QTL MAPPING OF SYMBIOTIC NITROGEN FIXATION IN DRY BEAN; DRY BEAN PERFORMANCE UNDER ORGANIC PRODUCTION SYSTEMS

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Plant Breeding, Genetics and Biotechnology, Crop and Soil Sciences
- Doctor of Philosophy

2015

ABSTRACT

QTL MAPPING OF SYMBIOTIC NITROGEN
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Michigan has been a leader in organic dry bean (*Phaseolus vulgaris* L) production. Previous research has found that dry bean yields were substantially lower under organic conditions compared with adjacent conventional production. Since pests are controlled with approved methods in each respective system, fertility appears to be an issue where the two systems may differ. Seventy-nine black and navy bean elite breeding lines and commercial checks, and a non-nodulating check were evaluated for yield under organic conditions in 3 MI locations in 2011 through 2013. These same genotypes were also assayed for nodulation characteristics, N fixation, and shoot and root growth in the greenhouse under N free conditions. Several traits measured in the greenhouse were significantly correlated to traits measured in the field. In particular, percent N derived from the atmosphere (%Ndfa) in the greenhouse was correlated with seed yield, N yield, and %Ndfa in the field for most site years, suggesting that enhancing symbiotic nitrogen fixation (SNF) traits could improve productivity in organic bean systems.

Variability for SNF ability has been reported within *P. vulgaris*. The black bean landrace selection ‘Puebla 152’ has been identified as having high SNF ability, however is poorly adapted to cultivation in northern latitudes due to long season maturity and indeterminate type III growth habit. The recombinant inbred line (RIL) population developed by crossing Puebla 152 with the commercial black bean cultivar ‘Zorro’ was used to investigate the inheritance of enhanced SNF ability. The RIL population consisted of 122 lines and was evaluated in the greenhouse under N free conditions, and under low N conditions in the field in East Lansing (EL), MI and in Isabela, Puerto Rico (PR). The %Ndfa averaged between 12.7 % up to 66.6 %, although individual RILs ranged up to 90.5 %Ndfa. Traits measured in the greenhouse such as shoot biomass and biomass difference correlated moderately with yield and %Ndfa traits measured in the field.

A quantitative trait loci (QTL) analysis of the phenotypic data from the field and greenhouse was conducted using single-nucleotide polymorphism (SNP) markers developed through the BeanCAP. The phenotypic data included traits for yield, nodule rating, biomass growth, agronomic traits, and N fixation. A total of 19 QTL associated with SNF traits were identified on all 11 chromosomes except Pv02 and large clusters of QTL were discovered on Pv01, Pv06, and Pv08. Many of the QTL associated with %Ndfa, N harvest index, and %N in biomass were also associated with candidate genes expressed in the nodules and roots. Candidate genes such as Phvul.006G146400, which is a chitin elicitor receptor kinase is involved in recognition of rhizobia in the early establishment of the symbiotic relationship. Other candidate genes are transcription factors, such as Phvul.006G034400 that is associated with %Ndfa determined by natural abundance $\delta^{15}N$ analysis, is a MADS-box family gene and is expressed in young and mature green pods. The majority of QTL associated with genes expressed in the root or nodule are derived from Puebla 152 while QTL associated with genes with enhanced expression in stems and pods are associated with Zorro. This follows a pattern where Puebla 152 has superior SNF ability, whereas Zorro is highly efficient in partitioning the fixed-N into the seed. The QTL described serve as potential targets for improvement of SNF characteristics in adapted commercial dry bean genotypes.

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CHAPTER 1

LITERATURE REVIEW

Introduction

Dry bean (*Phaseolus vulgaris* L.) is an important food crop providing a nutrient dense, high protein, and low calorie staple while delivering up to 35% of global dietary protein (Broughton et al. 2003). Production worldwide has increased from 17.5 M tonnes in 1990 to 23.1 M tonnes in 2013 (FAOSTAT, 2015a) representing a 32% increase over the time period with 35.4% of total production being in the Americas. The United States ranked 6th in production in 2013 (FAOSTAT, 2015b) behind China, India, Myanmar, Brazil and Mexico; however, this may be misleading as “dry bean” may include pulse crops other than *Phaseolus vulgaris*. Michigan is one of 18 states with major production of dry beans and ranks 2nd in total dry bean production, after North Dakota (USDA-ERS, 2015). The two major market classes grown in Michigan are black beans and navy beans and Michigan is the leading producer of black beans in the country and the second leading producer of navy beans after North Dakota (USDA-ERS, 2015). In the United States, black beans, 95,590 ha planted, rank behind the leading market classes of pintos with 246,170 ha planted and navy beans 100,240 ha planted (USDA-NASS, 2015). From 2008 to 2011 organic dry bean production in the U.S. has increased by 44.3% while area planted in Michigan increased from 1,960 ha to 3,545 ha (USDA-NASS, 2015). In both 2008 and 2011, Michigan was the leading producer of organic dry beans. The leading market class produced organically in Michigan is black beans, with an increasing interest in other market classes (Findlay and Sattelberg personal communication). Heilig and Kelly (2012) showed that beans grown under certified organic conditions yielded on average 20% lower than those grown on adjacent conventional fields. Those genotypes belonging to the Andean gene pool performed poorly compared to genotypes from Middle American gene pool and yielded 25% less overall.

Heilig and Kelly (2012) noted that those genotypes performing poorly in organic production systems also yielded poorly in conventional production systems. Organic production systems rely on addition of nutrients to the soil through amendments such as composts and manure (Hill, 2014). Cover crops are used in these systems to both fix nitrogen (legume cover crops) and retain nutrients in the soil (non-leguminous cover crops) which prevent leaching form the soil.

Dry bean was domesticated in a region from Central America south to the Andes region of South America (Bellucci et al., 2014; Schmutz et al., 2014). Prior to domestication, *P. vulgaris* had begun to diverge into two distinct populations with partial reproductive barriers (Gepts, 1998). The Middle American Gene Pool originated in Mexico and Central America while the Andean Gene Pool originated in the Andes region of South America (Schmutz, et al., 2014: Singh, et al., 1991). Each gene pool is further divided into multiple races based on morphological, allozyme and molecular differences (Blair et al., 2013). Singh et al. (1991) divided the Middle American Gene Pool into Races Jalisco, Durango, and Mesoamerica and Beebe et al. (2000) later adding Race Guatemala. The Andean gene pool is divided into three races, Peru, Nueva Granada, and Chile.

Symbiotic Nitrogen Fixation in Beans

Nearly a century ago Sevey (1918) said dry beans "offer to man one of the richest heritages known to agriculture" a reference to the ability of dry bean to acquire N from the atmosphere through the association with soil bacteria, *Rhizobium*. Yet, in the 21st Century, why do dry bean producers still need to apply N fertilizers and other soil amendments to achieve competitive yields in dry bean? Nitrogen application recommendations range from 11 kg ha^{-1} without irrigation in Michigan (MSU, 2015) up to 23 kg ha^{-1} under irrigated conditions in Nebraska (Hergert and Schild, 2013).

Dry bean is often considered a poor N fixer (Piha and Munns, 1987; Fageria et al., 2014) in comparison to soybean (*Glycine max* (L.) Merr.) and chickpea (*Cicer arietanum* L.). Piha and Munns (1987) conducted an acetylene reduction assay to determine the activity of the nitrogenase in the nodules of the species. They noted that dry bean evolved more H_2 during fixation than soybean or chickpea representing a reduction in efficiency of symbiotic nitrogen fixation (SNF) for bean. They also compared the size and number of the nodules and discovered that the nodules of dry bean were smaller but more numerous than soybean or chickpea. Piha and Munns (1987) also noted that the period between germination and flowering of dry bean was much shorter, 27 days in their study, compared to chickpea, which averaged 34 days to flowering. In addition, the interval between flowering and physiological maturity was much shorter for chickpea suggesting that the chickpea simply had more time in a vegetative state to establish nodules and fix N before the strong sink strength of the seed for photosynthate competes with the nodules for resources (Piha and Munns, 1987). In an effort to better calculate the contribution of pulse crops to soil N levels, Walley et al. (2007) used published data from the Northern Great Plains area to investigate the contributions of N fixation in pulse crops such as pea (*Pisum sativum* L.), lentil (*Lens culinaris* Medik.), chickpea, dry bean and faba bean (*Vicia fabia* L.). Similar to other findings Walley et al. (2007) determined that dry bean had the lowest average percent nitrogen derived from the atmosphere (%Ndfa) (40 %Ndfa) and also had the highest amount of variability year to year and by location among the pulse crops studied. Faba bean was the highest fixer achieving 84 %Ndfa.

