerned a higher mean value of the cross realized utilizing HH NIL as the mother, so it could reflect a maternal or cytoplasmic effect in this case.

4.2.1.5 Overall considerations concerning NILs' families tested in Experiment 1

Despite the significant differences among EN underlined in the previous chapter, the evidences reported for the NILs' families tested in Experiment 1 indicated that the interactions with EN were significant only in few cases (13%). This result was due at least partly to irrigation, that was supplied during summer season, thus reducing the effects of the rainfall casualty. Moreover, it should be considered that the investigated genotypes were derived from inbreds well adapted to our environments.

Concerning the comparison between NILs, the ones carrying the B73 allele for the QTL of interest had higher mean values for PH in three of the four tested NILs' families; HH NILs had significantly higher mean values for KW in two and GYP in one out of the four tested NILs' families.

Significant differences between crosses and NILs *per se* were always consistent, showing higher mean values of the heterozygous material as compared to homozygous materials at the QTL of interest. Significant differences were detected for SD, PH, KP and KE in three out of the four tested NILs' families, for GYP in two cases and for EP in one case only.

Significant differences for RC were evidenced in two different traits and NILs, corresponding to two cases out of 40 (5%). This evidence indicated that maternal and/or reciprocal effects are negligible.

4.2.2 EXPERIMENT 2

The six NILs' families (3.05_R08, 3.05_R40, 4.10_R40, 4.10_R55, 7.03_R35, 10.03_R63) tested in Experiment 2 as testcrosses to parental lines (B73 and H99) are discussed hereafter. The interactions of genotypic sources of variation with the EN were mostly non significant for all NILs' families tested in Experiment 2. So, the mean values over EN of the two PD are reported for all Nils' families.

4.2.2.1 NILs' family 3.05_R08

Table 11a shows the ANOVA concerning the testcrosses of NILs 3.05_R08 to parental lines.

Table 11a. ANOVA: significance of plant density (PD), testers (TS) and genotypes source of variation and interaction by environments (EN) in Experiment 2, NILs' 3.05_R08 testcrosses to parental lines.

Source of variation	JV	PS	ASI	PH	SD	GYP	KW	KP	EP	KE
PD	**	*	ns	**	**	**	ns	**	**	**
TS	**	**	**	**	**	**	**	ns	**	**
PS x TS	ns	ns	ns	ns	**	*	ns	ns	*	ns
BB *vs*. HH [a]	ns	ns	ns	ns	**	ns	ns	ns	ns	ns
PD x (BB *vs*. HH)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
TS x (BB *vs*. HH) [b]	**	ns	ns	ns	ns	**	**	*	ns	ns
PD x [TS x (BB *vs*. HH)]	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
EN x PD	**	**	ns	*	**	**	ns	**	**	**
EN x TS	**	**	ns	ns	**	ns	ns	ns	ns	ns
EN x PD x TS	ns	**	ns	**	ns	**	**	ns	ns	ns
EN x (BB *vs*. HH)	*	ns	ns	*	**	ns	*	ns	ns	**
EN x PD x (BB *vs*. HH)	ns	ns	ns	ns	*	ns	ns	ns	ns	ns
EN x [TS x (BB *vs*. HH)]	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
EN x PD x [TS x (BB *vs*. HH)]	ns	ns	ns	ns	ns	ns	ns	ns	ns	**

**, ** : significant at P ≤ 0.05 and P ≤ 0.01, respectively; ns: non significant.*

[a] *variation due to additive effect at the QTL of interest.*

[b] *variation due to dominance effect at the QTL of interest.*

The difference between plant densities (PD) was significant (PS) or highly significant for all traits except ASI and KW. As to the comparison between testers (TS), all traits showed highly significant differences, except KP. The interaction between PD and TS was mostly non significant, except for SD, GYP and EP.

The comparison between BB and HH NILs testcrosses, indicating the variation due to additive effect (*a*) at the QTL of interest, was significant for SD only, whereas the interaction PD x (BB *vs*. HH) was never significant. TS by (BB *vs*. HH), indicating the variation due to

dominance effect (*d*) at the QTL of interest, was significant for KP and highly significant for JV, GYP and KW; again, the interaction of this source of variation and PD was never significant. Table 11b reports the mean values of the four testcrosses of 3.05_R08 NILs.

Table 11b. Mean values across three Environments of NILs' 3.05_R08 testcrosses to parental lines evaluated at low (4.5 plants m^{-2}, LPD) and high (4.5 plants m^{-2}, HPD) plant density in Experiment 2.

Genotype	JV (cm)	PS (d)	ASI (d)	PH (cm)	SD (mm)	GYP (g)	KW (mg)	KP (no.)	EP (no.)	KE (no.)
					LPD					
BB × B73	126	63.2	0.0	218	26.0	182	272	562	1,66	334
BB × H99	124	58.8	-0.8	159	23.6	162	227	606	1,89	315
BB mean [a]	*125*	*61.0*	*-0.4*	*188*	*24.8*	*172*	*249*	*584*	*1,78*	*324*
HH × B73	140	63.2	0.0	221	27.7	202	270	634	1,71	359
HH × H99	121	59.1	-0.5	159	24.9	159	217	612	1,91	318
HH mean [b]	*130*	*61.2*	*-0.2*	*190*	*26.3*	*180*	*244*	*623*	*1,81*	*338*
LPD [c]	*128*	*61.1*	*-0.3*	*189*	*25.5*	*176*	*246*	*603*	*1,79*	*331*
					HPD					
BB × B73	140	63.5	0.3	233	23.0	120	255	468	1.01	460
BB × H99	129	59.3	-1.0	175	22.1	107	236	453	1.07	425
BB mean [a]	*135*	*61.4*	*-0.4*	*204*	*22.6*	*113*	*246*	*460*	*1.04*	*443*
HH × B73	147	64.1	0.4	238	24.9	125	260	480	1.03	469
HH × H99	127	60.0	-0.9	173	23.7	100	223	445	1.09	406
HH mean [b]	*137*	*62.1*	*-0.3*	*205*	*24.3*	*112*	*242*	*462*	*1.06*	*437*
HPD [c]	*136*	*61.7*	*-0.3*	*205*	*23.4*	*113*	*244*	*461*	*1.05*	*440*

[a] *mean of BB NIL testcrosses with the two testers.*

[b] *mean of HH NIL testcrosses with the two testers.*

[c] *mean across the four testcrosses in LPD and HPD, respectively.*

HPD showed higher mean values for JV, PS, and KE, and lower mean values for SD, GYP, KP and EP as compared to LPD mean values. As to TS, B73 testcrosses had higher mean values for all the traits showing significant differences, except for EP, as compared to H99 mean values. The significant interactions among PD and TS for SD, GYP and EP was due to a higher decline in the performance of B73 testcrosses, while H99 had a slighter decline in performance.

HH testcrosses revealed higher mean values for SD as compared to BB testcrosses. Significant differences between heterozygous and homozygous materials at the QTL of interest for JV, GYP, KW and KP always showed higher mean values for heterozygotes.

4.2.2.2 NILs' family 3.05_R40

The ANOVA concerning the testcrosses of NILs 3.05_R40 to parental lines is reported in Table 12a.

Table 12a. ANOVA: significance of plant density (PD), testers (TS) and genotype components source of variation and interaction by environments (EN) in Experiment 2, NILs' 3.05_R40 testcrosses to parental lines.

Source of variation	JV	PS	ASI	PH	SD	GYP	KW	KP	EP	KE
PD	**	**	**	**	**	**	**	**	**	**
TS	**	**	**	**	**	**	**	**	**	**
PS x TS	**	**	ns	ns	ns	*	ns	ns	**	ns
BB *vs.* HH [a]	ns	ns	**	ns	**	*	**	ns	ns	ns
PD x (BB *vs.* HH)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
TS x (BB *vs.* HH) [b]	ns	ns	ns	ns	ns	**	ns	**	ns	**
PD x [TS x (BB *vs.* HH)]	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
EN x PD	**	**	*	**	**	**	**	**	**	**
EN x TS	**	*	ns	ns	**	**	**	**	ns	ns
EN x PD x TS	ns	*	ns	*	ns	*	ns	*	ns	*
EN x (BB *vs.* HH)	ns	ns	ns	ns	**	**	ns	**	ns	ns
EN x PD x (BB *vs.* HH)	ns	ns	ns	ns	ns	*	ns	ns	*	ns
EN x [TS x (BB *vs.* HH)]	**	ns	ns	**	ns	ns	ns	ns	ns	ns
EN x PD x [TS x (BB *vs.* HH)]	ns	ns	ns	*	ns	ns	ns	ns	ns	ns

**, ** : significant at P ≤ 0.05 and P ≤ 0.01, respectively; ns: non significant.*

[a] variation due to additive effect at the QTL of interest.

[b] variation due to dominance effect at the QTL of interest.

All differences between PD as well as between TS were highly significant for all traits. The interaction between PD and TS was mostly non significant, except for JV, PS, GYP and EP.

The comparison between BB and HH was significant for GYP and highly significant for ASI, SD and KW; the interaction of this source of variation and PD was always non significant.

The comparison between heterozygous and homozygous materials at the QTL of interest was highly significant for GYP, KP and KE; again, the interaction of this source of variation and PD was never significant.

The mean values over EN of the two PD and the four testcrosses are reported in Table 12b.

Table 12b. Mean values across three Environments of NILs' 3.05_R40 testcrosses to parental lines evaluated at low (LPD) and high (HPD) plant density in Experiment 2.

Genotype	JV (cm)	PS (d)	ASI (d)	PH (cm)	SD (mm)	GYP (g)	KW (mg)	KP (no.)	EP (no.)	KE (no.)
					LPD					
BB × B73	142	61.5	0.1	210	26.0	181	252	600	1.49	397
BB × H99	113	60.5	-1.3	147	23.8	150	229	545	1.76	304
BB mean [a]	*127*	*61.0*	*-0.6*	*179*	*24.9*	*166*	*241*	*573*	*1.63*	*350*
HH × B73	137	61.8	1.0	214	27.7	198	264	636	1.49	415
HH × H99	111	60.6	-0.4	146	26.4	150	236	534	1.85	279
HH mean [b]	*124*	*61.2*	*0.3*	*180*	*27.1*	*174*	*250*	*585*	*1.67*	*347*
LPD [c]	*126*	*61.1*	*-0.1*	*180*	*26.0*	*170*	*245*	*579*	*1.65*	*349*
					HPD					
BB × B73	120	63.8	1.8	232	23.7	106	233	456	0.96	475
BB × H99	109	60.8	-0.9	169	23.1	93	224	413	1.03	401
BB mean [a]	*114*	*62.3*	*0.4*	*200*	*23.4*	*99*	*228*	*434*	*0.99*	*438*
HH × B73	118	64.1	1.8	235	25.6	118	244	485	0.97	499
HH × H99	107	60.9	0.2	167	24.8	88	221	395	1.02	388
HH mean [b]	*113*	*62.5*	*1.0*	*201*	*25.2*	*103*	*233*	*440*	*0.99*	*443*
HPD [c]	*114*	*62.4*	*0.7*	*201*	*24.3*	*101*	*231*	*437*	*0.99*	*441*

[a] *mean of BB NIL testcrosses with the two testers.*

[b] *mean of HH NIL testcrosses with the two testers.*

[c] *mean across the four testcrosses in LPD and HPD, respectively.*

LPD showed higher mean values than HPD for JV, SD, GYP and its components except KE. As to TS, H99 testcrosses had higher mean values for EP, while all other traits revealed higher mean values in B73 testcrosses. The significant interactions between PD and TS resulted

from a more pronounced decrease in performance of B73 testcrosses for JV, PS, GYP, while EP revealed a more evident decrease in performance of H99 testcrosses.
The comparison between BB and HH showed higher values of HH NIL for all traits showing significant differences. Moreover, heterozygous materials at the QTL of interest had higher mean values than homozygous materials for all significant traits.

4.2.2.3 NILs' family 4.10_R40

Table 13a shows the ANOVA concerning the testcrosses of NILs 4.10_R40 to parental lines.

Table 13a. ANOVA: significance of plant density (PD), testers (TS) and genotype components source of variation and interaction by environments (EN) in Experiment 2, NILs' 4.10_R40 testcrosses to parental lines.

Source of variation	JV	PS	ASI	PH	SD	GYP	KW	KP	EP	KE
PD	ns	**	ns	**	**	**	**	**	**	**
TS	**	**	**	**	**	**	**	ns	**	**
PS x TS	ns	**	ns	**	**	ns	ns	*	**	*
BB *vs*. HH [a]	**	**	ns	**	**	**	**	**	ns	**
PD x (BB *vs*. HH)	ns	ns	*	ns	ns	ns	ns	ns	ns	ns
TS x (BB *vs*. HH) [b]	ns	**	ns	**	ns	**	ns	**	**	*
PD x [TS x (BB *vs*. HH)]	ns	ns	ns	ns	ns	**	*	**	ns	ns
EN x PD	**	ns	ns	**	**	**	*	**	**	**
EN x TS	**	ns	ns	ns	ns	**	**	**	**	ns
EN x PD x TS	ns	ns	ns	ns	ns	ns	ns	ns	**	*
EN x (BB *vs*. HH)	**	ns	**	**	**	ns	**	*	*	**
EN x PD x (BB *vs*. HH)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
EN x [TS x (BB *vs*. HH)]	**	ns	ns	ns	ns	ns	*	*	ns	ns
EN x PD x [TS x (BB *vs*. HH)]	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

**, ** : significant at P ≤ 0.05 and P ≤ 0.01, respectively; ns: non significant.*

[a] variation due to additive effect at the QTL of interest.

[b] variation due to dominance effect at the QTL of interest.