Symbiotic nitrogen fixation (SNF) is a complex trait. Not only must the plant be able to form compatible symbioses with the appropriate rhizobacteria, it must also form sufficient nodule mass and effectively move fixed N through the plant to the seeds. Nodule number has been shown to vary among dry bean genotypes (Pereira et al., 1993). There was a significant correlation (r^2=0.64, p<0.01) between nodule number and N fixed in a population of dry beans bred for enhanced N fixation (Pereira et al., 1993). There is considerable variation in this trait within dry bean germplasm (Wolyn et al., 1991; Pereira et al., 1993; Fageria et al., 2014). Improvement should be possible as dry bean appears to be responsive to selection for improved SNF by selecting directly or indirectly for fixed N (Wolyn et al., 1991; Elizondo Barron et al., 1999).

St. Clair and Bliss (1991) selected four inbred backcross lines from their Puebla 152/'Sanilac' population which showed superior acetylene reduction assay (ARA) levels. These plants were intercrossed and the F_3 progeny were tested for their ability to fix N. The majority of the 25 resultant progenies were superior N fixers when compared to Sanilac. Several of the lines studied fixed N similar to high N-fixing parent Puebla 152 while having agronomic traits similar to Sanilac which would make them more amenable to direct harvest (St. Clair and Bliss, 1991).

In addition to the ability to fix N, efficient use of N is important. Fageria et al. (2013) noted variability among the 20 dry bean genotypes for nitrogen use efficiency (NUE). Values ranged from 7.3 mg mg^{-1} seed in genotype 'BRS Valente' for each mg N applied to 21.2 mg mg^{-1} for line CNFP 7624. However, Fageria et al. (2013) did not mention if the potting mix used was sterilized nor if the plants were nodulated, as their N-free treatment yielded nearly as much seed N (43.6 g kg^{-1} for the zero N treatment compared to 46.9 g kg^{-1} in the fertilized treatment) though none was intentionally added. The source of this N was fixation. Thus, traits associated with partitioning likely interact with SNF to achieve enhanced yield.

Phaseolus vulgaris L. is considered a "promiscuous" nodulator since it can form associations with many different strains of rhizobacteria from several different species and genera (Michiels et al., 1998; Herrera-Cervera, et al., 1999; Ribeiro et al., 2013). Using 100 different rhizobacterial strains isolated from nodules of a wide range of host plants in Fabacea, Michiels et al. (1998) discovered that the majority of the strains were able to form nodules in either or both dry bean lines ‘Carioca’ (Mesoamerican) and ‘Limburgse Vroege’ (Andean). The rhizobial genera included *Rhizobium, Bradyrhizobium, Azorhizobium, Mesorhizobium,* and *Sinorhizobium* (Michiels et al., 1998). Not all strains were equally able to form nodules on the two dry bean genotypes studied nor were all strains forming nodules able to fix N (fewer than 70% were able to fix N) on either or both dry bean genotypes. Michiels et al. (1998) concluded that Carioca had higher level of nodulation compared to Limburgse Vroege. This may indicate variability within *P. vulgaris* itself to form nodules and fix N and perhaps suggesting that there may be differences in fixation between members of the Andean and Mesoamerican gene pool.

Herrera-Cervera et al., (1999) investigated the diversity of rhizobia inhabiting nodules on dry bean genotype ‘Contender’ and identified strains belonging to five different rhizobial species. Most strains identified belonged to *Rhizobium etli, R. girardinii, R. gallicum, R. leguminosarum,* and *Sinorhizobium fredii* (Herrera-Cervera et al., 1999). These strains were isolated from nodules formed on plants grown in Spain. One of the species, *R. etli,* is an American species nodulating *P. vulgaris* while *Sinorhizobium* are generally known to nodulate soybean. The authors speculate that the *R. etli* strains must have been transported to Spain with bean seeds imported from South or Central America (Herrera-Cervera et al., 1999).

Rhizobacteria are typically found in soils where beans are grown, though not all rhizobacteria are able to effect nodulation on dry bean or are able to fix N in symbiosis with dry bean. Mora et al. (2014) investigated rhizobial strains that could form nodules, but not fix N in association with dry bean. What at first appeared to be contamination of their non-fixing bacterial cultures with fixing bacteria led to a very interesting finding: several strains of *R. phaseoli* and *R. leguminosarum* persisted within the seed tissue protected by the seed coat. These endophytic rhizobial strains were able to multiply and form nodules during development of the bean plant (Mora et al., 2014). While seeds are inoculated with rhizobia following various media such as peat, clay, or liquid, the discovery of endophytic rhizobial strains offer an alternate method. These “autoinoculated” seeds might be a more useful method in regions of the world where producing or obtaining Rhizobium inoculant is difficult or impossible (Mora et al., 2014).

Aside from the variation noted in SNF ability in dry bean and in the efficiency with which different rhizobia strains fix N, dry bean nodule development is variable resulting in genotypes producing nodules of different size. Rodino et al. (2011) identified two main groups of nodules-one they named “Big-Nodules Phenotype” (BNO) and the other “Small-Nodules Phenotype” (SNO) while investigating the SNF ability of a diverse group of 128 dry bean lines. Plants producing nodules over 2 mg nodule^{-1} were considered BNO while plants producing nodules less than 1.5 mg nodule^{-1} were considered SNO (Rodino et al., 2011). Nodules of BNO plants were concentrated on the crown roots and were lower than average in number while nodules of the SNO plants were spread throughout the root system. BNO was associated with plants that produced a greater above ground biomass which was interpreted to mean that the BNO plants were more efficient fixers (Rodino et al., 2011). Interestingly, the small size and diffuse distribution of nodules on the high N-fixing Puebla 152 would best be described as SNO under the parameters described by Rodino et al. (2011) (personal observation). Wolyn et al. (1989) similarly associated small nodules distributed diffusely throughout the root system with improved SNF.

Role of *Rhizobia*

The interaction of N level and inoculation (+ or -) of 15 Brazilian dry bean genotypes was investigated by Fageria et al. (2014). They found that there was considerable variation among the different genotypes for yield under different N levels and whether the plants were inoculated or not. Aside from seed yield, traits measured included shoot biomass, root biomass, 100 seed weight, and seeds per pod, all of which may be considered components of yield. They concluded that dry beans were poor nitrogen fixers and maximum yields could only be obtained by the addition of high rates of fertilizer N. Apparently this conclusion was based solely on the average seed yield for all 15 genotypes in each treatment taken together. Looking at the individual lines it becomes apparent that the yield response varies considerable among the genotypes as some, such as CNFC 10408 yielded more without additional fertilizer N with rhizobial inoculation than with the addition of 200 mg N kg^{-1} soil yielding 10.0 g seed $plant^{-1}$ vs 7.7 g seed $plant^{-1}$, respectively. For comparison, the average yield per plant for the 15 genotypes without N fertilizer but with rhizobium was 9.83 g and 11.66 g for the 200 mg N kg^{-1} soil treatment. The genotype CNFC 10408 performed similarly for shoot dry weight, seeds per pod, 100 seed weight, and root dry weight. A more appropriate conclusion would be that there is substantial variability for performance under various N and rhizobia rates. Fageria et al. (2014) also concluded that rhizobia inoculant with the addition of a small amount of N fertilizer was detrimental to yield when compared to the high N treatment without rhizobia. Perhaps this suggests that there is a diversion of resources to either the nodules themselves or the rhizobia since root dry weight of the N plus rhizobia inoculant was lower than the control (no N or rhizobia). Contrary to these findings, Muller et al. (1993) found that mineral N application increased N fixation though some SNF traits were affected differently, such as an increase in nodule size and biomass with the application of N early allowing for enhanced N fixation after flowering. Puebla 152 cultivar was not as responsive to mineral N application as the other high fixing dry bean genotype ‘Negro Argel’ (Muller et al., 1993). The form of the N supplied in the soil may have an impact on the extent to which nodulation and fixation is reduced. Hine and Sprent (1987) used nitrate, ammonia, and urea to study the impact of the source of N on the growth of bean plants. Both nitrate and ammonia application resulted in a significantly lower number of nodules, whereas urea did not depress the number of nodules formed. Hine and Sprent (1987) tested urea levels from 0 mol m^{-3} to 10 mol m^{-3} and found that nodule levels were not affected with application rates up to 4 mol m^{-3}. Application of any N source, as well as the increasing levels of urea resulted in increased biomass.

Najareddy et al. (2014) found that higher levels of nitrate did not reduce the number of infection sites but did reduce the development of those infection sites into nodules. Higher levels of nitrate resulted in taller shoots and more biomass while roots were shorter with increased nitrate levels regardless if the nitrate was provided for the first 5 d after germination or continuously until flowering (Najareddy et al., 2014). Application of a starter fertilizer at planting might actually help improve N fixation especially if levels are low enough to not hinder the development of the nodule after initial infection. It seems that some amount of soil N early in development is actually beneficial to the establishment and growth of nodules. The source of N may vary throughout the growth cycle of dry bean with vegetative N early in development being primarily soil N which is depleted during growth of the plant and establishment of nodules and SNF when fixed N becomes the dominant source (Thomas et al., 1984; St Clair et al., 1988; Lynch and White, 1992). The dependence on fixed N during the reproductive cycle may explain why Hungria and Neves (1987) found that 60 to 64% of N in seed was fixed N. Soil N is used to grow the early vegetative portions of the plant. As soil N levels decline and the nodules are fully developed and functioning the seed is beginning to be the sink for any N fixed. Only as the plant approaches physiological maturity is N in leaves, stems, roots, and pods remobilized to the seed.

Nitrogen fixing plants invest a considerable amount of resources into establishing symbioses and subsequent N fixation which consumes up to 30% of the photosynthate produced by the plant (Schubert, 1986).

Other crops belonging to the Fabacea are able to fix N similar to dry bean. Kim et al. (2013) suggest that SNF is a basic and integral characteristic of legume species. Utilizing a set of 20 SNF related genes in soybean, chickpea, *Lotus japonicas*, *Medicago truncatula*, pigeon pea (*Cajanus cajan*), and dry bean, Kim et al. (2013) found that there was a high level of conservation among these six species. Soybean and chickpea were the most closely related based on sequence of the 20 genes investigated, followed by soybean and dry bean whereas dry bean and *M. truncatula* were the most distantly related pair (Kim et al., 2013). In field pea early SNF is linked to the developing the plant's demand for N while later in development photosynthate availability drives SNF (Liu et al., 2013). The C supplied to nodules is relatively constant during early development, but is reduced during the transition to the reproductive stage, and then rises during pod fill through maturity. This pattern seems to follow the pattern observed in dry bean, with highest demand for N occurring during pod fill. A QTL analysis of 207 RILs derived from the pea cultivars 'Cameo' and 'Ballet' identified many regions of the genome associated with SNF characteristics (Bourion et al., 2010). Many of the QTL that were related to traits such as shoot and root biomass production, %Ndfa, accumulated C, and nodule number colocalized within the genome. Soybean is often cited as superior to dry bean in N fixation ability. As a result of this enhanced ability supplemental N is not typically provided to soybean which relies completely on SNF for N requirements. Although SNF is usually sufficient to achieve maximum yields in soybean the high-yielding modern genotypes may be reaching their maximum SNF ability according to Nicolas et al. (2006). This group found several QTL associated with nodule number, nodule dry weight, and shoot dry weight which are all traits often associated with SNF. Nicolas et al. (2006) found several interactions between unlinked loci for shoot dry weight, nodule number, and nodule dry weight and an epistatic interaction between loci for nodule number and nodule dry weight. These results demonstrate the complexity of SNF while offering several QTL which may be utilized in marker assisted selection (MAS) to further improve SNF ability in soybean to meet the N demands of higher performing genotypes.

Rhizobia and Other Benefits

Beneficial effects of the symbiosis of rhizobia and dry bean go beyond SNF resulting in indirect benefits such as nutrient acquisition and control of disease (Yadegari et al., 2010; Abbaszadeh-dehaji et al., 2012; Neila et al., 2014; Ahemad and Kibret, 2014). The interaction between rhizobia and other plant growth promoting rhizobacteria (PGPR) such as coinoculation of dry bean plants with other rhizobacteria, such as *Pseudomonas fluorescens* and *Azospirillum lipoferum* further increased N fixation, biomass accumulation and protein content (Yadegari et al., 2010). These benefits are not limited to legume crops but extend to other crops such as corn (*Zea mays*) and wheat (*Triticum aestivum*) (Ahemad and Kibret, 2014). The diversity of the soil microbial community may be affected by inoculation with rhizobia. Trabelsi et al. (2011) noted an increase in the microbial diversity of soil around dry bean ‘Coco’ plants which had been inoculated with different strains of rhizobium: *Sinorhizobium* (*Ensifer*) *meliloti* strain 4H41 and *Rhizobium gallicum* strain 8a3. There was little effect on the soil content of nitrate, phosphate, or ammonium; however, there was a significant increase in the number of bacterial species present in the soil whether the inoculants were applied individually or combined especially as the season progressed with greatest differences being seen at harvest (Trabelsi et al., 2011). Not all of the bacteria were identified, as many belong to groups known for their benefit to plants, including many rhizobia and actinorhyzal species. The control, which had no inoculation but fertilizer showed only a modest growth in the diversity of soil bacteria (Trabelsi et al., 2011). While an increase in soil bacterial diversity would be considered an indirect benefit it could clearly improve productivity as many of the bacteria may prove beneficial to the growth of plants.

Soil nutrient availability may also be enhanced by some strains of rhizobium. Abbaszadeh-dehaji et al. (2012) found that 14 rhizobium strains selected for their high symbiotic effectiveness were also able to produce growth enhancing phytohormones such as auxin, solubilize P and Zn, and produce siderophores which are involved in chelating soil Fe and mobilizing the Fe into plant roots. Improved availability of soil nutrients is not only beneficial to plant growth but also to SNF activity of the plant. Under P deficient conditions, SNF may be reduced (Lazali et al., 2014). When P was sufficient the nodules were effective at excluding O_2, which is necessary for the proper function of nitrogenase responsible for reducing N_2 in the nodule. Under P deficiency the nodules were more permeable to O_2 thus reducing SNF (Lazali et al., 2014). Studies by Neila et al. (2014) using both soluble and insoluble P showed that nodule number on dry bean variety ‘Coco-blanc’ was reduced in plants with insufficient P and that different rhizobial strains from Tunisia were able to solubilize P at different rates. Under soluble P conditions, *Rhizobium sp.* strain P.Bj.09 was inferior to *Rhizobium tropici* strain CIAT899, whereas *R. sp. strain* P.Bj.09 formed more nodules on dry bean variety Coco-blanc when only insoluble P was applied (Neila et al., 2014). Thus P supply can impact nodule formation, development, and function. Piha and Munns (1987) suggested that the release of H^+ represented a loss of energy from the symbiotic system in dry bean and might explain the reduced SNF ability of dry bean compared to other legume crops. However, Alkama et al. (2012) noted that the release of H^+ was effective at acidifying the rhizosphere and thus solubilize P from the soil especially when P was limiting. While the efflux of H^+ may represent a loss of energy it may serve a greater purpose in making available P thus the energy cost may be offset by the P obtained. The presence of rhizobia and other rhizobacteria have real benefit to not only N fixing legumes but also to a diverse range of crop plants, whether through direct action such as forming a symbiosis with the plant to fix N, production of siderophores which chelate metals like Fe, soil acidification for P availability, providing competition to pathogenic organisms, or producing plant hormones such as IAA, (as reviewed in Ahemed and Kibret, 2014).

The benefits of rhizobial inoculation go beyond nutrient availability and into defense of pests such as Mexican bean beetles (*Epilachna varivestis* Mulsant). Comparing lima bean (*Phaseolus lunatus* L.) which had been inoculated with *Bradyrhizobium* sp. to uninoculated controls, Ballhorn et al. (2013) found that the volatile organic compounds (VOCs) released by colonized lima bean plants were more repellent to the Mexican bean beetle than the VOCs released by non-colonized plants. Once the jasmonic acid (JA) pathway was induced by mechanical damage, insect damage, or application of JA those plants which were colonized by the rhizobial strain produced a “bouquet” of various VOCs which caused the bean beetles to avoid them, whereas the same repellent bouquet was not produced by the non-colonized plants (Ballhorn et al., 2013).

Nod factors, signaling molecules produced by rhizobia living free in the soil, when extracted from rhizobacteria have plant growth enhancing characteristics. Pea seed treated with an extract of liquid culture of *R. leguminosarum* bv. *viciae* GR09 germinated in 50% less time that the water control (Podlesny et al., 2014). In addition, leaf area and green pods were increased significantly in plants sprayed with the extract over plants not sprayed (Podlesny et al., 2014). Other benefits of the nod factor extract included an increase in chlorophyll levels, which is correlated with the N status of the plant implying that though not inoculated with rhizobia, plants sprayed with the Nod factor extract had higher levels of N in their tissue than plants not sprayed (Podlesny et al., 2014).