Differences between PD were highly significant for all traits except JV and ASI. Differences between TS were highly significant for all traits except KP. The interaction between PD and TS was mostly significant, except for JV, ASI, GYP and KW.

The comparison between BB and HH was highly significant for all traits except ASI, and EP; the interaction PD x (BB *vs*. HH) was non significant except for ASI. The comparison between heterozygous and homozygous materials at the QTL of interest was significant for

KW and highly significant for GYP and KP; the interaction of this source of variation and PD was non significant in most traits, except for GY, KW and KP.

The mean values of the two PD and the four testcrosses over EN are reported in Table 13b.

Table 13b. Mean values across three Environments of NILs' 4.10_R40 testcrosses to parental lines evaluated at low (LPD) and high (HPD) plant density in Experiment 2.

Genotype	JV (cm)	PS (d)	ASI (d)	PH (cm)	SD (mm)	GYP (g)	KW (mg)	KP (no.)	EP (no.)	KE (no.)
					LPD					
BB × B73	141	62.8	1.1	206	26.6	158	243	550	1.27	424
BB × H99	123	59.5	-0.4	153	24.0	175	225	652	1.73	376
BB mean [a]	*132*	*61.2*	*0.3*	*180*	*25.3*	*167*	*234*	*601*	*1.50*	*400*
HH × B73	138	60.8	0.6	205	28.2	173	257	562	1.34	415
HH × H99	117	60.2	-1.5	147	24.9	139	250	465	1.53	295
HH mean [b]	*127*	*60.5*	*-0.4*	*176*	*26.5*	*156*	*254*	*513*	*1.44*	*355*
LPD [c]	*130*	*60.8*	*-0.1*	*178*	*25.9*	*161*	*244*	*557*	*1.47*	*377*
					HPD					
BB × B73	137	65.1	1.1	221	22.8	110	235	470	0.95	488
BB × H99	123	60.5	-0.7	164	21.7	103	220	464	1.03	454
BB mean [a]	*130*	*62.8*	*0.2*	*192*	*22.2*	*107*	*228*	*467*	*0.99*	*471*
HH × B73	136	62.7	1.1	220	24.8	113	257	440	0.99	439
HH × H99	116	59.6	-0.2	154	24.0	89	238	376	0.99	385
HH mean [b]	*126*	*61.2*	*0.5*	*187*	*24.4*	*101*	*247*	*408*	*0.99*	*412*
HPD [c]	*128*	*62.0*	*0.3*	*190*	*23.3*	*104*	*237*	*437*	*0.99*	*442*

[a] *mean of BB NIL testcrosses with the two testers.*

[b] *mean of HH NIL testcrosses with the two testers.*

[c] mean across the four testcrosses in LPD and HPD, respectively.

Significantly higher mean values of PS, PH and KE were noted in HPD as compared to LPD. As to the comparison between TS, B73 testcrosses had higher mean values than H99 testcrosses, except for SD and KW. Considering the change of performance of testcrosses from LPD to HPD, B73 testcrosses revealed a higher increase of PS and PH mean values, and a higher decrease of SD mean values as compared to H99 testcrosses; moreover, H99 testcrosses revealed a higher decrease in mean values of KP and EP, and higher increase of KE mean values as compared to B73 testcrosses from LPD to HPD.

Significant differences between BB and HH NILs resulted from higher mean values of BB in all traits, except SD and KW. Mean values of the heterozygotes at the QTL of interest were higher than homozygotes', again for all significant traits. Considering the significant interaction of *d* effect and PD, from LPD to HPD the heterozygotes showed a higher decrease in performance for GYP and KP as compared to the homozygotes, while the reverse was true for KW.

4.2.2.4 NILs' family 4.10_R55

The ANOVA concerning NILs 4.10_R55 testcrosses to parental lines is reported in Table 14a.

Table 14a. ANOVA: significance of plant density (PD), testers (TS) and genotype components source of variation and interaction by environments (EN) in Experiment 2, NILs' 4.10_R55 testcrosses to parental lines.

Sources of variation	JV	PS	ASI	PH	SD	GYP	KW	KP	EP	KE
PD	ns	**	**	**	**	**	**	**	**	**
TS	**	**	**	**	**	**	**	**	**	**
PS x TS	ns	ns	ns	**	ns	**	ns	*	**	ns
BB *vs.* HH [a]	ns	ns	ns	**	**	**	*	**	ns	**
PD x (BB *vs.* HH)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
TS x (BB *vs.* HH) [b]	*	**	ns	ns	ns	**	ns	*	*	ns
PD x [TS x (BB *vs.* HH)]	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
EN x PD	ns	**	*	**	**	**	ns	**	**	**
EN x TS	**	*	**	**	**	ns	*	ns	**	ns
EN x PD x TS	ns	ns	ns	**	**	ns	ns	ns	ns	ns
EN x (BB *vs.* HH)	**	ns	*	ns	**	ns	ns	ns	ns	ns
EN x PD x (BB *vs.* HH)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
EN x [TS x (BB *vs.* HH)]	ns	ns	ns	ns	*	ns	ns	ns	ns	ns
EN x PD x [TS x (BB *vs.* HH)]	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

**, ** : significant at P ≤ 0.05 and P ≤ 0.01, respectively; ns: non significant.*

[a] variation due to additive effect at the QTL of interest.

[b] variation due to dominance effect at the QTL of interest.

The differences between PD were highly significant for all traits except JV. Differences between TS were highly significant for all traits. The interaction between PD and TS was mostly non significant, except for PH, GYP, KP, and EP

The comparison between BB and HH was significant for KW and highly significant for PH, SD, GYP, KP and KE; the interaction between this source of variation and PD was non significant for all traits. The comparison between heterozygous and homozygous materials

at the QTL of interest was significant for JV, KP and EP, and highly significant for PS and GYP; the interaction of this source and PD was non significant for all traits.

Table 14b reports the mean values over EN of the two PD and 4.10_R55 NILs' four testcrosses are reported in Table 14b.

Table 14b. Mean values across three Environments of NILs' 4.10_R55 testcrosses to parental lines evaluated at low (LPD) and high (HPD) plant density in Experiment 2.

Genotype	JV (cm)	PS (d)	ASI (d)	PH (cm)	SD (mm)	GYP (g)	KW (mg)	KP (no.)	EP (no.)	KE (no.)
					LPD					
BB × B73	137	61.0	-0.2	227	23.5	175	241	606	1.35	441
BB × H99	124	58.1	-0.7	146	21.3	125	208	509	1.59	315
BB mean [a]	*131*	*59.5*	*-0.4*	*186*	*22.4*	*150*	*225*	*557*	*1.47*	*378*
HH × B73	140	60.8	0.0	219	25.1	178	240	620	1.44	423
HH × H99	117	59.0	-0.9	136	23.0	107	201	451	1.55	288
HH mean [b]	*129*	*59.9*	*-0.5*	*177*	*24.0*	*142*	*220*	*535*	*1.49*	*356*
LPD [c]	*130*	*59.7*	*-0.4*	*182*	*23.2*	*146*	*223*	*546*	*1.48*	*367*
					HPD					
BB × B73	134	62.8	1.1	242	21.9	113	231	487	0.95	511
BB × H99	123	58.7	-0.1	155	20.1	83	204	403	0.99	409
BB mean [a]	*128*	*60.7*	*0.5*	*198*	*21.0*	*98*	*218*	*445*	*0.97*	*460*
HH × B73	140	61.9	1.0	236	23.8	109	232	469	0.97	482
HH × H99	123	60.2	-0.5	147	22.6	72	195	367	0.98	374
HH mean [b]	*131*	*61.1*	*0.3*	*192*	*23.2*	*91*	*213*	*418*	*0.98*	*428*
HPD [c]	*130*	*60.9*	*0.4*	*195*	*22.1*	*94*	*216*	*432*	*0.97*	*444*

[a] *mean of BB NIL testcrosses with the two testers.*

[b] *mean of HH NIL testcrosses with the two testers.*

[c] *mean across the four testcrosses in LPD and HPD, respectively.*

HPD exhibited higher mean values for PS, ASI, PH and KE, and lower values for the other traits with significant differences for PD. In the comparison between TS, higher mean values were measured for B73 testcrosses as compared to H99 testcrosses for all traits except EP. The cases of significant PD x TS interaction revealed that from LPD to HPD the increase of PH and the decrease of GYP and KP mean values were more evident in B73 testcrosses than H99 testcrosses. H99 testcrosses revealed a higher decrease than B73 testcrosses for EP from LPD to HPD.

Concerning significant differences between BB and HH, HH NIL mean value was higher than BB NIL for SD, while for PH, GYP, KW, KP and KE BB NIL mean values were higher than HH NIL mean values. Significant difference between heterozygous and homozygous materials at the QTL of interests exhibited higher values of heterozygotes for all traits, except for PS.

4.2.2.5 NILs' family 7.03_R35

The ANOVA concerning the testcrosses of NILs 7.03_R35 to parental lines is reported in Table 15a.

Table 15a. ANOVA: significance of plant density (PD), testers (TS) and genotype components source of variation and interaction by environments (EN) in Experiment 2, NILs' 7.03_R35 testcrosses to parental lines.

Source of variation	JV	PS	ASI	PH	SD	GYP	KW	KP	EP	KE
PD	**	**	ns	**	**	**	**	**	**	ns
TS	**	**	*	**	**	ns	**	*	**	**
PS x TS	ns	**	ns	ns	**	ns	ns	ns	**	ns
BB *vs*. HH [a]	ns	ns	ns	**	**	**	**	ns	ns	ns
PD x (BB *vs*. HH)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
TS x (BB *vs*. HH) [b]	ns	ns	ns	*	ns	ns	ns	ns	ns	ns
PD x [TS x (BB *vs*. HH)]	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
EN x PD	ns	*	ns	**	**	**	**	**	ns	**
EN x TS	**	ns	*	**	**	**	**	**	**	ns
EN x PD x TS	ns	ns	*	**	ns	*	ns	*	**	**
EN x (BB *vs*. HH)	*	ns	ns	ns	**	ns	ns	ns	**	ns
EN x PD x (BB *vs*. HH)	ns	ns	ns	ns	ns	ns	ns	ns	**	ns
EN x [TS x (BB *vs*. HH)]	ns	ns	ns	ns	ns	ns	ns	ns	**	ns
EN x PD x [TS x (BB *vs*. HH)]	ns	ns	ns	ns	**	ns	ns	ns	ns	ns

**, ** : significant at P ≤ 0.05 and P ≤ 0.01, respectively; ns: non significant.*

[a] variation due to additive effect at the QTL of interest.

[b] variation due to dominance effect at the QTL of interest.

The differences between PD were highly significant for all traits except ASI and KE. Differences between TS were significant for all traits except for GYP. The interaction between PD and TS was mostly non significant, except for PS, SD and EP.

The differences between BB and HH were highly significant for PH, SD, GYP, KW; the interaction of this source of variation and PD was non significant for all traits. Differences between heterozygotes and homozygotes at the QTL of interest were significant for PH only; the interaction of this source of variation and PD was non significant for all traits. The

mean values over EN of the two PD and the four testcrosses of 7.03_R35 NILs are reported in Table 15b.

Table 15b. Mean values across three Environments of NILs' 7.03_R35 testcrosses to parental lines evaluated at low (LPD) and high (HPD) plant density in Experiment 2.

Genotype	JV (cm)	PS (d)	ASI (d)	PH (cm)	SD (mm)	GYP (g)	KW (mg)	KP (no.)	EP (no.)	KE (no.)
					LPD					
BB × B73	147	63.1	1.9	249	24.9	195	278	595	1.34	449
BB × H99	129	58.8	1.9	194	23.6	199	310	537	1.33	410
BB mean [a]	*138*	*60.9*	*1.9*	*221*	*24.3*	*197*	*294*	*566*	*1.33*	*429*
HH × B73	145	62.8	1.9	238	27.8	184	271	576	1.26	464
HH × H99	126	59.0	0.9	182	26.1	184	301	517	1.32	393
HH mean [b]	*135*	*60.9*	*1.4*	*210*	*27.0*	*184*	*286*	*546*	*1.29*	*429*
LPD [c]	*137*	*60.9*	*1.6*	*216*	*25.6*	*190*	*290*	*556*	*1.31*	*429*
					HPD					
BB × B73	151	65.6	2.1	253	21.5	109	267	410	0.85	473
BB × H99	133	59.6	1.1	206	21.5	122	293	416	0.98	427
BB mean [a]	*142*	*62.6*	*1.6*	*230*	*21.5*	*115*	*280*	*413*	*0.92*	*450*
HH × B73	150	65.1	2.1	252	24.4	108	250	435	0.89	484
HH × H99	133	59.5	0.8	195	23.7	109	277	392	0.98	401
HH mean [b]	*141*	*62.3*	*1.4*	*224*	*24.0*	*108*	*263*	*413*	*0.93*	*442*
HPD [c]	*142*	*62.4*	*1.5*	*227*	*22.8*	*112*	*272*	*413*	*0.92*	*446*

[a] *mean of BB NIL testcrosses with the two testers.*

[b] *mean of HH NIL testcrosses with the two testers.*

[c] *mean across the four testcrosses in LPD and HPD, respectively.*

The significant comparison between PD was due to higher mean values in HPD for JV, PS and PH, and higher mean values in LPD for SD, GYP, KW, KP and EP. Significant differences between TS revealed higher mean values of B73 testcrosses as compared to H99

testcrosses for all traits except KW and EP. Considering the interaction between PD and TS, from LPD to HPD the increase of PS mean values and the decrease of SD and EP mean values for B73 testcrosses were higher than for H99 testcrosses.