Puebla 152 Genotype

Dry bean genotype Puebla 152 is a mid-sized type III black bean belonging to the Middle American gene pool (Singh et al., 1991) that originated as a landrace selection from Puebla, Mexico. Several studies have found the dry bean genotype Puebla 152 to be superior in nodule development and subsequent N fixation (St. Clair et al., 1988; Bliss et al., 1989; Park and Buttery, 1989; Pereira et al., 1989; Chaverra and Graham, 1992; Thomas et al., 1984; Wolyn et al., 1991; Tsai et al., 1998). Puebla 152 was the donor parent of five high N fixing dry bean germplasm lines (WBR22-3, WBR22-8, WBR22-34, WBR22-50, and WBR22-55) released by Bliss et al. (1989). ‘ICA Pijao’ was the recurrent parent. An estimated 44% of the shoot Ndfa, compared to 35% Ndfa for their high fixing check, ‘Rio Tibagi’ (Bliss et al., 1989). Chaverra and Graham (1992) studied early nodule formation and different inoculation rates on 40 dry bean genotypes, including Puebla 152. They found that Puebla 152 formed a high number of nodule initials by day 8 after inoculation and that it was responsive to increase inoculation rates while other genotypes seemed to have reduced nodule formation at higher rates of inoculation. For plants harvested 51 days after planting, Puebla 152 had a high nodule dry weight, high shoot weight, and accumulated a moderate level of N per plant. Puebla 152 was also shown to have superior SNF characteristics such as nodule number and dry weight with the application of varying rates of fertilizer N (Park and Buttery, 1989). Since agricultural soils have different levels of soil N it is beneficial to grow dry bean genotypes that are not inhibited or reduced in their ability to fix N when supplemental N is available. Puebla 152 has been useful in the study of many dry bean characteristics. It has been used as a parent in several QTL studies including selection for sugar levels in snap bean pods (Vandenlangenberg et al., 2012) and for root rot resistance in snap bean (Navarro et al., 2009; Ronquillo-Lopez et al., 2010).

Methods to Measure SNF

Several methods exist to measure the amount of nitrogen derived from the atmosphere (%Ndfa). These include: the difference method (Pereira et al., 1993; Muller et al., 1993), ureide levels in stem sap (Thomas et al., 1984; Hungria and Neves, 1987; Diatloff et al., 1991), acetylene reduction assay (Hungria and Neves, 1987; Piha and Munns, 1987; Boddey et al., 1996), ^{15}N enrichment (Hungria and Neves, 1987; St.Clair et al., 1988; Boddey et al., 1996), ^{15}N depletion (St. Clair et al., 1988; Pereira et al., 1989; Wolyn et al., 1991), and ^{15}N natural abundance method (Pereira et al., 1989). The simplest method to measure SNF is the difference method, which relies on calculating N fixed with the use of a non-nodulating reference plant. Pereira et al. (1993) used the non-nodulating soybean genotypes 'Harosoy' and 'Clay' as reference plants. The following equation is used to calculate %Ndfa:

%Ndfa=Nfixer-Nno-nod/Nfixer

Where Nfixer is the total N in the seed of the fixing crop and Nno-nod is the total N in the seed of the non-nodulating reference. It must be assumed that both the reference plant and fixing crop plant access similar layers of the soil.

Use of a fertilizer enriched, or depleted, in ^{15}N from the standard atmospheric content of 0.368 atom%, meaning 0.368 % of all N atoms in the atmosphere are ^{15}N vs. 99.632 atom% ^{14}N, has been utilized to determine which fraction of the N in a fixing plant was derived from the atmosphere or the soil. Pereira et al. (1989) utilized ammonium sulfate $(NH_4)_2SO_4$ containing 0.01 atom% ^{15}N applied throughout the growth cycle to both fixing dry bean lines and non-fixing soybean lines. At different growth stages and seed ^{15}N atom% were then measured, Pereira et al. (1989) used the following equation to determine %Ndfa.

%Ndfa = ((atom% ^{15}N(nfs)-atom%^{15}N(fs)) X 100)/(0.368 atom% ^{15}N – atom% ^{15}N(nfs))
Where nfs=non fixing system (non-nodulating soybean) and fs=the fixing system (the dry bean studied).

Using this method Pereira et al. (1989) determined that Puebla 152 obtained 31.6% of seed Ndfa whereas the navy bean Sanilac obtained only 5.7% of its N from the atmosphere. These two genotypes were the high and low fixers, respectively, for this study. The 5.5 fold increase in %Ndfa for Puebla 152 translated into an increase in seed yield (4,457 kg ha^{-1}) by a factor of 5.4 times the seed yield of Sanilac (818 kg ha^{-1}). One drawback of the ^{15}N depleted or enriched fertilizers is availability and cost. In addition, it is important that the fertilizer be adequately mixed throughout the root profile and is equally available to all test plants. The non-fixing reference plant must be a similar root type as the species being evaluated, and preferably of the same species (Boddey et al., 1996). Wheat has traditionally been used as a reference crop in such studies since it is assumed to access the same soil profile as dry beans. However, Boddey et al. (1996) determined that was not the case when comparing the N acquisition from soil of the non nodulating bean 'NORH 54' and wheat 'BR 33.'

Ramaekers et al. (2013) utilized a modification of the ^{15}N depletion method to determine %Ndfa. Relying on the natural abundance of ^{15}N in the soil, a non-fixing reference crop is used to determine the level of ^{15}N in the soil, which would be high in a non-fixing plant and low in a plant fixing N from the air. They used the following equation to determine %Ndfa:

%Ndfa=($\delta^{15}N$ non-fixing reference- $\delta^{15}N$ fixing line)/($\delta^{15}N$ non-fixing reference-B)

Where B is the $\delta^{15}N$ of the fixing line when it is relying completely on SNF. Ramaekers et al. (2013) averaged the $\delta^{15}N$ of several fixing genotypes grown under greenhouse conditions in N free media and N free nutrient solution. They do not specify which lines were included to calculate the B value nor do they provide the B value.

Evaluation of SNF levels earlier in the growth cycle prior to harvest would be beneficial to more rapidly screen dry bean lines for SNF ability. St. Clair et al. (1988) discovered that there was little agreement among the rank of the genotypes studied at R3 and R9, except Puebla 152 which fixed the most nitrogen at both stages. Thus, determining SNF levels at harvest would be advantageous compared to earlier time points. Lynch and White (1992) found that different organs are sinks for N at different developmental stages. Initially, N was partitioned in vegetative portions of the plant while later in the season pods and seeds were the destination of plant N, which was likely being relocated from vegetative tissues to reproductive tissues (seeds) (Lynch and White, 1992). Similarly, Boddey et al. (1996) and Wolyn et al. (1991) determined that early measurements of SNF levels were not necessarily related to SNF levels in seed at maturity.

Differences in a genotype's partitioning and discrimination of ^{15}N can result in over or underestimation of %Ndfa. Lazali et al. (2014) looked at six RILs selected from a population developed by crossing BAT477 and DOR364. The individual lines were selected for their tolerance or sensitivity to P deficiency. They found that there were differences across P levels and among the genotypes in their discrimination against ^{15}N (Lazali et al., 2014). Looking at different portions of the plant-roots, shoots, and nodules, they found that a higher proportion of ^{15}N remained in the roots, specifically the nodules, while the proportion of $^{15}N/^{14}N$ was lower in the shoots. Thus, measuring ^{15}N in the shoot might cause an overestimation of %Ndfa. Lazali et al. (2014) did find a significant correlation between ^{15}N in nodules and P sensitivity and N fixed by the plant. In addition to the genotypes discriminating against ^{15}N, different strains of Rhizobia also discriminate differently for ^{15}N. Yoneyama et al. (1986) found that not only did the 10 Rhizobium strains studied vary in their SNF ability, the amount of ^{15}N in the shoot of the three genotypes tested (‘Himetebou,’ ‘Daifuku,’ and ‘Toramame’) varied considerably when inoculated by different strains. In the laboratory, it is possible to control the specific strain of Rhizobium, however, in the field nodule occupancy is likely to vary even within the same plant. Yoneyama et al. (1984) noted that the amount of ^{15}N varied by the plant organ with stems and petioles having considerably less ^{15}N than leaves. Studying the kidney bean genotypes ‘Shakugosum’ and ‘Nagauzura’ $\delta^{15}N$ was +9.3 and +8.5 in the nodules, +1.5 and -1.0 for pods, and -0.6 and +2.8 for stems, respectively (Yoneyama et al., 1984). The selection of the plant part in calculations could cause a very different estimation of %Ndfa.

Dry bean is a ureide transporting legume, meaning that Ndfa is often translocated through the plant as alontoin and alontoic acid (Thomas et al., 1984). Thus, SNF levels could be inferred from the composition of the bleeding stem sap. During early developmental stages the predominant form of N is nitrate, which is derived from the soil. As plants mature and advance into pod filling phase ureides become a much larger portion of the N in plant sap (Thomas et al., 1984; Diatloff et al., 1991). Surprisingly, Thomas et al. (1984) found that among genotypes Puebla 152 and Sanilac, and seven lines derived from an inbred backcross of Sanilac (the recurrent parent) and Puebla 152, little variation in the sap composition, especially early in development was observed. As pods of Puebla 152 began to fill the ureide content in the N- treatment also increased relative to other forms of N, including nitrate. When sap flow rate is considered, however, the differences become more dramatic with Puebla 152 clearly fixing more N than Sanilac and most of the inbred lines. Diatloff et al. (1991) found the same pattern with other navy bean genotypes.

QTL Analysis and SNF

Several studies have been conducted to map QTL for various SNF traits (Nodari et al., 1993; Tsai et al., 1998). In an attempt to look for QTL involved in the interaction between host and bacteria, Nodari et al. (1993) used an F_3 population from 'BAT93' (Mesoamerican derived genotype with fewer nodules and resistance to common bean blight (CBB)) and 'Jalo EEP558' (Andean selection with high nodule number, susceptible to CBB.) Four QTL which explained a total of 52% of the phenotypic variation for nodule number were discovered. One locus appeared to have an effect on both nodule number and CBB resistance which is not surprising since many stages in the interaction with pathogenic bacteria are similar to interactions with beneficial bacteria. This region, on Pv07 contributed by the BAT93 parent, was associated with CBB resistance but with low nodule number (Nodari et al., 1993). Tsai et al. (1998) used a similar population by crossing the high nodulating dry bean, Jalo EEP558 with the low nodulating BAT93 to investigate nodule number and CBB resistance inheritance under contrasting N conditions. Both parents contributed positive alleles to nodule number and CBB resistance in the F_2 derived F_3 RILs. Given that the low nodulating parent (BAT93) contributed alleles with a positive effect on nodule number and the CBB susceptible parent similarly contributed positive alleles for CBB resistance. Ramaekers et al. (2013) used 85 RILs developed from G2333 x G19839 to investigate characteristics associated with SNF in both the greenhouse under N free conditions and in the field. They measured traits such as leaf chlorophyll content, shoot dry weight, total biomass N, seed yield and total N in seed. Many QTL were discovered for SNF traits, such as SPAD (a measure of chlorophyll, and hence N level in leaves) at different growth stages (R^2= 11.49% to 35.53%), %N in the shoot, root, and plant (R^2= 16.3% to 21.01%), and total N in the shoot, root, and plant (R^2= 14.69% to 20.87%), in the greenhouse along with to nodule number QTL (R^2= 17.25% and 16.72%), two nodule dry weight QTL (R^2= 12.97% and 19.07%), and one %Ndfa at harvest QTL (R^2=18.79%), in the field. They found different QTL between the field and greenhouse experiment but there were QTL that overlapped between both experiments such as a SPAD QTL on Pv01 and two QTL on Pv07 for SPAD at pod filling in the greenhouse and the field. The QTL reported have low to moderate effect on the phenotype but could prove useful in developing markers for MAS.

Summary

Dry bean is an important crop and an important source of protein for low income people, worldwide. It is grown over many regions, including the northern tier and intermountain states in the U.S. In Michigan, dry bean is an important component of crop rotations especially in the main growing region, often known as the “Thumb”, an area consisting of 4 major bean growing counties including Tuscola, Huron, Sanilac, and Bay. While a member of a plant family (Fabacea) known for SNF, dry bean is not the most efficient at fixing N especially when compared to related species such as soybean. The variability found within the species for improved N fixation, however, serves as important genetic material to improve SNF ability in commercially acceptable dry bean lines. This improvement could help to reduce dependence on N inputs which will also help to reduce production costs and damage to the environment caused by runoff of N from fields into ground water and adjacent waterways.

Use of dry bean genotypes, such as Puebla 152, has been an important part of research conducted on SNF in dry bean with the focus on improving the SNF ability of dry bean. While not well adapted to commercial production at northern latitudes in the U.S. when used as a parent with a commercially adapted lines, Puebla 152 is a dependable source of traits relating to SNF. Using genomic tools, such as SNP markers and QTL analysis, traits associated with improved SNF may be moved from the poorly adapted Puebla 152 to commercially acceptable dry bean lines. This information may also be useful in developing genotypes with improved SNF characteristics in other market classes. The objective of this study was to integrate the superior SNF traits of Puebla 152 into genotypes better adapted to current productions methods in northern latitudes as well as to elucidate genomic regions associated with SNF with the goal of identifying markers useful in selecting for enhanced SNF in future dry bean breeding programs.

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CHAPTER 2

EVALUATION OF SYMBIOTIC N FIXATION OF BLACK AND NAVY BEAN ADVANCED BREEDING LINES UNDER ORGANIC PRODUCTION SYSTEMS

Abstract

Michigan has been a leader in organic dry bean production. Organic production involves the use of certified inputs which are derived from natural sources. Fertility is managed through crop rotation, cover crops, and addition of composts and manures instead of the application of synthetically produced fertilizers. Previous research has found that dry bean yields were substantially lower under organic conditions compared to adjacent conventional production. Since pests are controlled with approved methods in each respective system, fertility appears to be an issue where the two systems may differ. Seventy-nine black and navy bean elite breeding lines, commercial checks, and a non-nodulating check were evaluated for yield under organic conditions in Frankenmuth, Caro, and Wisner, MI in 2011 through 2013. These same genotypes were also assayed for nodulation characteristics, N fixation, and shoot and root growth in the greenhouse under N free conditions. Several traits measured in the greenhouse were significantly correlated to traits measured in the field. In particular, percent N derived from the atmosphere (%Ndfa) in the greenhouse was correlated with seed yield, N yield, and %Ndfa in the field in most site years. Measuring N in seed or plant tissue may not be necessary to estimate symbiotic nitrogen fixation (SNF) characteristics when plants are grown under limited N fertility as “biomass difference” from the greenhouse was significantly correlated with seed yield and N yield in the field.

Introduction

Michigan is one of 18 states with major production of dry beans (*Phaseolus vulgaris* L.) and ranks 2nd in total dry bean production, after North Dakota (USDA-ERS, 2014). The two major market classes in Michigan are black beans and navy beans with Michigan being the leading producer of black beans in the country and the second leading producer of navy beans, after North Dakota (USDA-ERS, 2014). In the United States, black beans rank behind the leading market classes of pintos and navy beans in overall production (USDA ERS, 2014).
Michigan is the leading producer of organic dry beans in the U.S. (Table 2.01). According to the 2008 report of the National Agricultural Statistics Service with 1,960 ha harvested at a value of $3.9 million. In that year, Michigan accounted for 38.5% of the acres of organic dry beans harvested (USDA ERS, 2008). Organic dry bean production grew 81% by 2011 with Michigan accounting for 48% of U.S. production (Table 2.01). According to the USDA National Organic Program (NOP) “organic” is a label used to designate that an agricultural product was produced in an approved manner consistent with the standards set by the program. Inputs are limited to those approved by the Organic Materials Review Institute (OMRI). Generally, synthetic pesticides and fertilizers commonly utilized in “conventional” production are banned for use in certified organic production. Inputs, such as manure, composts and other approved soil additives as well as certain “natural” pesticides are allowed in the production of certified organic agricultural products. A producer must work through a certifying agency which reviews records and certifies that acceptable materials are used and practices are followed.

In comparative studies of dry beans grown under both organic and conventional production systems, Heilig and Kelly (2012) found that on average, yields in organic production were 20% lower compared with those raised under conventional systems in adjacent trials. Weeds are often a problem in organic production systems as there are few approved controls aside from delayed planting and multiple mechanical cultivations. Weeds were controlled with mechanical methods including hand removal. Insects were similarly controlled with approved insecticides. Aside from potential varying efficacies of organic control methods, soil fertility was identified as a possible source of the differences in yield (Heilig and Kelly, 2012). Conventionally produced beans were provided artificial fertilizer at the recommended rate of 55 kg N ha^{-1} (Warncke et al., 2009) which is readily available while fertility in the organic system relies on the natural processes involved in breaking down organic components to release nutrients potentially leading to reduced availability, in particular nitrogen. Dry bean genotypes that performed well in conventional production systems were also the best suited to organic production due to resistance to disease as well as adaptation to modern agricultural practices (Heilig and Kelly, 2012). Genotypes developed under conventional management, however, are not selected for their ability to fix N from the atmosphere as artificial fertilizer is applied to all conventional trial plots. Comparing 4 dry bean cultivars, Oliveira et al. (1998) found that beans without supplemental N were able to fix as much N as plants fertilized with 60 kg N ha^{-1}. The genotype 'Serro Azul B' accumulated 198.8 mg N $plant^{-1}$ through fixation compared to 184.7 mg N $plant^{-1}$ with the addition of 60 kg N ha^{-1}.