Significant differences between BB and HH resulted from higher mean values of BB NILs for PH, GYP and KW; on the contrary, higher mean values of HH NILs were detected for SD. Higher mean values of heterozygotes as compared to homozygotes were noted for PH.

4.2.2.6 NILs' family 10.03_R63

The ANOVA concerning the testcrosses of NILs 10.03_R63 to parental lines is reported in Table 16a.

Table 16a. ANOVA: significance of plant density (PD), testers (TS) and genotype components source of variation and interaction by environments (EN) in Experiment 2, NILs' 10.03_R63 testcrosses to parental lines.

Source of variation	JV	PS	ASI	PH	SD	GYP	KW	KP	EP	KE
PD	**	**	**	**	ns	**	**	**	**	**
TS	**	**	**	**	**	ns	**	**	**	**
PS x TS	ns	ns	**	ns	ns	**	**	ns	*	**
BB *vs.* HH [a]	ns	ns	*	ns	**	ns	**	ns	ns	ns
PD x (BB *vs.* HH)	ns	ns	ns	ns	**	ns	ns	ns	ns	ns
TS x (BB *vs.* HH) [b]	**	**	**	ns	ns	**	*	**	**	**
PD x [TS x (BB *vs.* HH)]	ns	ns	ns	ns	ns	ns	ns	ns	ns	*
EN x PD	**	ns	*	**	**	**	**	**	**	**
EN x TS	**	*	**	ns	**	ns	ns	ns	**	**
EN x PD x TS	ns	ns	**	ns	**	ns	ns	ns	ns	ns
EN x (BB *vs.* HH)	ns	ns	ns	ns	**	ns	ns	ns	ns	ns
EN x PD x (BB *vs.* HH)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
EN x [TS x (BB *vs.* HH)]	**	ns	ns	ns	ns	ns	ns	*	ns	ns
EN x PD x [TS x (BB *vs.* HH)]	ns	ns	**	ns	ns	ns	ns	*	ns	ns

**, ** : effect significant at P ≤ 0.05 and P ≤ 0.01, respectively; ns: non significant.*

[a] variation due to additive effect at the QTL of interest.

[b] variation due to dominance effect at the QTL of interest.

The differences between PD were highly significant for all traits except for SD. Differences among TS were highly significant for all traits except GYP. The interaction between PD and TS was significant for EP and highly significant for ASI, GYP, KW and KE.

BB *vs.* HH was significant for ASI and highly significant for SD and KW; the interaction between this source of variation and PD was highly significant for SD only. The comparison between heterozygotes and homozygotes at the QTL of interest was significant for KW, and

highly significant for JV, PS, ASI, GYP, KP, EP and KE; the interaction of this source of variation and PD was significant for KE only.

The mean values over EN of the two PD of the four testcrosses of 10.03_R63 NILs are reported in Table 16b.

Table 16b. Mean values across three Environments of NILs' 10.03_R63 testcrosses to parental lines evaluated at low (LPD) and high (HPD) plant density in Experiment 2.

Genotype	JV (cm)	PS (d)	ASI (d)	PH (cm)	SD (mm)	GYP (g)	KW (mg)	KP (no.)	EP (no.)	KE (no.)
					LPD					
BB × B73	117	65.2	0.4	193	26.4	103	282	310	1.11	283
BB × H99	123	60.6	-0.2	149	24.1	122	258	402	1.68	228
BB mean [a]	*120*	*62.9*	*0.1*	*171*	*25.2*	*113*	*270*	*356*	*1.40*	*255*
HH × B73	128	63.9	-0.4	197	27.2	132	272	412	1.25	325
HH × H99	104	62.1	0.1	147	24.1	98	239	368	1.55	233
HH mean [b]	*116*	*63.0*	*-0.2*	*172*	*25.7*	*115*	*255*	*390*	*1.40*	*279*
LPD [c]	*118*	*62.9*	*0.0*	*172*	*25.4*	*114*	*263*	*373*	*1.40*	*267*
					HPD					
BB × B73	109	66.7	4.3	218	24.9	41	250	163	0.56	293
BB × H99	114	62.1	-0.1	170	23.3	76	252	304	0.91	338
BB mean [a]	*111*	*64.4*	*2.1*	*194*	*24.1*	*59*	*251*	*234*	*0.73*	*315*
HH × B73	118	65.5	2.4	218	27.6	62	252	247	0.73	340
HH × H99	104	62.3	-0.1	170	26.0	62	240	254	0.95	267
HH mean [b]	*111*	*63.9*	*1.2*	*194*	*26.8*	*62*	*246*	*251*	*0.84*	*303*
HPD [c]	*111*	*64.2*	*1.6*	*194*	*25.4*	*60*	*249*	*242*	*0.79*	*309*

[a] mean of BB NIL testcrosses with the two testers.

[b] mean of HH NIL testcrosses with the two testers.

[c] mean across the four testcrosses in LPD and HPD, respectively.

The comparison between PD pointed out higher mean values at LPD for most traits except PS, ASI, PH and KE. The comparison between TS revealed higher mean values of B73 testcrosses for all significant traits except KP and EP. Considering the significant interaction between PD and TS, B73 testcrosses showed a higher increase of mean value for ASI from LPD to HPD; for GYP, KW and EP B73 testcrosses showed a higher decrease in performance from LPD to HPD. Finally, considering KE, H99 testcrosses showed a higher increase in performance than B73 testcrosses again from LPD to HPD.

As to the comparison between BB and HH, BB NIL testcrosses had higher mean values for ASI and KW, and lower mean values for SD as compared to HH NIL testcrosses. The significant interaction BB *vs*. HH and PD for SD was due to a decrease in BB NIL testcrosses performance and to an increase of HH NIL testcrosses mean value from LPD to HPD. Many significant differences between heterozygotes and homozygotes at the QTL of interest, *i.e.*, for JV, GYP, KW, KP, EP and KE, were characterized by higher mean values for the heterozygotes than the homozygotes, except for PS and ASI. The significant interaction of *d* effect and PD for KE revealed that heterozygotes showed a higher increase in performance as compared to the homozygotes from LPD to HPD.

4.2.2.7 Overall considerations concerning NILs' families tested in Experiment 2

Considering all the evidences reported above, the difference between PD within each family was significant in most cases (87% considering all families and traits). In particular, in five cases out of six, LPD showed higher mean values for GYP, KP and EP, while HPD showed higher mean values for PS and KE. Moreover, in four cases out of six LPD showed higher mean values for SD, and KW, while HPD showed higher mean values for PH.

To summarize, the mean values of the two PD across all genotypes are given in Table 17.

Table 17. Mean values in Experiment 2 for the two plant densities (PD) across three environments, six families of NILs and two testers.

PD	JV (cm)	PS (d)	ASI (d)	PH (cm)	SD (mm)	GYP (g)	KW (mg)	KP (no.)	EP (no.)	KE (no.)
LPD	128	61.1	0.1	186	25.3	159	252	536	1.52	353
HPD	127 ¤	62.3	0.7	202	23.6	97	241	404	0.95	420

¤ comparison between mean values highly significant (P ≤ 0.01) for all traits except JV (non significant).

The data reported in all previous Tables and in this last Table 17 are consistent with the stress condition realized in HPD. Actually, the increase noted for PH, PS and ASI from LPD to HPD indicate that closer plants tend to grow more in height to reach light, thus leading also to later flowering; moreover, the same stress causes a more pronounced difference in flowering time (ASI). The increase in height also causes a reduction of SD, which is actually smaller in HPD. GYP and its components again reflect the stressful situations, being lower in HPD. All these findings thus indicate that, as compared to LPD, HPD led to a stress level appreciable for all the traits of the adult plant. Interestingly, GYP, *i.e.*, a trait whose expression is affected throughout all plant's life cycle, showed the most pronounced decline due to the increase of PD. This decline, however, was lower than 50% (*i.e.*, 39%) and, hence, the higher mean value for yield as expressed per unit area (not shown) was detected in HPD (8.78 and 7.19 Mg ha^{-1} for HPD and LPD, respectively).

The differences among TS within each family were in most instances significant. For all six NILs' families, B73 testcrosses had higher mean values for JV, PS, ASI and SD, while H99 testcrosses had higher mean values for EP. Moreover, in the comparison between TS, in all cases B73 testcrosses had higher mean values for JV, PS, ASI, PH and KE; in five cases out of six B73 testcrosses had higher mean values for SD, GYP and KW, while H99 testcrosses

showed higher mean values for EP. To summarize, the mean values of the two TS across all genotypes are given in Table 18.

Table 18. Mean values in Experiment 2 for the two related testers (TS) across three environments, six families of NILs and two plant densities.

TS	JV (cm)	PS (d)	ASI (d)	PH (cm)	SD (mm)	GYP (g)	KW (mg)	KP (no.)	EP (no.)	KE (no.)
B73	135	63.4	1.1	224	25.3	139	255	492	1.17	422
H99	120 [a]	60.0	-0.3	164	23.5	118	238	447	1.30	352

[a] comparison between mean values highly significant ($P \leq 0.01$) for all traits.

Considering the overall mean values, the comparison between TS was highly significant for all traits; in particular, the mean values of B73 testcrosses were always higher than those of H99, with the exception of EP.

The interaction involving PD and TS within each family was significant in most cases; this interaction was always of size, *i.e.*, the increase or decrease of one TS was higher than the increase or decrease of the other TS. In most cases, B73 proved to be a more sensitive tester, showing a more pronounced increase or decrease in performance from LPD to HPD than H99. This finding could be connected with the fact that B73 is of greater size than H99, thus the former tended to be more responsive to PD change.

Considering the comparisons between HH and BB materials (*i.e.*, additive effect), it is interesting to note that for PH and SD significant differences were always consistent. Considering the comparison between heterozygous materials at the QTL of interest as compared to homozygotes (*i.e.*, dominance effect), it should be noted that results were always consistent. Actually, even those traits (PS and ASI) showing higher mean values of homozygotes as compared to heterozygotes were consistent with what observed for the

other traits from a biological point of view, since an anticipation of flowering time or a better synchronization of male and female flowering (lower ASI) is typical of more vigorous individuals.

4.2.3 EXPERIMENT 3

The six NILs' families (3.05_R08, 3.05_R40, 4.10_R40, 4.10_R55, 7.03_R35, 10.03_R63) tested as testcrosses to the four unrelated testers A632, Lo1016, Mo17 and Va26 in Experiment 3 are discussed hereafter. It should be recalled that A632 and Lo1016 belong to SSS heterotic group, the same as B73 inbred line, while Mo17 and Va26 belong to LAN heterotic group, the same as H99 inbred line. Therefore, testcrosses involving BB NILs and SSS lines, or involving HH NILs and LAN lines, are realized within the same heterotic group; testcrosses involving BB NILs and LAN lines, or involving HH NILs and SSS lines are realized between opposite heterotic groups.

4.2.3.1 NILs' family 3.05_R08

The ANOVA concerning the testcrosses of NILs 3.05_R08 to unrelated tester lines is reported in Table 19a.

Table 19a. ANOVA: significance of genotype, of the unrelated testers (considering if the testers belong to SSS (A632 and Lo1016) or LAN (Mo17 and Va26) heterotic group) sources of variation and interaction by environments (EN) in Experiment 3, NILs' 3.05_R08 testcrosses to four unrelated testers.

Source of variation	JV	PS	ASI	PH	GYP	KW	KP	EP	KE
BB *vs*. HH	ns	ns	ns	ns	ns	ns	ns	*	ns
SSS *vs*. LAN	ns	*	**	ns	**	**	**	**	ns
Within SSS, within LAN	ns	**	ns	**	**	**	**	**	**
(BB *vs*. HH) x (SSS *vs*. LAN)	ns	ns	ns	ns	ns	*	*	ns	*
(BB *vs*. HH) x (within SSS, within LAN)	ns	ns	ns	ns	ns	ns	ns	ns	*
EN x (BB *vs*. HH)	ns	ns	ns	ns	ns	ns	ns	ns	ns
EN x (SSS *vs*. LAN)	ns	ns	ns	ns	ns	ns	ns	**	ns
EN x (within SSS, within LAN)	ns	ns	*	ns	ns	ns	ns	ns	ns
EN x (BB *vs*. HH) x (SSS *vs*. LAN)	ns	ns	ns	ns	ns	ns	ns	ns	ns
EN x (BB *vs*. HH) x (within SSS, within LAN)	ns	ns	ns	ns	ns	ns	ns	ns	ns

**, ** : significant at P ≤ 0.05 and P ≤ 0.01, respectively; ns: non significant.*

Considering the comparison between BB and HH NILs, the only significant difference was noted for EP. The comparison between SSS and LAN revealed a significant difference for PS and highly significant differences for ASI, GYP, KW, KP, and EP. Considering the comparison within SSS and within LAN, highly significant differences were detected for PS, PH, GYP, KW, KP, EP and KE. Actually, these differences were expected, since tester lines are different from each other even if belonging to the same heterotic group. However, the evaluation of the differences between testers is not an objective of the present research, so they will not be commented according to mean values results. The interaction NILs' by (SSS *vs*. LAN)

was significant for KW, KP and KE. The residual variation due to the interaction NILs by (within SSS, within LAN) was significant for KE only.

Interactions with EN were non significant in most instances for any of the tested NILs' families, so for all NILs' families the mean values over EN of the eight testcrosses is reported. Table 19b reports the mean values of 3.05_R08 testcrosses. .

Table 19b. Mean values across three environments of the testcrosses among NILs' family 3.05_R08 the four unrelated testers evaluated in Experiment 3.

Genotype	JV (cm)	PS (d)	ASI (d)	PH (cm)	GYP (g)	KW (mg)	KP (no.)	EP (no.)	KE (no.)
BB × A632	116	59.0	0.7	240	167	320	525	1.19	446
BB × Lo1016	113	59.8	1.3	257	209	305	685	1.38	502
BB × SSS	*115*	*59.4*	*1.0*	*249*	*188*	*313*	*605*	*1.29*	*474*
BB × Mo17	108	59.5	0.0	236	188	331	586	1.05	560
BB × Va26	120	58.8	-0.2	249	164	315	522	1.14	458
BB × LAN	*114*	*59.2*	*-0.1*	*243*	*176*	*323*	*554*	*1.10*	*509*
BB mean	*114*	*59.3*	*0.5*	*245*	*182*	*318*	*580*	*1.19*	*492*
HH × A632	116	58.7	1.0	241	170	297	571	1.26	454
HH × Lo1016	111	61.0	1.3	259	211	283	746	1.42	531
HH × SSS	*114*	*59.9*	*1.2*	*250*	*191*	*290*	*659*	*1.34*	*493*
HH × Mo17	110	59.2	-0.2	238	184	345	533	1.16	462
HH × Va26	107	58.8	0.2	261	159	307	522	1.14	459
HH × LAN	*109*	*59.0*	*0.0*	*250*	*172*	*326*	*528*	*1.15*	*461*
HH mean	*111*	*59.4*	*0.6*	*250*	*181*	*308*	*593*	*1.24*	*477*

[a] *mean value of the testcrosses of BB NIL and SSS testers (BB × SSS) and of BB NIL and LAN testers (BB × LAN).*

[b] *mean value of the testcrosses of BB NIL and the four testers.*

[c] *mean value of the testcrosses of HH NIL and SSS testers (HH × SSS) and of HH NIL and LAN testers (HH × LAN).*

[d] *mean value of the testcrosses of HH NIL and the four testers.*

The comparison between BB and HH NIL revealed a higher mean value of HH NIL testcrosses for EP. Considering the comparison between SSS and LAN, SSS testcrosses showed higher mean values for PS, ASI, GYP, KP and EP, while LAN testcrosses showed higher mean values for KW. Considering the interaction NILs' by (SSS *vs*. LAN), the testcrosses between heterotic groups (*i.e.*, BB × LAN and HH × SSS) showed higher values than those of the four testcrosses within heterotic groups (BB × SSS and HH × LAN) for KW, KP and KE.

4.2.3.2 NILs' family 3.05_R40

The ANOVA concerning the testcrosses of NILs 3.05_R40 to unrelated tester lines is reported in Table 20a.

Table 20a. ANOVA: significance of genotype, of the unrelated testers (considering if the testers belong to SSS (A632 and Lo1016) or LAN (Mo17 and Va26) heterotic group) sources of variation and interaction by environments (EN) in Experiment 3, NILs' 3.05_R40 testcrosses to four unrelated testers.

Source of variation	JV	PS	ASI	PH	GYP	KW	KP	EP	KE
BB *vs*. HH	ns	ns	**	ns	*	*	ns	ns	*
SSS *vs*. LAN	ns	ns	**	**	**	**	ns	ns	ns
Within SSS, within LAN	*	ns	**	**	**	**	**	**	**
(BB *vs*. HH) x (SSS *vs*. LAN)	ns	ns	**	ns	**	ns	**	ns	*
(BB *vs*. HH) x (within SSS, within LAN)	ns	ns	ns	ns	ns	ns	ns	ns	ns
EN x (BB *vs*. HH)	ns	ns	ns	*	ns	ns	ns	ns	ns
EN x (SSS *vs*. LAN)	ns	ns	**	ns	*	ns	**	ns	*
EN x (within SSS, within LAN)	ns	ns	ns	ns	*	ns	ns	ns	ns
EN x (BB *vs*. HH) x (SSS *vs*. LAN)	ns	ns	ns	ns	ns	ns	ns	ns	ns
EN x (BB *vs*. HH) x (within SSS, within LAN)	ns	ns	ns	ns	ns	ns	ns	ns	ns

**, ** : significant at P ≤ 0.05 and P ≤ 0.01, respectively; ns: non significant.*

Considering the comparison between BB and HH NILs, significant differences were noted for GYP and KW, and a highly significant difference was noted for ASI. Considering the comparison between SSS and LAN, highly significant differences were found for ASI, PH, GYP and KW. Considering the comparison within SSS and within LAN, a significant difference was detected for JV, and highly significant differences were detected for ASI, PH, GYP, KW, KP, EP and KE. The interaction NILs' by (SSS *vs*. LAN) was significant for KE and highly significant for ASI, GYP and KP. The residual variation due to the interaction NILs by (within SSS, within LAN) was non significant for any trait.

Table 20b shows the mean values over EN of the eight testcrosses of 3.05_R40 NILs.

Table 20b. Mean values across three environments of the testcrosses among NILs' family 3.05_R40 the four unrelated testers evaluated in Experiment 3.

Genotype	JV (cm)	PS (d)	ASI (d)	PH (cm)	GYP (g)	KW (mg)	KP (no.)	EP (no.)	KE (no.)
BB × A632	117	60.0	0.8	238	130	282	462	1.07	432
BB × Lo1016	110	61.8	0.0	251	138	269	507	1.00	508
BB × SSS	*114*	*60.9*	*0.4*	*245*	*134*	*276*	*485*	*1.04*	*470*
BB × Mo17	117	60.8	0.8	240	171	342	504	1.00	505
BB × Va26	115	59.7	0.3	231	139	283	492	1.07	461
BB × LAN	*116*	*60.3*	*0.6*	*236*	*155*	*313*	*498*	*1.04*	*483*
BB mean	*115*	*60.6*	*0.5*	*240*	*144*	*294*	*491*	*1.03*	*477*
HH × A632	119	60.3	2.3	235	142	296	482	1.06	456
HH × Lo1016	105	61.5	1.5	249	166	281	588	1.00	591
HH × SSS	*112*	*60.9*	*1.9*	*242*	*154*	*289*	*535*	*tity*	*524*
HH × Mo17	112	60.5	0.7	237	167	338	494	0.95	525
HH × Va26	109	59.8	-0.2	226	140	300	468	1.05	447
HH × LAN	*111*	*60.2*	*0.3*	*232*	*154*	*319*	*481*	*1.00*	*486*
HH mean	*111*	*60.5*	*1.1*	*237*	*154*	*304*	*508*	*1.01*	*505*

[a] *mean value of the testcrosses of BB NIL and SSS testers (BB × SSS) and of BB NIL and LAN testers (BB × LAN).*

[b] *mean value of the testcrosses of BB NIL and the four testers.*

[c] *mean value of the testcrosses of HH NIL and SSS testers (HH × SSS) and of HH NIL and LAN testers (HH × LAN).*

[d] *mean value of the testcrosses of HH NIL and the four testers.*

The comparison between BB and HH NIL pointed out a higher mean value of HH NIL testcrosses for all significant traits. Considering the comparison between SSS and LAN, SSS testcrosses showed higher mean values for ASI and PH, while LAN testcrosses showed higher mean values for GYP and KW. Considering the interaction NILs' by (SSS *vs*. LAN), the

testcrosses between heterotic groups (*i.e.*, BB × LAN and HH × SSS) showed higher values than those of the four testcrosses within heterotic groups (BB × SSS and HH × LAN) for KW, KP and KE.

4.2.3.3 NILs' family 4.10_R40

The ANOVA concerning the testcrosses of NILs 4.10_R40 to unrelated tester lines is reported in Table 21a.

Table 21a. ANOVA: significance of genotype, of the unrelated testers (considering if the testers belong to SSS (A632 and Lo1016) or LAN (Mo17 and Va26) heterotic group) sources of variation and interaction by environments (EN) in Experiment 3, NILs' 4.10_R40 testcrosses to four unrelated testers.

Source of variation	JV	PS	ASI	PH	GYP	KW	KP	EP	KE
BB *vs*. HH	ns	**	ns	ns	ns	**	**	ns	**
SSS *vs*. LAN	ns	**	*	**	ns	**	**	*	**
Within SSS, within LAN	ns	**	ns	**	**	**	ns	ns	**
(BB *vs*. HH) x (SSS *vs*. LAN)	ns	ns	ns	ns	ns	ns	ns	ns	ns
(BB *vs*. HH) x (within SSS, within LAN)	ns	ns	ns	ns	ns	ns	ns	ns	ns
EN x (BB *vs*. HH)	ns	ns	*	ns	ns	ns	ns	ns	ns
EN x (SSS *vs*. LAN)	ns	ns	ns	ns	ns	ns	ns	ns	ns
EN x (within SSS, within LAN)	ns	ns	ns	ns	ns	ns	ns	ns	ns
EN x (BB *vs*. HH) x (SSS *vs*. LAN)	ns	ns	ns	ns	ns	ns	ns	ns	ns
EN x (BB *vs*. HH) x (within SSS, within LAN)	ns	ns	ns	ns	ns	ns	ns	ns	ns

**, ** : significant at P ≤ 0.05 and P ≤ 0.01, respectively; ns: non significant.*

Considering the comparison between BB and HH NILs, highly significant difference were found for PS, KW, KP and KE. Considering the comparison between SSS and LAN, significant differences were found for ASI and EP, and highly significant differences for PS, PH, KW, KP and KE. Considering the comparison within SSS and within LAN, highly significant differences were detected for PS, PH, GYP, KW and KE. The interaction NILs' by (SSS *vs*. LAN) and the interaction NILs by (within SSS, within LAN) were non significant for all traits.

The mean values over EN of the eight testcrosses of 4.10_R40 NILs are reported in Table 21b.

Table 21b. Mean values across three environments of the testcrosses among NILs' family 4.10_R40 the four unrelated testers evaluated in Experiment 3.

Genotype	JV (cm)	PS (d)	ASI (d)	PH (cm)	GYP (g)	KW (mg)	KP (no.)	EP (no.)	KE (no.)
BB × A632	106	61.3	0.3	234	145	263	552	1.02	540
BB × Lo1016	103	62.3	0.7	244	162	266	612	1.03	594
BB × SSS	*105*	*61.8*	*0.5*	*239*	*154*	*265*	*582*	*1.03*	*567*
BB × Mo17	109	61.3	-0.3	234	162	313	520	0.97	537
BB × Va26	110	59.3	0.2	229	147	269	547	1.01	542
BB × LAN	*110*	*60.3*	*-0.1*	*232*	*155*	*291*	*534*	*0.99*	*540*
BB mean	*107*	*61.1*	*0.2*	*236*	*154*	*278*	*558*	*1.01*	*553*
HH × A632	115	59.3	1.0	231	148	299	500	1.06	469
HH × Lo1016	105	62.0	0.7	242	157	284	555	1.02	545
HH × SSS	*110*	*60.7*	*0.9*	*237*	*153*	*292*	*528*	*1.04*	*507*
HH × Mo17	111	59.2	0.2	228	161	354	455	1.00	456
HH × Va26	113	59.0	-0.2	232	130	294	445	0.99	447
HH × LAN	*112*	*59.1*	*0.0*	*230*	*146*	*324*	*450*	*1.00*	*452*
HH mean	*111*	*59.9*	*0.4*	*233*	*149*	*308*	*489*	*1.02*	*479*

[a] *mean value of the testcrosses of BB NIL and SSS testers (BB × SSS) and of BB NIL and LAN testers (BB × LAN).*

[b] *mean value of the testcrosses of BB NIL and the four testers.*

[c] *mean value of the testcrosses of HH NIL and SSS testers (HH × SSS) and of HH NIL and LAN testers (HH × LAN).*

[d] *mean value of the testcrosses of HH NIL and the four testers.*

The comparison between BB and HH NIL pointed out a higher mean value of BB NIL testcrosses for PS, KP and KE, while HH NIL testcrosses had higher mean values for KW. Considering the comparison between SSS and LAN, SSS testcrosses showed higher mean values for PS, ASI, PH, KP, EP and KE, while LAN testcrosses showed a higher mean value for KW.

4.2.3.4 NILs' family 4.10_R55

The ANOVA concerning the testcrosses of NILs 4.10_R55 to unrelated tester lines is reported in Table 22a.

Table 22a. ANOVA: significance of genotype, of the unrelated testers (considering if the testers belong to SSS (A632 and Lo1016) or LAN (Mo17 and Va26) heterotic group) sources of variation and interaction by environments (EN) in Experiment 3, NILs' 4.10_R55 testcrosses to four unrelated testers.

Source of variation	JV	PS	ASI	PH	GYP	KW	KP	EP	KE
BB *vs*. HH	ns	ns	ns	ns	ns	*	ns	ns	ns
SSS *vs*. LAN	ns	ns	ns	**	ns	**	**	*	**
Within SSS, within LAN	*	**	ns	*	**	**	**	ns	**
(BB *vs*. HH) x (SSS *vs*. LAN)	**	ns	ns	ns	ns	ns	ns	ns	ns
(BB *vs*. HH) x (within SSS, within LAN)	**	ns	ns	ns	ns	ns	ns	ns	ns
EN x (BB *vs*. HH)	*	*	ns	ns	ns	ns	ns	ns	ns
EN x (SSS *vs*. LAN)	ns	ns	ns	**	ns	ns	ns	ns	ns
EN x (within SSS, within LAN)	ns	ns	ns	ns	ns	ns	ns	ns	ns
EN x (BB *vs*. HH) x (SSS *vs*. LAN)	ns	ns	ns	ns	ns	ns	ns	ns	ns
EN x (BB *vs*. HH) x (within SSS, within LAN)	ns	ns	ns	ns	ns	ns	ns	ns	ns

**, ** : significant at P ≤ 0.05 and P ≤ 0.01, respectively; ns: non significant.*

Considering the comparison between BB and HH NILs, a significant difference was found for KW only. Considering the comparison between SSS and LAN, a significant difference was found for EP, and highly significant differences emerged for PH, KW, KP and KE.