Cover crops are often used in organic production to manage fertility and control weeds. Different cover crops contribute different amounts of N to total inorganic N in soil (Hill, 2014). Legume cover crops such as medium red clover (*Trifolium pratense* L.) contributed less than 20 kg ha^{-1} to over 100 kg ha^{-1} through the growing season following incorporation, depending on location and year (Hill, 2014). Without a cover crop, total inorganic N available in the soil ranged from less than 20 kg ha^{-1} to 80 kg ha^{-1} (Hill, 2014). Other cover crops studied, cereal rye

(*Secale cereale* L.), and oilseed radish (*Raphanus sativus* L.) soil N rates were intermediate (Hill, 2014). The higher levels of soil N observed are above recommendations (Warncke et al., 2009), however, the amount of N in the soil is extremely variable depending on location and cover crop used. Additionally, total soil N measurements do not represent the N available to the plant at any given time. In those situations where soil N is too low to produce a competitive dry bean crop, SNF should provide the balance. Improving the SNF ability of future dry bean cultivars should be a useful benefit for organic producers and commercial producers who want to use reduce fertilizer inputs.

The current study was designed to investigate the importance of symbiotic N fixation (SNF) ability in performance of dry bean genotypes grown under organic production systems and compare performance with traits associated with SNF measured under N-free conditions in the greenhouse.

Materials and Methods

Plant material

Seventy-nine black and navy bean genotypes including elite breeding lines, commercial checks and one non-nodulating genotype were grown under organic conditions over three growing seasons (2011, 2012, and 2013) on certified organic ground in Caro, Michigan, Wisner, Michigan and Frankenmuth, Michigan. Each season 18 black bean and 18 navy bean genotypes were planted but entries differed annually based on prior performance. As the study progressed, some lines were dropped from the study while newer breeding lines were added to replace them. Black and navy bean lines included cultivars and elite breeding lines from the bean breeding program at MSU along with selections from the a black bean recombinant inbred line population developed from crossing the black bean cultivars ‘Zorro’ (Kelly et al., 2009) and the landrace selection ‘Puebla 152’ (see following chapter). Puebla 152 was selected as the donor parent for enhanced SNF ability (St. Clair et al., 1988; Bliss et al., 1989; Park and Buttery, 1989; Pereira et al., 1989; Chaverra and Graham, 1992; Thomas et al., 1984; Wolyn et al., 1991; Tsai et al. 1998). Puebla 152 was not included in the test due to a lack of adaptation to local growing conditions. The non-nodulating ‘R99’ (Park and Buttery, 2006) genotype was planted as a reference cultivar to calculate nitrogen fixation.

Inoculation

Prior to planting seed was treated with rhizobial inoculant consisting of fine buffered peat (American Peat, Technology, Aitkin, MN, USA) carrying *Rhizobium tropici* strain CIAT899 which was prepared by culturing the rhizobia in yeast mannitol broth (as described in Somasegaran and Hoben, 1994) for three d prior to mixing with peat at a rate to allow sufficient wetting of the peat. Selection of *Rhizobium tropici* strain CIAT899 as the inoculant was based on its widespread use in research and ability to form a symbiosis with a wide range of dry bean genotypes (Graham et al., 2003). The resulting inoculant was incubated in the dark for 8 to 12 wk at room temperature. Seed was mixed with the peat inoculant and a small amount of water to adhere the peat inoculant to the seed. Treated seed was stored in a cooler until planting.

Planting and Cultivation

Seed was planted into two-row plots 6.1 m long with rows spaced 51 cm apart at a rate of approximately 14 seed per m in a lattice design with 4 repetitions. Two locations were planted each season at the Saginaw Valley Research and Extension Center (SVREC), Frankenmuth, MI and Wisner, MI in 2011; Wisner, MI, and Caro MI in both 2012 and 2013. The Wisner location was abandoned in 2013 due to excessive moisture throughout the season and at harvest. Field conditions are reported in Table 2.02. Weed control was by mechanical cultivation as needed early in the season and supplemented with hand weeding to control weeds especially within the plant row where the cultivator was unable to reach. Plots were cultivated 2 to 3 times each season, as needed. No fertilizer was applied at planting nor during the growing season. Potato leaf hopper was controlled as needed with Pyganic Crop Protection EC 5.0 (McLaughlin Gormley King Company, Minneapolis, MN) at a rate of 586 ml ha^{-1} resulting in 30 ml ha^{-1} active ingredient.

Data Collection

Days to flower was recorded as the number of days after planting when 50% of the plants in each plot had one open flower. Maturity was determined when 50% or more of the plants had reached physiological maturity, at which time plant height (cm), lodging (1=upright, 5=prostrate) and agronomic desirability (1=not desirable, 6=highly desirable) were recorded.

Harvest

When plants reached maturity, plots were direct harvested with a Wintersteiger AG plot combine (Winterstieger AG, Austria). Seed was air dried and cleaned with a Clipper Mill (A.T. Ferrell Company, Bluffton, IN, USA) before weighing. Seed moisture content at weighing was measured with a Dickey-john GAC 2500 moisture meter (Churchill Industries, Minneapolis, MN). Yield was calculated by adjusting values to 18% moisture content. Seed size was determined by weighing a random sample of 100 seeds, adjusted to 18% moisture.

Nitrogen Analysis

A 30 g seed subsample from each plot was placed in an envelope and placed into a dryer at 60º C for 1 wk. Seed was then ground in a Wiley Mill pass through a 1 mm mesh screen. Seed samples were then stored at room temperature until sent to the Stable Isotope Facility at UC Davis, Davis CA for N analysis where a “PDZ Europa ANCA-GSL elemental analyzer (Sercon Ltd., Cheshire, UK)” was used to measure N.

Percent nitrogen derived from the atmosphere (%Ndfa) using the difference method by the following equation (Boddey, 1987):

%Ndfa = (N yield-Fixer – N yield-non-Fixer)/N yield-Fixer

Where N yield = Seed Yield (kg) * %N of the respective fixer and non-fixer (R99).

Greenhouse Assay

To study the SNF ability of individual genotypes at flowering, the navy and black bean genotypes along with commercial checks were grown in the absence of N under greenhouse conditions. Seeds of the genotypes being studied were sterilized by soaking in a 10% bleach solution for 2 min followed by two 2-min rinses with sterile water. Six seeds were planted into each plastic 5.7 l nursery container which had been filled with a 2:1 mix, v:v of perlite to vermiculite which had been autoclaved. Seeds were watered in with tap water. At 3 d after germination, 500 ml YMB culture of *R. tropici* CIAT899 was diluted in 20 l of tap water which had been adjusted to a pH of approximately 6.5 which resulted in a concentration of approximately 10^3 cells per ml. Each nursery container received 250 ml of the final rhizobial dilution. Inoculation was repeated 10 d after planting in the same manner to ensure sufficient population levels of rhizobia to effect symbiosis. Pots were placed randomly on a greenhouse bench with day length extended to 16 h with high pressure sodium lights. Two to three times weekly each pot was watered with 500 ml full strength Broughton and Dilworth N free solution (Broughton and Dilworth, 1970) as needed to avoid drought stress. Plants were thinned to two plants per pot before emergence of the first trifoliate. The experiment was repeated three times. When one or both of the plants had at least one flower open, plant shoots were measured and cut with a razor blade at soil level. The perlite and vermiculite was carefully removed from the roots which were measured for maximum length and scored from nodulation on a scale of 0 to 6, with 0 representing roots without nodules and 6 being roots with a large number of fully developed and functioning nodules. Both root and shoot biomass samples were dried in a dyer at 60º C for 7 d at which time they were weight and then ground to pass through a 1mm screen on a Wiley mill. Samples were also sent the U.C. Davis SIF for N analysis as described previously for field grown seed. Total biomass was calculated by adding root biomass and shoot biomass. Similar to the calculation for %Ndfa, total biomass difference, shoot biomass difference, and root biomass difference were calculated by the following equation for the respective trait:

Difference = (mass (g) of fixer-mass (g) of non-fixer)/mass (g) of fixer

Statistical Analysis

A PROC GLM analysis using SAS 9.4 (SAS Institute Inc. 100 SAS Campus Drive, Cary, NC 27513-2414, USA) to generate an ANOVA determined that there was a significant difference among years and sites. Consequently, each site/year was analyzed separately. PROC CORR was used to generate Pearson Correlations in SAS 9.4.