Considering the comparison within SSS and within LAN, significant differences were detected for JV and PH, and highly significant differences were detected for PS, GYP, KW, KP and KE. The interaction NILs' by (SSS *vs*. LAN) and the interaction NILs by (within SSS, within LAN) were significant for JV only.

The mean values over EN of the eight testcrosses of 4.10_R55 NILs are reported in Table 22b.

Table 22b. Mean values across three environments of the testcrosses among NILs' family 4.10_R55 the four unrelated testers evaluated in Experiment 3.

Genotype	JV (cm)	PS (d)	ASI (d)	PH (cm)	GYP (g)	KW (mg)	KP (no.)	EP (no.)	KE (no.)
BB × A632	105	58.5	0.0	230	147	268	549	1.10	498
BB × Lo1016	99	60.0	0.0	241	175	258	681	1.13	606
BB × SSS	*102*	*59.3*	*0.0*	*236*	*161*	*263*	*615*	*1.12*	*552*
BB × Mo17	111	59.7	0.2	229	157	307	511	0.97	531
BB × Va26	107	58.8	0.0	233	142	284	498	1.04	480
BB × LAN	*109*	*59.3*	*0.1*	*231*	*150*	*296*	*505*	*1.01*	*506*
BB mean	*106*	*59.3*	*0.0*	*233*	*155*	*280*	*560*	*1.06*	*529*
HH × A632	108	58.8	0.5	236	151	257	585	1.09	535
HH × Lo1016	105	60.2	0.8	241	166	261	631	1.07	587
HH × SSS	*107*	*59.5*	*0.7*	*239*	*159*	*259*	*608*	*1.08*	*561*
HH × Mo17	93	59.3	0.2	229	175	299	580	1.08	538
HH × Va26	108	59.2	0.0	229	139	266	524	1.06	495
HH × LAN	*101*	*59.3*	*0.1*	*229*	*157*	*283*	*552*	*1.07*	*517*
HH mean	*103*	*59.4*	*0.4*	*234*	*158*	*271*	*580*	*1.08*	*539*

[a] *mean value of the testcrosses of BB NIL and SSS testers (BB × SSS) and of BB NIL and LAN testers (BB × LAN).*

[b] *mean value of the testcrosses of BB NIL and the four testers.*

[c] *mean value of the testcrosses of HH NIL and SSS testers (HH × SSS) and of HH NIL and LAN testers (HH × LAN).*

[d] *mean value of the testcrosses of HH NIL and the four testers.*

The comparison between BB and HH NIL showed a higher mean value of BB NIL testcrosses for KW. Considering the comparison between SSS and LAN, SSS testcrosses showed higher mean values for PH, KP, EP and KE, while LAN testcrosses showed a higher mean value for KW only. Considering the interaction NILs' by (SSS *vs*. LAN), the testcrosses between heterotic groups (*i.e.*, BB × LAN and HH × SSS) showed higher mean value than that of the four testcrosses within heterotic groups (BB × SSS and HH × LAN) for JV.

4.2.3.5 NILs' family 7.03_R35

The ANOVA concerning the testcrosses of NILs 7.03_R35 to unrelated tester lines is reported in Table 23a.

Table 23a. ANOVA: significance of genotype, of the unrelated testers (considering if the testers belong to SSS (A632 and Lo1016) or LAN (Mo17 and Va26) heterotic group) sources of variation and interaction by environments (EN) in Experiment 3, NILs' 7.03_R35 testcrosses to four unrelated testers.

Source of variation	JV	PS	ASI	PH	GYP	KW	KP	EP	KE
BB *vs*. HH	ns	ns	ns	**	**	ns	**	ns	**
SSS *vs*. LAN	*	**	**	**	**	**	**	**	**
Within SSS, within LAN	*	**	ns	**	**	**	**	**	**
(BB *vs*. HH) x (SSS *vs*. LAN)	ns	ns	ns	*	*	ns	ns	ns	ns
(BB *vs*. HH) x (within SSS, within LAN)	ns	ns	ns	ns	ns	ns	ns	ns	ns
EN x (BB *vs*. HH)	ns	ns	ns	ns	ns	ns	ns	ns	ns
EN x (SSS *vs*. LAN)	ns	**	ns	ns	**	ns	**	ns	ns
EN x (within SSS, within LAN)	ns	*	ns	ns	ns	ns	ns	ns	ns
EN x (BB *vs*. HH) x (SSS *vs*. LAN)	ns	ns	ns	ns	ns	ns	ns	ns	ns
EN x (BB *vs*. HH) x (within SSS, within LAN)	ns	ns	ns	ns	ns	ns	ns	ns	ns

**, ** : significant at P ≤ 0.05 and P ≤ 0.01, respectively; ns: non significant.*

Considering the comparison between BB and HH NILs, highly significant differences were found for PH, GYP, KP and KE. Considering the comparison between SSS and LAN, a significant difference was detected for JV, and highly significant differences were found for all other measured traits. Considering the comparison within SSS and within LAN, significant difference were detected for JV and highly significant differences were detected for all other traits except ASI (non significant). The interaction NILs' by (SSS *vs*. LAN) was significant for PH and GYP; the interaction NILs by (within SSS, within LAN) were non significant for all traits.

The mean values over EN of the eight testcrosses of 7.03_R35 NILs are reported in Table 23b.

Table 23b. Mean values across three environments of the testcrosses among NILs' family 7.03_R35 the four unrelated testers evaluated in Experiment 3.

Genotype	JV (cm)	PS (d)	ASI (d)	PH (cm)	GYP (g)	KW (mg)	KP (no.)	EP (no.)	KE (no.)
BB × A632	112	60.0	0.7	264	178	306	580	1.27	460
BB × Lo1016	111	61.8	0.8	286	215	306	702	1.08	652
BB × SSS	*112*	*60.9*	*0.8*	*275*	*197*	*306*	*641*	*1.18*	*556*
BB × Mo17	113	61.0	0.2	267	173	354	490	0.95	518
BB × Va26	117	59.8	-0.5	262	145	329	439	0.99	445
BB × LAN	*115*	*60.4*	*-0.2*	*265*	*159*	*342*	*465*	*0.97*	*482*
BB mean	*113*	*60.7*	*0.3*	*270*	*177*	*324*	*553*	*1.07*	*519*
HH × A632	107	60.2	0.8	257	172	304	566	1.30	437
HH × Lo1016	110	62.0	1.0	278	206	308	671	1.11	607
HH × SSS	*109*	*61.1*	*0.9*	*268*	*189*	*306*	*619*	*1.21*	*522*
HH × Mo17	107	60.3	-0.2	237	144	347	415	0.92	449
HH × Va26	121	59.5	-0.3	247	130	335	391	0.96	407
HH × LAN	*114*	*59.9*	*-0.3*	*242*	*137*	*341*	*403*	*0.94*	*428*
HH mean	*111*	*60.5*	*0.3*	*255*	*163*	*324*	*511*	*1.07*	*475*

[a] *mean value of the testcrosses of BB NIL and SSS testers (BB × SSS) and of BB NIL and LAN testers (BB × LAN).*

[b] *mean value of the testcrosses of BB NIL and the four testers.*

[c] *mean value of the testcrosses of HH NIL and SSS testers (HH × SSS) and of HH NIL and LAN testers (HH × LAN).*

[d] *mean value of the testcrosses of HH NIL and the four testers.*

The comparison between BB and HH NIL showed a higher mean value of BB NIL testcrosses for all significant traits. Considering the comparison between SSS and LAN, SSS testcrosses showed higher mean values for PS, ASI, PH, GYP, KP, EP and KE, while LAN testcrosses showed higher mean values for JV and KW. Considering the interaction NILs' by (SSS *vs.*

LAN), the testcrosses between heterotic groups (*i.e.*, BB × LAN and HH × SSS) showed higher mean value than those of the four testcrosses within heterotic groups (BB × SSS and HH × LAN) for PH and GYP.

4.2.3.6 NILs' family 10.03_R63

The ANOVA concerning the testcrosses of NILs 10.03_R63 to unrelated tester lines is reported in Table 24a.

Table 24a. ANOVA: significance of genotype, of the unrelated testers (considering if the testers belong to SSS (A632 and Lo1016) or LAN (Mo17 and Va26) heterotic group) sources of variation and interaction by environments (EN) in Experiment 3, NILs' 10.03_R63 testcrosses to four unrelated testers.

Source of variation	JV	PS	ASI	PH	GYP	KW	KP	EP	KE
BB *vs*. HH	ns	ns	ns	ns	ns	ns	ns	ns	*
SSS *vs*. LAN	ns	*	ns	**	*	ns	**	ns	**
Within SSS, within LAN	*	**	ns	**	**	**	**	ns	**
(BB *vs*. HH) x (SSS *vs*. LAN)	ns	ns	ns	ns	ns	ns	ns	ns	ns
(BB *vs*. HH) x (within SSS, within LAN)	ns	ns	ns	ns	ns	ns	*	ns	ns
EN x (BB *vs*. HH)	ns	ns	ns	ns	ns	ns	ns	ns	ns
EN x (SSS *vs*. LAN)	ns	ns	ns	ns	**	ns	**	**	ns
EN x (within SSS, within LAN)	ns	ns	ns	ns	ns	ns	ns	ns	ns
EN x (BB *vs*. HH) x (SSS *vs*. LAN)	ns	ns	ns	ns	ns	ns	ns	ns	ns
EN x (BB *vs*. HH) x (within SSS, within LAN)	ns	ns	ns	ns	ns	ns	ns	ns	ns

**, ** : significant at P ≤ 0.05 and P ≤ 0.01, respectively; ns: non significant.*

Considering the comparison between BB and HH NILs, a significant difference was detected for KE only. Considering the comparison between SSS and LAN, significant differences were found for PS and GYP, and highly significant differences for PH, KP and KE. Considering the comparison within SSS and within LAN, ASI and EP were non significant, JV showed a significant difference and all other traits showed a highly significant difference. The interaction NILs' by (SSS *vs*. LAN) was non significant for all traits. The residual interaction NILs by (within SSS, within LAN) was significant for KP.

The mean values over EN of the eight testcrosses of 10.03_R63 NILs are reported in Table 24b.

Table 24b. Mean values across three environments of the testcrosses among NILs' family 10.03_R63 the four unrelated testers evaluated in Experiment 3.

Genotype	JV (cm)	PS (d)	ASI (d)	PH (cm)	GYP (g)	KW (mg)	KP (no.)	EP (no.)	KE (no.)
BB × A632	101	61.8	0.3	232	108	336	319	0.96	332
BB × Lo1016	102	63.0	-0.2	259	160	343	463	0.97	477
BB × SSS	*102*	*62.4*	*0.1*	*246*	*134*	*340*	*391*	*0.97*	*405*
BB × Mo17	97	62.3	0.0	234	138	357	392	1.01	392
BB × Va26	111	60.8	-0.8	232	111	316	353	1.03	341
BB × LAN	*104*	*61.6*	*-0.4*	*233*	*125*	*337*	*373*	*1.02*	*367*
BB mean	*103*	*62.0*	*-0.2*	*239*	*129*	*338*	*382*	*0.99*	*386*
HH × A632	106	61.3	1.0	243	133	332	399	1.01	395
HH × Lo1016	92	63.8	0.2	258	145	330	438	0.94	469
HH × SSS	*99*	*62.6*	*0.6*	*251*	*139*	*331*	*419*	*0.98*	*432*
HH × Mo17	95	62.5	-0.2	229	134	346	389	0.93	418
HH × Va26	112	60.5	-0.2	233	109	322	340	0.97	353
HH × LAN	*104*	*61.5*	*-0.2*	*231*	*122*	*334*	*365*	*0.95*	*386*
HH mean	*101*	*62.0*	*0.2*	*241*	*130*	*332*	*392*	*0.96*	*409*

[a] *mean value of the testcrosses of BB NIL and SSS testers (BB × SSS) and of BB NIL and LAN testers (BB × LAN).*

[b] *mean value of the testcrosses of BB NIL and the four testers.*

[c] *mean value of the testcrosses of HH NIL and SSS testers (HH × SSS) and of HH NIL and LAN testers (HH × LAN).*

[d] *mean value of the testcrosses of HH NIL and the four testers.*

The comparison between BB and HH NIL showed a higher mean value of HH NIL testcrosses for KE. Considering the comparison between SSS and LAN, SSS testcrosses showed higher mean values for all significant traits.

4.2.3.7 Overall considerations concerning NILs' families tested in Experiment 3

As to Experiment 3, the differences between BB and HH NILs effects in the crosses with the four unrelated testers were significant in some cases, but did not evidence a particular trend. Considering the differences between the two heterotic groups, in five cases out of six SSS testers had higher mean values for PH and KP. In four cases out of six SSS testers had higher mean values for PS, ASI, EP and KE and LAN testers showed higher mean values for KW. A large part of the variation among testcrosses was due to differences among the four inbred testers, with Lo1016 being always later and taller and often more productive than the other three inbreds, but, as previously mentioned, the differences among tester were not a specific interest of this work. It is noteworthy that in all cases of significant NILs' by (SSS *vs*. LAN) interaction (δ), the testcrosses between heterotic groups (*i.e.*, BB × LAN and HH × SSS) showed higher mean value than that of the four testcrosses within heterotic groups (BB × SSS and HH × LAN).

4.3 ANALYSIS OF THE QTL EFFECTS

4.3.1 QTL 3.05

The analysis of QTL 3.05 effects in the three Experiments is presented in Table 25 and 26, according to the two backgrounds R08 and R40.

Table 25. Effects and dominance ratios of the QTL 3.05_R08 investigated in Experiments 1, 2 and 3.

Trait	Experiment 1			Experiment 2 [a]			Experiment 3	
	a [b]	*d* [b]	|*d*/*a*|	*a* [c]	*d* [c]	|*d*/*a*|	α [d]	δ [e]
JV (cm)	-	-	-	3.8	6.9 **	1.8	-3.3	1.9
PS (d)	-	-	-	0.4	-0.1	0.2	0.1	0.3
ASI (d)	-	-	-	0.1	0.0	0.2	0.1	0.0
PH (cm)	-	-	-	1.6	2.3	1.5	4.4	-2.7
SD (mm)	-	-	-	1.6 **	0.2	0.1	-	-
GYP (g)	-	-	-	3.8	9.2 **	2.4	-1.1	3.6
KW (mg)	-	-	-	-4.7	6.7 **	1.4	-10.0	-12.6
KP (no.)	-	-	-	20.3	21.6 *	1.1	13.7	39.8 *
EP (no.)	-	-	-	0.03	0.01	0.3	0.06 *	0.00
KE (no.)	-	-	-	4.5	12.6	2.8	-15.2	33.7 *

**, ** : effect significant at P ≤ 0.05 and P ≤ 0.01, respectively; -: trait non estimated.*

[a] mean values across two plant densities.

[b] a: additive effect calculated as (HH – BB)/2; d: dominance effect calculated as the difference between the mean value of the two RC and the mean value of the two NILs.

[c] a: additive effect calculated as the difference between the testcrosses' mean value of HH and the testcrosses' mean value of BB [i.e., (HH × B73 + HH × H99)/2 - (BB × B73 + BB × H99)/2]; d: dominance effect calculated as the difference between the mean value (BB × H99 + HH × B73)/2 and the mean value (BB × B73 + HH × H99)/2.

[d] α: average effect of the QTL allele substitution calculated as the difference between the testcrosses' mean value of HH and the testcrosses' mean value of BB.

[e] δ :effect of the (SSS vs. LAN) × NILs interaction, calculated as the difference between the mean value of the four testcrosses between heterotic groups (i.e., BB × LAN and HH × SSS) and the mean value of the four testcrosses within heterotic groups (BB × SSS and HH × LAN).

QTL 3.5_R08 was not investigated in Experiment 1. In Experiment 2, additive effect (*a*) was significant for SD only. Dominance effect (*d*) was significant for JV, GYP and its components KW and KP. For all these latter traits, |*d*/*a*| ratio was higher than 1, indicating overdominance; in particular, the value of |*d*/*a*| for GYP was 2.4. In Experiment 3, the average effect of the QTL allele substitution (α) was significant for EP; the interaction (SSS

vs. LAN) × (BB *vs*. HH) (δ) was significant for KP and KE and the effect was positive, indicating that the NILs BB and HH performed relatively better with the two inbred testers of the opposite heterotic group.

The effects of the QTL in bin 3.05 was also studied in the family R40 (*i.e.*, 3.05_R40) and in all the three Experiments (Table 26).

Table 26. Effects and dominance ratios of the QTL 3.05_R40 investigated in Experiments 1, 2 and 3.

Trait	Experiment 1			Experiment 2 [a]			Experiment 3	
	a [b]	*d* [b]	|*d*/*a*|	*a* [c]	*d* [c]	|*d*/*a*|	α [d]	δ [e]
JV (cm)	-0.9	2.1	2.3	-2.4	-1.1	0.5	-3.7	2.0
PS (d)	0.3	-0.7	2.3	0.2	0.1	0.4	-0.1	0.0
ASI (d)	0.1	-0.2	1.9	0.7 **	-0.3	0.4	0.6 **	0.9 **
PH (cm)	-2.5	6.0 **	2.4	0.8	2.7	3.4	-3.2	0.7
SD (mm)	0.9	1.0 *	1.1	2.0 **	-0.2	0.1	-	-
GYP (g)	9.4 *	13.2 **	1.4	6.0 *	8.3 **	1.4	9.4 **	10.8 **
KW (mg)	13.8 *	5.5	0.4	6.9 **	4.7	0.7	9.7 **	3.1
KP (no.)	27.1	59.0 **	2.2	8.8	23.7 **	2.7	16.6	33.7 **
EP (no.)	0.06	0.05	0.8	0.02	-0.02	0.9	-0.02	0.02
KE (no.)	6.8	28.6 **	4.2	0.8	20.3 **	24.1	28.4 **	25.0 *

**, ** : effect significant at P ≤ 0.05 and P ≤ 0.01, respectively; -: trait non estimated.*

[a] mean values across two plant densities.

[b] a: additive effect calculated as (HH – BB)/2; d: dominance effect calculated as the difference between the mean value of the two RC and the mean value of the two NILs.

[c] a: additive effect calculated as the difference between the testcrosses' mean value of HH and the testcrosses' mean value of BB [i.e., (HH × B73 + HH × H99)/2 - (BB × B73 + BB × H99)/2]; d: dominance effect calculated as the difference between the mean value (BB × H99 + HH × B73)/2 and the mean value (BB × B73 + HH × H99)/2.

[d] α: average effect of the QTL allele substitution calculated as the difference between the testcrosses' mean value of HH and the testcrosses' mean value of BB.

[e] δ :effect of the (SSS vs. LAN) × NILs interaction, calculated as the difference between the mean value of the four testcrosses between heterotic groups (i.e., BB × LAN and HH × SSS) and the mean value of the four testcrosses within heterotic groups (BB × SSS and HH × LAN).

In both Experiments 1 and 2, *a* effect for GYP was significant, and it was mainly due to the component KW; moreover, the *a* effect was positive in both Experiments, indicating that the increasing allele was provided by H99. Significant and positive *a* effects were also found for ASI and SD in Experiment 2. The *d* effect was significant in both experiments for several traits and, in particular, for GYP and its components KP and KE. The |*d*/*a*| ratio for

GYP was in the overdominance range, being 1.4 for both Experiments 1 and 2. In Experiment 3, α was significant and positive for ASI as well as for GYP and its components KW and KE; δ was significant for ASI, GYP, KE and KP and the effect was always positive.

A combined analysis of the QTL effects over the two families was conducted for Experiments 2 and 3 (not shown). The results confirmed the significance of both the *a* and *d* effects in Experiment 2 for GYP; the overdominant gene action for GYP was confirmed, with $|d/a|$ ratio of 1.6. The interactions of *a* and *d* effects with families was not significant in almost all instances, suggesting that the gene action of the QTL 3.05 was not much affected by the genetic background in testcrosses with related testers.

For Experiment 2, the combined ANOVA also revealed the significance of the interaction PD × *a* effect for GYP. This interaction (Fig.10) was of size, since *a* showed a high value in LPD and a low value in HPD; in addition, *d* effect did not vary significantly from LPD to HPD. As a consequence, $|d/a|$ ratio proved to be much higher at 9.0 rather than at 4.5 plants m^{-2} (3.0 and 1.2, respectively) (Fig.10).

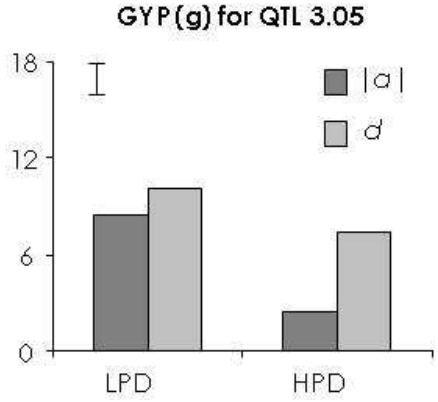

Fig.10 |a| and d value of QTL 3.05 over genetic background in low (LPD) and high (HPD) plant density.

As to Experiment 3, the significance of the interaction (SSS *vs*. LAN) × (BB *vs*. HH) was confirmed for GYP, for KE and KP.

4.3.2 QTL 4.10

The analysis of effects of QTL 4.10 in the three Experiments is presented in Table 27 and 28, according to the two backgrounds R40 and R55.

Table 27. Effects and dominance ratios of the QTL 4.10_R40 investigated in Experiments 1. 2 and 3.

Trait	Experiment 1			Experiment 2 [a]			Experiment 3	
	a [b]	*d* [b]	|*d*/*a*|	*a* [c]	*d* [c]	|*d*/*a*|	α [d]	δ [e]
JV (cm)	-4.0	5.4	1.4	-4.3 **	2.4	0.6	3.7	1.6
PS (d)	-1.9	-0.3	0.1	-1.2 **	-1.0 **	0.9	-1.2 **	0.0
ASI (d)	-0.8	0.1	0.2	-0.3	0.1	0.2	0.2	0.1
PH (cm)	-9.8 **	8.1 **	0.8	-4.1 **	3.6 **	0.9	-2.4	-0.8
SD (mm)	-0.4	1.5 **	4.4	1.7 **	0.1	0.1	-	-
GYP (g)	2.9	20.1 **	7.0	-8.2 **	16.5 **	2.0	-5.1	4.1
KW (mg)	9.1 **	-7.0	0.8	19.7 **	-1.3	0.1	30.1 **	-3.1
KP (no.)	4.1	118.3 **	28.7	-73.1 **	64.3 **	0.9	-68.8 **	14.5
EP (no.)	-0.04	0.02	0.5	-0.03	0.09 **	3.0	0.01	0.00
KE (no.)	16.6	103.5 **	6.2	-51.8 **	23.2 *	0.4	-73.6 **	13.8

**, ** : effect significant at P ≤ 0.05 and P ≤ 0.01, respectively; -: trait non estimated.*

[a] mean values across two plant densities.

[b] a: additive effect calculated as (HH – BB)/2; d: dominance effect calculated as the difference between the mean value of the two RC and the mean value of the two NILs.

[c] a: additive effect calculated as the difference between the testcrosses' mean value of HH and the testcrosses' mean value of BB [i.e., (HH × B73 + HH × H99)/2 - (BB × B73 + BB × H99)/2]; d: dominance effect calculated as the difference between the mean value (BB × H99 + HH × B73)/2 and the mean value (BB × B73 + HH × H99)/2.

[d] α: average effect of the QTL allele substitution calculated as the difference between the testcrosses' mean value of HH and the testcrosses' mean value of BB.

[e] δ :effect of the (SSS vs. LAN) × NILs interaction, calculated as the difference between the mean value of the four testcrosses between heterotic groups (i.e., BB × LAN and HH × SSS) and the mean value of the four testcrosses within heterotic groups (BB × SSS and HH × LAN).

Considering QTL 4.10 in background R40 (Table 27), *a* effect in Experiment 1 was significant for PH and KW; *a* effect had different signs, being negative for the former trait (*i.e.*, the allele leading to an increase in trait came from B73) and positive for the latter (*i.e.*, the allele leading to an increase in trait came from H99). Dominance effect was significant for

several traits; in particular, it was highly significant for GYP. In Experiment 2, the *a* effect was significant in most instances, including GYP, and generally negative, indicating that the increasing allele was provided by B73. The *d* effect was significant for ASI (*d* negative), PH, GYP, KP, EP and KE (*d* positive). The $|d/a|$ ratio for GYP was superior to 1. In Experiment 3, α was significant for some traits but not for GYP, whereas δ was not significant for any trait.

Table 28. Effects and dominance ratios of the QTL 4.10_R55 investigated in Experiments 1. 2 and 3.

Trait	Experiment 1			Experiment 2			Experiment 3	
	a [b]	*d* [b]	|*d*/*a*|	*a* [c]	*d* [c]	|*d*/*a*|	α [d]	δ [e]
JV (cm)	-3.8	7.1	1.9	0.6	4.2 *	7.2	-2.2	6.4 **
PS (d)	-0.4	-0.3	0.7	0.4	-0.9 **	2.4	0.1	0.1
ASI (d)	0.1	-0.1	1.0	-0.1	0.2	1.2	0.3	0.3
PH (cm)	-6.1 *	3.3	0.5	-7.7 **	1.0	0.1	0.6	2.5
SD (mm)	0.3	1.1	3.4	1.9 **	-0.2	0.1	-	-
GYP (g)	-8.8	18.8 **	2.1	-7.5 **	6.8 **	0.9	2.2	-5.0
KW (mg)	1.4	-0.8	0.6	-4.4 *	4.0	0.9	-8.8 *	4.9
KP (no.)	-64.1	128.3 **	2.0	-24.3 **	22.2 *	0.9	20.1	-27.3
EP (no.)	-0.10	0.21	2.2	0.02	0.04 *	2.4	0.02	-0.04
KE (no.)	-33.5	80.4 **	2.4	-27.3 **	3.5	0.1	10.4	-0.9

**, ** : effect significant at P ≤ 0.05 and P ≤ 0.01, respectively.*

[a] mean values across two plant densities.

[b] a: additive effect calculated as (HH – BB)/2; d: dominance effect calculated as the difference between the mean value of the two RC and the mean value of the two NILs.

[c] a: additive effect calculated as the difference between the testcrosses' mean value of HH and the testcrosses' mean value of BB [i.e., (HH × B73 + HH × H99)/2 - (BB × B73 + BB × H99)/2]; d: dominance effect calculated as the difference between the mean value (BB × H99 + HH × B73)/2 and the mean value (BB × B73 + HH × H99)/2.

[d] α: average effect of the QTL allele substitution calculated as the difference between the testcrosses' mean value of HH and the testcrosses' mean value of BB.

[e] δ :effect of the (SSS vs. LAN) × NILs interaction, calculated as the difference between the mean value of the four testcrosses between heterotic groups (i.e., BB × LAN and HH × SSS) and the mean value of the four testcrosses within heterotic groups (BB × SSS and HH × LAN).