Results and Discussion

Variability in precipitation and soil conditions at each site along with the rotation of genotypes as the study progressed made it difficult to make comparisons of yield performance of particular genotypes. Field stations were relied on for precipitation measurements, however, variable precipitation patterns with localized rain events caused a discrepancy between what was measured at the field station and what was observed on the actual plot. Checks planted in all site/years showed variability in yield across years and locations. In 2011 Zorro was the highest yielding line at both sites (2117 kg ha^{-1} and 1519 kg ha^{-1}) while breeding lines yielded more in 2012 and 2013 (Table 2.03). In 2012 the elite breeding line B11361 was the highest yielding line at the Wisner location while B11302 was the highest yielding line at the Caro location. The commercial black bean cultivar 'Zenith' (Kelly et al. 2015) was the highest yielding line at the Caro location in 2013. Zenith was evaluated as breeding line B10244 in this study prior to registration. Overall the navy bean genotypes were not as competitive as the black bean genotypes in these organic sites with the exception of the 'Medalist' cultivar at Wisner in 2012. Prior research on early nodulation found that while navy and black beans formed nodules in the same amount time, navy beans produced fewer nodules early in development than black beans (Heilig and Kelly, 2012b). Hungria and Philips (1993) found that dry beans with white seeds produced fewer flavonoids than genotypes with colored seed. These flavonoids are involved in early initiation of symbiosis and reducing the concentration may reduce the number of infection sites and thus nodule number. The non-nodulating genotype R99 was the lowest yielding line only in 2012 at both locations, possibly reflecting the nitrogen level in the fields (Table 2.03). R99 was at or below average for percent seed N in all sites and years. Low to moderate coefficients of variation for percent seed N suggest that this trait was not as affected by environmental variation as yield (Table 2.04).

Nitrogen yield reflects the amount of N harvested and is calculated by multiplying seed yield by percent seed N. R99 had the lowest N yield in all years and sites (Table 2.03). Nitrogen yield largely followed the pattern of seed yield except in 2012 at the Wisner site when B09175 had the highest N yield, whereas Zorro had the highest seed yield. %Ndfa was determined for all entries using R99 as a non-fixing reference check (Table 2.04). Overall, the highest %Ndfa was 71.7% for Zorro at the Frankenmuth site in 2011 while the lowest was 9.8% for the breeding line B10243 at the Wisner site in 2012 (Table 2.04). The relationship among yield over all site years appears to be rather limited as Pearson correlations show that yield in any particular site/year is not correlated to yield in any other site year (data not shown). The correlation was moderate between the Wisner site and Frankenmuth site in 2011. The same pattern was found for N yield, which was expected as seed yield was used to calculate N yield. Correlations between %Ndfa and %N in seed were much more consistent across locations and years (Table 2.05). There was a high correlation for %Ndfa between Caro site 2013 (r=0.96, p=0.0019) and Wisner and Frankenmuth in 2011 (r=0.94, p=0.0046). Similarly correlations were fairly high between Wisner 2012 and both sites in 2011 (r=0.88, p=0.0009 and r=0.82, p=0.0034) and Caro 2012 and both sites in 2011 (r=0.77, p=0.0009, and r=0.73, p=0.016). %Ndfa was moderately or strongly correlated with %N for most site/years, suggesting that there is an underlying genetic factor that controls this characteristic. Environmental variability had a higher impact on yield than it did on %Ndfa. There was no statistical difference among the site/years for percent seed N suggesting that this trait too is less dependent on environmental factors but genetically determined.

The values for traits measured in the greenhouse under N free conditions are shown in Table 2.06. As expected, the vast majority of N was derived from the atmosphere, ranging from 75.8 to 98.8 %Ndfa (Table 2.06). The only potential sources for N would be the N in the planted seed, N in tap water, or dust that fell on the surface of the media. Nodule ratings differed considerably with some genotypes, such as the black bean cultivar ‘Black Velvet,’ being highly nodulated (6.0) while another black bean cultivar ‘Shania’ had fewer, and less developed nodules (2.0) (Table 2.06). Many of the traits measured in the greenhouse were significantly correlated with each other (Table 2.07). Root N and shoot N are very highly correlated ($r=0.95$, $p\leq0.0001$) which is not surprising as N status of the plant depends on the roots acquiring N whether it originates in the potting media or the rhizobia in the nodules. Root biomass and shoot biomass are similarly highly correlated ($r=0.93$, $p\leq0.001$). Since N is a major nutrient which determines plant growth these parameters would be expected to be closely related. The visual score (0 to 6) of root nodules was moderately to highly correlated to all measured traits. A visual scoring of nodules is preferable to actual counts due to time restraints and cost. The root:shoot biomass ratio was inversely correlated to all other greenhouse traits (Table 2.07). As the shoot biomass increases, other traits, such as shoot N ($r=-0.3$, $p\leq0.05$) and %Ndfa ($r=-0.69$, $p\leq0.0001$) decrease. While N fixation is important for growth of dry bean, partitioning of N to the seed appears to be equally important.

Biomass, and the proportion of biomass in the roots versus the shoot seems to play a role in yield and SNF traits. The root:shoot ratio in the greenhouse showed a significant moderate negatively correlation with seed yield and N yield in the field for three of five site years (Table 2.08). As the amount of root biomass increased, seed yield and N yield decreased. This partitioning among various organs may account for the differences seen in the field with respect to seed yield and N yield and likely are not directly involved in the SNF process but more involved in the movement of N, whether fixed or from the soil, within the plant.

Developing a screening method for traits related to SNF that requires less time and space than a full field evaluation would be advantageous for breeders selecting genotypes with enhanced SNF. Assays conducted in the greenhouse require less time to complete, can be conducted during the off-season, and can be controlled to a much greater degree than field studies. Correlations between the traits measured in the greenhouse and those in the field can help to determine which traits are useful for making selections for enhanced SNF. One trait that was fairly consistently correlated to the field traits measured at all site/years was %Ndfa (Table 2.05). There was a significant moderate to high correlation between greenhouse %Ndfa and seed yield in three out of five site/years. The field %Ndfa was significantly correlated with greenhouse %Ndfa for all site/years whereas N yield in the field was moderately correlated with greenhouse %Ndfa in three of five site/years.

The %N in either the seed from the field trials or %N in shoot biomass is not significantly associated with yield traits. It is likely that %N in plant tissue is genetically independent of the processes involved in acquiring N. Since there is no storage form of N in the plant, N is found in the structural components of the biomass, with biomass growth being dependent on availability of N. A suitable proxy for SNF may be "biomass difference" of plants grown in the greenhouse which is less costly and easier to measure than measuring actual N content. Shoot biomass difference was significantly correlated with seed yield in four of five site years; moderately to highly correlated with field %Ndfa in five out of five site years; and moderately correlated to N yield in four of five site years. The only site year where the shoot biomass difference was not correlated with seed yield or N yield was the Wisner site in 2012. Drought stress was severe in 2012 and may be confounding the results for this site year. Other greenhouse traits showing promise for predicting field performance include nodule rating, shoot biomass, and root:shoot ratio. Given that SNF traits in the greenhouse correlate with yield parameters in the field, N may be a limiting factor in organic dry bean production and should be considered in crop rotation choices. In the field studies %Ndfa ranged from 23.5% to 71.2% for commercial genotypes whereas %Ndfa for advanced breeding lines was more variable ranging from 11.1% to 71.7% (Table 2.04). Genotypes such as Zorro and Zenith had relatively high yield along with higher levels of %Ndfa suggesting that there may be little advantage to enhanced SNF in these genotypes. The range in %Ndfa seen in the advanced breeding lines indicates that there is considerable potential to select lines with enhanced SNF ability and retain yield potential.

Conclusions

Variability in performance of genotypes as well as variable environmental conditions make it necessary to evaluate dry bean genotypes over several seasons and multiple locations to better determine their yield and SNF potential. Not all dry bean genotypes have potential to fix a high %Ndfa, but variation in this trait is present in elite breeding lines that exceed fixation levels seen in commercial genotypes. These genotypes may prove to be worthy of release, such as Zenith, which surpassed the previously released cultivar Zorro in yield and %Ndfa in 2 of 3 years tested.