As for background R55 for QTL 4.10 (Table 28), *a* effect, when significant, was negative for all traits (except for SD) in both Experiments 1 and 2, confirming that the increasing allele for QTL 4.10 derived from B73. Significant *d* effects were positive, except for PS. Considering GYP, *a* effect was significant only in Experiment 2, whereas *d* effect was significant in both

Experiments. GYP |*d*/*a*| ratio was slightly lower than 1 in Experiment 2. In Experiment 3, significant effects were found for JV (δ) and KW (α) only.

Considering the two families of QTL 4.10, a combined ANOVA over families (not shown) was made. The results in both Experiments 1 and 2 confirmed that significant effects were generally negative for *a*, while were always positive for *d,* except for PS. Focusing on GYP, *d* effect was significant in both experiments, with the |*d*/*a*| ratio higher than 1, especially in Experiment 1. The combined ANOVA revealed a significant interaction PD × *d* for GYP. Also in this case, the interaction was of size, since *d* showed a higher value in HPD than LPD. In addition, *a* value was not significantly affected by PD; consequently, the |*d*/*a*| ratio was reduced from LPD to HPD (being 1.9 in the former case and 0.9 for the latter). For a better insight, *a* (as absolute values) and *d* effects at the two PD are presented in Fig.11.

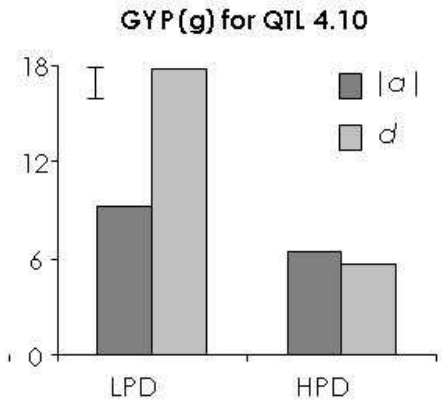

Fig.11: |a| and d value of QTL 4.10 over genetic background in low (LPD) and high (HPD) plant density.

No significant effects were found in the combined analysis over families in Experiment 3.

Considering the interactions with families, those involving *a* effects weres significant in five cases out of ten for both Experiment 1 (ASI, GYP, KW, KE and KP) and Experiment 2 (EV, PS, PH, KW, KP). The interactions involving *d* effect were not significant in Experiment 1 and

significant for two traits in Experiment 2 (GYP and KP). Considering in particular GYP, *a* and *d* effects revealed significant interaction with families (in Experiment 1 and 2, respectively), indicating an important role of the genetic background.

4.3.3 QTL 7.03

The analysis of effects of QTL 7.03_R35 in the three Experiments is presented in Table 29.

Table 29. Effects and dominance ratios of the QTL 7.03_R35 investigated in Experiments 1. 2 and 3.

Trait	Experiment 1			Experiment 2			Experiment 3	
	a [b]	d [b]	$\|d/a\|$	a [c]	d [c]	$\|d/a\|$	α [d]	δ [e]
JV (cm)	-8.1	2.3	0.3	-1.9	-0.4	0.2	-1.8	-0.7
PS (d)	-0.6	0.4	0.6	-0.2	-0.2	1.3	-0.2	0.3
ASI (d)	0.1	-0.9	7.0	-0.3	0.3	1.0	0.0	0.1
PH (cm)	-7.7 *	15.7 **	2.0	-8.6 **	2.9 *	0.3	-14.8 **	7.5 *
SD (mm)	0.4	-0.69	1.8	2.6 **	0.3	0.1	-	-
GYP (g)	-3.1	7.5	2.4	-10.1 **	4.0	0.4	-14.4 **	7.3 *
KW (mg)	-9.4 **	8.3 *	0.9	-12.1 **	0.1	0.0	-0.2	0.1
KP (no.)	-6.6	23.2	3.5	-9.9	12.5	1.3	-42.3 **	19.3
EP (no.)	0.05	0.13	2.4	-0.01	-0.01	0.9	0.00	0.03
KE (no.)	-15.9	1.2	0.1	-4.1	17.3	4.3	-43.8 **	9.6

**, ** : effect significant at P ≤ 0.05 and P ≤ 0.01, respectively; -: trait non estimated.*

[a] mean values across two plant densities.

[b] a: additive effect calculated as (HH – BB)/2; d: dominance effect calculated as the difference between the mean value of the two RC and the mean value of the two NILs.

[c] a: additive effect calculated as the difference between the testcrosses' mean value of HH and the testcrosses' mean value of BB [i.e., (HH × B73 + HH × H99)/2 - (BB × B73 + BB × H99)/2]; d: dominance effect calculated as the difference between the mean value (BB × H99 + HH × B73)/2 and the mean value (BB × B73 + HH × H99)/2.

[d] α: average effect of the QTL allele substitution calculated as the difference between the testcrosses' mean value of HH and the testcrosses' mean value of BB.

[e] δ :effect of the (SSS vs. LAN) × NILs interaction, calculated as the difference between the mean value of the four testcrosses between heterotic groups (i.e., BB × LAN and HH × SSS) and the mean value of the four testcrosses within heterotic groups (BB × SSS and HH × LAN).

In both Experiments 1 and 2, significant *a* effects were always negative, whereas the significant *d* effects were always positive. For this QTL, PH is a trait of peculiar interest, since this was the main trait for which this QTL was selected. So, considering PH, *a* and *d* effects were significant in both Experiments, with the $|d/a|$ ratio largely superior to 1 in Experiment 1 but lower than 1 in Experiment 2. Considering GYP, *a* effect was significant in Experiment

2 only, whereas *d* effect was not significant. The interactions of PD and *a* and *d* effects were both significant for PH. From LPD to HPD, *a* showed a clear decline, thus accounting for PD x *a* interaction, while *d* showed an opposite trend, accounting for PD x *d* interaction. As a result, |*d*/*a*| ratio was close to 0 at LPD and 0.8 at HPD (Fig.12).

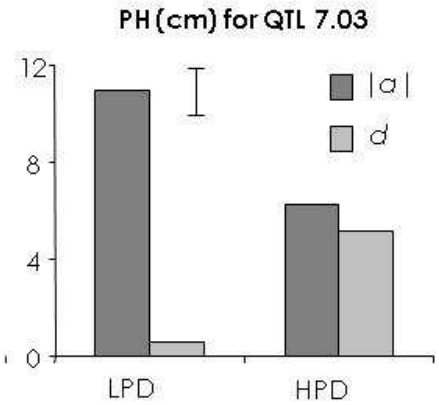

Fig.12: |a| and d value of QTL 7.03 in low (LPD) and high (HPD) plant density.

In Experiment 3, the average effect of allele substitution was significant for several traits, including PH and GYP, and was always negative. The interaction (SSS *vs*. LAN) × (BB *vs*. HH) was significant for PH and GYP with positive effects in both cases.

4.3.4 QTL 10.03

The analysis of effects of QTL 10.03_R35 in the three Experiments is presented in Table 30.

Table 30. Effects and dominance ratios of the QTL 10.03_R63 investigated in Experiments 1. 2 and 3.

Trait	Experiment 1			Experiment 2 [a]			Experiment 3	
	a [b]	*d* [b]	|*d*/*a*|	*a* [c]	*d* [c]	|*d*/*a*|	α [d]	δ [e]
JV (cm)	-	-	-	-2.4	11.7 **	4.9	-1.8	-0.9
PS (d)	-	-	-	-0.2	-1.1 **	5.9	0.0	0.1
ASI (d)	-	-	-	-0.6 *	-0.8 **	1.2	0.4	0.1
PH (cm)	-	-	-	0.5	1.3	2.4	1.8	3.5
SD (mm)	-	-	-	1.6 **	0.2	0.1	-	-
GYP (g)	-	-	-	2.8	22.4 **	8.0	1.2	4.0
KW (mg)	-	-	-	-9.8 **	5.9 *	0.6	-5.6	-3.1
KP (no.)	-	-	-	25.3	67.4 **	2.7	10.0	17.3
EP (no.)	-	-	-	0.06	0.10 **	1.8	-0.03	0.04
KE (no.)	-	-	-	5.8	38.6 **	6.7	23.3 *	4.3

**, ** : effect significant at P ≤ 0.05 and P ≤ 0.01, respectively; -: trait non estimated.*

[a] mean values across two plant densities.

[b] a: additive effect calculated as (HH – BB)/2; d: dominance effect calculated as the difference between the mean value of the two RC and the mean value of the two NILs.

[c] a: additive effect calculated as the difference between the testcrosses' mean value of HH and the testcrosses' mean value of BB [i.e., (HH × B73 + HH × H99)/2 - (BB × B73 + BB × H99)/2]; d: dominance effect calculated as the difference between the mean value (BB × H99 + HH × B73)/2 and the mean value (BB × B73 + HH × H99)/2.

[d] α: average effect of the QTL allele substitution calculated as the difference between the testcrosses' mean value of HH and the testcrosses' mean value of BB.

[e] δ :effect of the (SSS vs. LAN) × NILs interaction, calculated as the difference between the mean value of the four testcrosses between heterotic groups (i.e., BB × LAN and HH × SSS) and the mean value of the four testcrosses within heterotic groups (BB × SSS and HH × LAN).

As for QTL 10.03_R63, *a* effect in Experiment 2 was significant for ASI, SD and KW, having different signs. Dominance effect was significant for PS and ASI, with a negative sign, and for JV, GYP and all its components with a positive sign. In particular, *d* effect observed for GYP was very high while the corresponding *a* effect was not significant, suggesting an

overdominant action of the QTL. In Experiment 3, the effects were not significant in almost all instances.

DISCUSSION

Science is simply common sense at its best.
Thomas Huxley

5.1 CHARACTERIZATION OF HETEROTIC QTL AND IMPORTANCE OF THEIR EFFECTS

The effects of the QTL chosen for the present study, reported in Tables 25 – 30, proved to be consistent with the effects that the same QTL had exhibited in the previous studies conducted by Frascaroli *et al.*(2007) and by Pea *et al.*(2009). In fact, the QTL showed important *d* effects, especially referring to the traits they were selected for: QTL 3.05, 4.10 and 10.03 showed important d effects for GYP and for its evaluated components, and QTL 7.03 showed a sizable *d* effect for PH. The consistency of the effects and of the trait they have effect on is a very important result at first instance, because this consistency confirms the results previously observed, being relevant the risk of obtaining false positive and/or inflated estimates of QTL effects. This mentioned risk becomes important especially when the reference mapping population is not large in size (e.g., N < 200), when QTL mapping and estimates of effects are based on the same data, and when dealing with complex traits (Beavis *et al.*, 1994; Kearsey and Farquhar, 1998; Melchinger *et al.*, 1998; Schön *et al.*, 2004). Moreover, considering QTL 7.03 for PH, the present research allowed the estimate of a significant and negative *a* effect, that was not detected in the previous QTL analysis of Frascaroli *et al.* (2007). The result observed for QTL 7.03 emphasizes the relevance of the use of genetic materials like NILs for the analysis of QTL main effects, given the absence of biases due to the genetic background.

In addition, *a* effects estimated for GYP were positively associated with *a* effects of its component KP and also with PH, especially in the two NILs' families of QTL 4.10 and of QTL 7.03. This positive associations among *a* effects were also found in previous QTL studies like Stuber *et al.* (1992) and also in the previous work of Frascaroli *et al.* (2007) and were ascribed to close linkage and/or to pleiotropy. Considering *d* effects, they were consistent when considering GYP, its component KP and, to some extent, the other component KE. In all other cases the consistency of the *d* effects was weaker or even negligible, mainly because of the modest importance of the *d* effects for the other two GYP components KW and EP. It should be noted that GYP and its main component KP are the result of a multiplicative function of their simpler components, which can show from negligible to complete dominance. These findings are consistent with the results noted in other studies conducted in maize (Lu *et al.*, 2003) or in other species like tomato (Semel *et al.*, 2006) and even in animals; such studies underline the association of high $|d/a|$ ratio of the QTL and fitness-related traits. This particular association seems to have had a role in evolution, since it is reasonable to hypothesize that natural selection for reproductive fitness acted on QTL comprising single genes or multiple linked genes acting as a complex Mendelian locus (Semel *et al.*, 2006). Moreover, the findings of the present research concerning GYP and its components indicate that the high heterotic level of complex traits is important both considering the whole contribution of many loci in crosses between different inbreds (Tollenaar *et al.*, 2004; Yan *et al.*, 2006) but also in crosses of NILs families differing for just one QTL (Melchinger *et al.*, 2007a; Semel *et al.*, 2006). Concerning this topic, Falconer and Mckay (1996) pointed out that heterosis for a complex trait can arise even in case of a single gene acting additively on trait's components and affecting them in a pleiotropic way and in opposite directions. As an example, let us consider the complex trait *XY* (e.g., plant height) and its two components *X* (e.g., average number of internode) and *Y* (e.g.,

average length of the internodes); let us also assume that for trait *X* the three genotypes *A1A1*, *A1A2* and *A2A2* show 13, 14 and 15, respectively, whereas for trait *Y* the three genotypes perform in the opposite way, *i.e.*, 15 cm, 14 cm and 13 cm, respectively. As a result, despite the clear additivity concerning both component traits *X* and *Y*, there is overdominance for the complex trait *XY*, the performance being 195 cm for both homozygotes and 196 cm for the heterozygote.

The present research also shows that significant *d* effects were always negative for PS and ASI, and always positive for all other traits. These findings are in accordance, because the negative *d* effects for PS and ASI are indicative of a more rapid growth and of a better synchronization between male and female flowering. The data reported in the present research thus confirmed that the dominant alleles are the ones more favorable and that unidirectional *d* effects (*i.e.*, either all positive or all negative in algebraic terms) are an essential prerequisite to attain a high heterotic level in hybrids.

5.2 ROLE OF GENETICK BACKGROUND (FAMILIES) ON QTL EFFECTS

In this study, QTL 3.05 and 4.10 were available in two families each. Consequently, the QTL effects were estimated in two different genetic backgrounds and the interactions FAM × QTL effects was analyzed. Considering QTL 3.05, the interactions proved to be negligible in almost all instances, thus suggesting that the QTL is quite stable across genetic backgrounds, *i.e.*, that epistatic interactions are not relevant. However, this hypothesis should be considered cautiously, since only two families were investigated. As for QTL 4.10, the interactions FAM × *a* and FAM × *d* were both significant for a number of traits, especially GYP. So, the possibility that marker-assisted selections (MAS) for this QTL might lead to unfavourable results (depending on the genetic background of the recipient

material) should not be ignored. The role of the genetic background in MAS was investigated in several studies on different species (Bouchez *et al.*, 2002; Chaïb *et al.*, 2006; Reyna and Sneller, 2001) and a lack of consistency of QTL effects was not rare. This inconsistency was evident especially when the QTL were inserted into unrelated genetic backgrounds and when complex traits were considered. However, inconsistent results in different NILs families could also arise from the contribution of small chromosome segments (relics) independent from the target QTL and fixed randomly in the genome (Paterson *et al.*, 1990). This last observation could be also true for the NILs investigated in the present work, and actually the effects of these relics could bias both the effect of the QTL of interest and its interaction with the genetic background. Pea *et al.* (2009) characterized the NILs investigated in the present work for 19 SSR markers present in each chromosome arm different from the one carrying the introgressed QTL. The results of that work of marker characterization pointed out that the pair of NILs of each family were always identical, with one only exception for family 4.10_R55. The two NILs of QTL 4.10_R55 differed for the marker alleles identifying the long arm of chromosome 2; therefore, this difference between NILs could have contributed, to some extent, to the significant interaction FAM × QTL effects detected for some traits for QTL 4.10.

5.3 ROLE OF INBREEDING LEVEL ON QTL EFFECTS

The different inbreeding levels in the three Experiments of the present work were meaningful, since they allowed the investigation of the QTL effects at both extremes (*i.e.*, $F = 1$ in Experiment 1 and $F = 0$ in Experiment 3) as well at an intermediate level (*i.e.*, $F = 0.5$ in Experiment 2). Comparing Experiment 1 and 2, the consistency of a effects was relevant, indicating that these effects were not much influenced by the inbreeding level. A different

situation was noted for *d* effects. The expectation was to note a higher *d* effect in the more vigorous material of Experiment 2, as compared to the inbred material of Experiment 1, at least because of a possible positive relationship between mean values and *d* effects (*i.e.*, scaling effects). Actually, the *d* effects were consistent in these two Experiments, but, unexpectedly, they were more pronounced for the inbred materials of Experiment 1, especially considering both families of QTL 4.10 for GYP and KP, and QTL 7.03 for PH. Also the $|d/a|$ ratio followed a similar trend, showing more often higher values in Experiment 1 than in Experiment 2. These findings can not be ascribed to scaling effects, since, as expected, the mean values of Experiment 1 were much lower than mean values of Experiment 2. The results thus suggest that the estimate of *d* for the QTL of interest can vary depending on the homozygosity level of the background. Such an influence of the background could be accounted for by assuming that the heterozygote target QTL in highly inbred material gives rise to a more appreciable phenotypic performance; on the contrary, the same heterozygote QTL in a more vigorous background, like in the testcrosses of Experiment 2, has a less pronounced effect in the phenotype, because the hybrids have greater biochemical versatility and, hence, may allow the attainment of the same QTL function by following different pathways. At least to some extent, this hypothesis recalls the concept of 'marginal contribution', being the relative contribution of a single heterozygote locus more pronounced in materials with *F* close to 1 than in materials having more heterozygous loci (*i.e.*, lower *F*). This hypothesis is also consistent with the observation that heterosis can be affected by dosage dependent regulatory genes operating in hierarchical networks and interacting with genes expressed downstream (Birchler *et al.*, 2010).

For all four investigated QTL, *a* effects detected in Experiment 1 and 2 showed a certain consistency with the average effects of allele substitution (α) detected for the same traits in

Experiment 3. When *a* effects and α were significant, they showed in most cases similar size and always the same sign. These findings are noteworthy, because *a* and α are comparable only when *p = q = 0.5* across the four testers, especially in those cases where *d* effects are not negligible, like for the heterotic QTL of the present study [$\alpha = a + d(q - p)$]. The consistency of *a* and α thus indicates that the four unrelated testers do not carry all the same dominant alleles at the QTL of interest. Actually, homozygosity for the same dominant alleles in all inbred testers would have implied *p = 1* and *q = 0*, and these allelic frequencies would have led to the cancellation of the effects of the QTL allele substitution (α) in case of complete dominance. The importance of the role of testers in affecting QTL effects detection was evaluated by Frascaroli *et al.* (2009). The role played by different testers proved to markedly influence the estimate of the QTL effects and also proved to vary depending on the tester used and on the investigated trait. In Frascaroli *et al.* (2009), an unrelated tester line seemed to be more effective in QTL mapping and estimating effects for traits with mainly additive control; in contrast, for traits characterized by prevailing dominant or overdominant gene action, the high performing related tester was extremely less effective. Actually, a change in tester can even lead to a change in sign of the effects, in case of QTL showing overdominance (Frascaroli *et al.*, 2009).

Moreover, the significant interaction TS × (BB *vs*. HH), detected especially for QTL 3.05 and 7.03, was mainly due to the component (SSS *vs*. LAN) × (BB *vs*. HH), since the other component (within SSS, within LAN) × (BB *vs*. HH) was a negligible residual. As mentioned, the interaction involving heterotic groups and NILs consisted on the comparison between the performance of crosses realized between materials belonging to the same heterotic groups and the performance of crosses between heterotic groups. The effect of (SSS *vs*. LAN) × (BB *vs*. HH), when significant, was always positive, thus indicating the relative superiority of the crosses that, at the QTL under study, carried alleles deriving from opposite

heterotic groups. Actually, the BB NIL, homozygous for the QTL allele coming from B73 (thus of SSS origin), performed relatively better when combined with testers of LAN group, whereas the HH NIL, homozygous for the QTL allele coming from H99 (thus of LAN origin), performed relatively better with SSS inbred testers. These results suggest that, for each QTL, the two unrelated inbred testers of a given heterotic group (e.g., A632 and Lo1016 for SSS) are homozygous for similar (or even the same) allele/s as the allele provided by the parental inbred of the same group (*i.e.*, B73). The same should be true for the other two inbred testers (Mo17 and Va26), which can be assumed to be homozygous for similar (or even the same) complementary allele/s as the allele provided by the other parental inbred (H99). This hypothesis is consistent with the hypothesis expressed by Schön *et al.* (2010), who studied the congruency of heterotic QTL detection and estimate of effects in three different mapping populations, including the one of the work of Frascaroli *et al.* (2007), all arising from the same heterotic pattern SSS × LAN. Schön *et al.* (2010) suggested that, for important loci affecting heterosis, complementary alleles are fixed in the two opposite heterotic groups, and that they remain essentially unchanged in the subsequent within-group selections, until new genetic variation is introduced with genetic material of different origin.

5.4 Role of competition level on QTL effects

Two different competition levels were realized with PD in Experiment 2. Despite the large effects of PD for almost all traits, the interactions between PD and QTL effects were often negligible, thus giving a further confirmation of QTL stability. A possible criticism that could be addressed to this finding is that the competition level among plants, even at 9.0 plants m^{-2} in our quite favorable environments, was not as high as needed to attain a rather

discriminative growing condition. Previous studies (Duvick, 2005; Liu and Tollenaar, 2009) reported an increase of heterosis observed as a response to increasing plant density. On the other hand, it must be emphasized that single QTL were evaluated in the present research, while the mentioned works of Duvick (2005) and Liu and Tollenaar (2009) studied the effects of a multitude of QTL acting together; hence, those studies also took into account the possible contribution of the complex interactions among all such QTL. However, some important exceptions were noted in our study; these exceptions were represented by *a* effect of QTL 3.05 and *d* effect of QTL 4.10 (both across families) for GYP and by both *a* and *d* effects of QTL 7.03_R35 for PH. Therefore, at least for these traits and QTL, the competition level among plants played a certain role in influencing their effects, and this aspect should not be neglected in possible future studies on such QTL. The role exerted by plant density on the single QTL effects was investigated by Gonzalo *et al.* (2006); they tested segmental introgression lines (derived from the cross between B73 and Tx303 inbred lines) and their hybrids in crosses with Mo17, and found that the QTL effects for inbreds and their crosses varied depending on PD. On the other hand, in a study conducted on a population of RILs derived from the cross B73 × Mo17, LeDeaux *et al.* (2006) found that heterotic QTL were rather stable at varying stress levels, including low and high plant density, with very few QTL being affected. A possible explanation for these contrasting findings could be that in the study of LeDeaux *et al.* (2006) both parents were well adapted to temperate climatic regions, like the materials tested for the present work, whereas in the study of Gonzalo *et al.* (2006) one parent was of subtropical origin.

5.5 OVERALL CONSIDERATIONS CONCERNING QTL EFFECTS

The importance of the effects of the investigated QTL provides the stimulus to conduct further studies on the materials herein presented. In particular, studies of fine-mapping could be made, so as to gain useful information on the causes of the association among traits (linkage *vs.* pleiotropy) and on the causes of QTL heterotic effect (true overdominance *vs.* pseudo-overdominance). McMullen *et al.* (2009) pointed out that centromeric regions are characterized by low recombination rate, and so can be associated with heterotic phenomena determined by linkage of favorable alleles in repulsion phase (pseudo-overdominance). In this connection, it is noteworthy that bins 3.05 and 10.03 are centromeric and that bin 7.03 is adjacent to the centromeric bin 7.02. Moreover, the average length of the introgressed chromosome segments was of ca. 22 cM (Pea *et al.*, 2009) and, hence, the possibility that two or even more linked genes controlling the same trait are included in these segments should not be neglected. This could be the case of GYP for QTL 10.03, which showed the highest *d* effect of all investigated QTL associated with a negligible *a* effect, suggesting genes linked in repulsion. Also QTL 3.05 and 4.10 are of great interest for fine mapping because of the importance of their dominance effects and because two different NILs' families are available for each QTL. In particular, for QTL 4.10 the significance of both interactions FAM × *a* and FAM × *d* for GYP suggests the choice of the NILs' family to be used as the base material for fine mapping should be made carefully. Following this consideration, family 4.10_R55 seems to be more suitable than 4.10_R40, because the former proved to be less prone to interactions with PD. As to QTL 7.03, it seems to be the most appealing of the investigated QTL for fine-mapping, because the phenotyping can be made on plant height, *i.e.*, a trait easily measurable, with high heritability and less affected by inbreeding depression as compared with grain

yield. Moreover, plant height is an interesting model trait for relating the expression of genes and the manifestation of heterosis, as pointed out by Uzarowska *et al.* (2007).

NILs' families developed for the present research can also represent a valuable base material to undertake studies aimed at elucidating the molecular bases of heterosis. Structural genome diversity between inbred lines, as well as gene and allelic expression diversity between parental lines and their corresponding F_1 hybrids, have been described in relation to heterosis (Hochholdinger and Hoecker, 2007; Springer and Stupar 2007a). In recent studies, high levels of structural genome diversity, which may contribute to heterosis, have been detected on the whole maize genome (Springer *et al.*, 2009; Beló *et al.*, 2010). Moreover, the application of high-throughput sequencing in next-generation has widened the possibilities of genome wide comparisons (Lai *et al.*, 2010). As recently pointed out, results of studies on gene expression diversity still do not allow a consensus view, since varying levels of additive as well as of non-additive gene actions were shown in heterotic hybrids (Birchler *et al.*, 2010). Such studies have been so far conducted by comparing parental lines of different origins and their hybrids, thus taking into account a multitude of possible causative genes and chromosomal regions spread all over the genomes. In this context, the NILs' families described in this work are unique since they carry heterotic QTL in near isogenic materials. Investigations upon expression diversity on these materials could clarify the complex picture by focusing on restricted chromosome regions carrying already validated and well-characterized heterotic QTL for specific phenotypic traits. This latter aspect might also help us to overcome the gap between genotype and phenotype allowing hypothesis-driven phenotypic validation of heterotic effects.

CONCLUSION

Lisa! In this house, we obey the laws of thermodynamics!
Homer J. Simpson

In the present study, four QTL were validated and characterized. These QTL showed sizable dominance effects especially for GYP, its main component KP and other important traits such as PH. QTL effects were estimated, and they proved to be consistent across genetic backgrounds, levels of inbreeding and of competition among plants as determined by low and high PD. In some cases, significant interactions of QTL effects were detected with genetic background and PD, especially for GYP, but these interactions were always of size and led to moderate changes of *a* and *d* effects. The *d* effects and the $|d/a|$ ratios tended to be higher in inbred materials of Experiment 1, suggesting the importance of the role played by the inbreeding level of the overall genetic background in modulating such effects. The importance of *d* effects at least for 3.05 and 7.03 QTL was also confirmed in crosses with unrelated inbred testers belonging to opposite heterotic groups, suggesting that complementary QTL alleles were fixed in these groups.

All the mentioned findings suggest to proceed on further studies on such QTL for their fine mapping, to widen information on the role played by true *vs.* pseudo-overdominance in affecting heterosis. Undoubtedly, these NILs' families and their crosses can represent a valuable material also for studies focused on elucidating the molecular bases of heterosis.

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Every experiment proves something.
If it doesn't prove what you wanted it to prove, it proves something else.
Prof. Anon

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Perfect.
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Miranda Priestly
from "The devil wears Prada"