APPENDICES

APPENDIX A

CHAPTER 2 TABLES

Table 2.01. Hectares planted and production of organically produced dry bean in the United States†.

State	Hectares Harvested	Hectares Harvested	Production (1,000 kg)	Production (1,000 kg)
	2008	2011	2008	2011
California	391	276	588	499
Colorado	629	506	284	659
Idaho	255	440	466	799
Iowa	72		126	
Michigan	1960	3545	3265	6951
Minnesota	163		318	
Nebraska	72	83	163	118
New York	140	260	183	380
North Dakota	588	399	983	523
Ohio		68		86
Oregon		39		35
Vermont		10		12
Washington	145	384		649
Wisconsin	22		37	
Wyoming		107		260
National	5087	7342	7975	12861

† Data from USDA-NASS, 2015. http://quickstats.nass.usda.gov/ accessed June 2015.

Table 2.02. Field location, year, planting date, precipitation, soil type and soil chemistry of fields where black and navy bean genotypes were evaluated under organic conditions in 2011, 2012, and 2013 in Michigan.

Locaton	Year	Date Planted	Predcipitation (mm)[†‡]	Weather Station[§]	Soil Type[ℓ]	pH	% N[ʒ]
Frankenmuth, MI	2011	6/9/2011	148	Richville	Tapan Loam	7.8	0.094
Wisner, MI	2011	6/20/2011	202	Munger	Tapan Loam	7.6	0.21
Caro, MI	2012	6/12/2012	240	Fairgrove	Tapan-Londo Loam	8.0	0.1
Wisner, MI	2012	6/15/2012	160	Munger	Tapan Loam	7.7	0.3
Caro, MI	2013	6/20/2013	109	Fairgrove	Tapan-Londo Loam	7.7	0.13

[†] Report generated at Enviro-Weather (http://www.agweather.geo.msu.edu/mawn/) for the months of June through September

[‡] The 30 year average for 1 June through 30 September is 362 mm

[§] Nearest weather station used for precipitation data

[ℓ] USDA Web Soil Survey Natural Resource Conservation Service

[ʒ] Total Kjeldahl Nitrogen measured by the Soil and Plant Nutrient Lab at Michigan State University

Table 2.03. Seed yield and N yield of 79 black and navy bean genotypes grown under organic conditions in Frankenmuth, Caro, and Wisner, MI in 2011, 2012, and 2013.

		Seed Yield					N yield				
Commercial	Market	2011		2012		2013	2011		2012		2013
Checks	Class	Wisner	SVREC†	Wisner	Caro	Caro	Wisner	SVREC†	Wisner	Caro	Caro
		-------kg ha^{-1}-------					-------kg ha^{-1}-------				
Black Velvet	Black	1773	1272				65	42			
Medalist	Navy	1038	1350	2187	1247	1683	36	37	83	42	29
Merlin	Navy					1759					
R99	No-Nod	847	421	1151	1077	1192	27	12	45	38	19
Shania	Black			1364	1752	1695			59	59	30
Vista	Navy	1706	1197	1735	1335	1873	65	38	68	47	31
Zenith	Black			1409	2331	2474			58	85	40
Zorro	Black	2117	1519	1652	1458	1740	76	44	66	53	29
Experimental Lines‡											
Highest Yield		1975	1515	2570	2151	2215	71	48	94	89	36
Lowest Yield		1328	934	907	877	1386	43	31	39	33	16
Test Mean		1601	1228	1711	1557	1762	58	37	67	54	29
LSD ($p \leq 0.05$)		313.1	283.0	730.1	747.7	416.3	11.8	11.3	27.5	27.2	6.7
CV (%)		14.0	16.4	26.2	32.7	16.8	14.7	21.4	25.0	34.6	16.3

† Saginaw Valley Research and Extension Center, Frankenmuth, MI.
‡ Highest and lowest yield in each site and year of advanced breeding lines. May not be the same line each year.

Table 2.04. Percent N derived from the atmosphere (%Ndfa) and percent N in seed of 79 black and navy bean genotypes grown under organic conditions in Frankenmuth, Caro, and Wisner, MI in 2011, 2012, and 2013.

	%Ndfa Difference Method					% N in Seed				
	2011		2012		2013	2011		2012		2013
Commercial Checks	Wisner	SVREC†	Wisner	Caro	Caro	Wisner	SVREC†	Wisner	Caro	Caro
Black Velvet	54.5	65.5				3.68	3.22			
Medalist	52.3	66.8	39.6	26.2	34.3	3.48	2.83	3.77	3.34	3.76
Merlin					37.1					3.64
R99 (no Nod)	0.0	0.0	0.0	0.0	0.0	3.20	2.89	3.95	3.58	3.37
Shania			23.5	42.2	44.0			4.32	3.37	3.80
Vista	52.3	61.8	30.6	32.0	39.3	3.80	3.16	3.95	3.52	3.61
Zenith			20.9	52.4	53.3			4.25	3.66	3.51
Zorro	46.8	71.3	29.6	31.0	35.1	3.60	2.86	4.00	3.67	3.62
Experimental Lines‡										
Highest Yield	65.1	71.7	52.8	62.3	48.6	4.18	3.89	4.67	3.81	3.16
Lowest Yield	33.4	49.9	9.8	11.6	11.1	3.18	2.68	3.46	2.91	3.85
Test Mean	50.4	63.3	32.6	31.1	35.8	3.60	3.10	4.00	3.50	3.60
LSD ($p \leq 0.05$)	20.8	9.3	25.0	25.0	15.1	0.2	0.5	ns	0.4	2.8
CV (%)	28.8	10.4	42.9	46.0	28.1	3.9	10.6	8.9	7.9	5.6

† Saginaw Valley Research and Extension Center, Frankenmuth, MI.

‡ Highest and lowest value in each site and year of advanced breeding lines. May not be the same line each year.

Table 2.05. Pearson correlations for %Ndfa (lower left) and %N (upper right) for 79 black and navy bean genotypes grown under organic conditions in Frankenmuth, Caro, and Wisner, MI in 2011, 2012, and 2013 †.

	Wisner 2011	SVREC‡ 2011	Wisner 2012	Caro 2012	Caro 2013
Wisner 2011		0.68***	0.63*	0.59*	0.65*
SVREC‡ 2011	0.57**		0.55*	0.58*	0.04
Wisner 2012	0.88**	0.82*		0.56**	0.30
Caro 2012	0.77*	0.73*	0.27		0.07
Caro 2013	0.96*	0.94*	0.38	0.50*	

† Correlation significant at * $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.0001$.
‡ Saginaw Valley Research and Extension Center, Frankenmuth, MI.

Table 2.06. Traits measured on 79 black and navy dry bean genotypes grown under N free conditions in the greenhouse in East Lansing MI in 2011 and 2012.

Commercial Checks	Shoot N /Root N[†]	Nodule Rating[‡]	Shoot Biomass	Root Biomass	Total Biomass	Root/Shoot Biomass	%Ndfa[§]	Shoot Biomass Difference[ʅ]	Root Biomass Difference[ʅ]	Biomass Difference[ʅ]
				-----g-----						
Black Velvet	2.81	6.0	5.85	1.80	7.65	0.31	94.2	93.2	72.2	88.2
Medalist	2.09	4.5	1.75	0.80	2.55	0.46	89.7	77.1	37.5	64.7
Merlin		3.5	9.45	3.56	13.00	0.38	93.3	95.8	85.9	93.1
R99 (no Nod)	0.67	0.0	0.40	0.50	0.90	1.25	0.0	0.0	0.0	0.0
Shania		2.0	2.07	1.56	3.63	0.75	91.9	80.7	68.0	75.2
Vista	2.89	2.5	1.85	0.70	2.55	0.38	90.5	78.4	28.6	64.7
Zenith		4.0	9.44	3.04	12.48	0.32	98.4	95.8	83.6	92.8
Zorro	2.70	3.5	2.50	0.80	3.30	0.32	92.5	84.0	37.5	72.7
Experimental Lines[ʒ]										
High	3.50	6.0	13.40	4.24	17.37	0.83	98.8	97.0	88.2	94.8
Low	1.64	2.0	0.75	0.30	1.05	0.23	75.8	46.7	0.0	14.3
Test Mean	2.59	3.52	4.92	1.78	6.50	0.39	91.6	83.2	53.2	75.1
LSD ($p \leq 0.05$)	n.s.	1.55	2.03	1.49	2.03	n.s.	22.7	22.7	34.5	28.9
CV (%)	30.1	21.9	30.7	36.1	29.2	27.9	14.1	14.7	30.1	22.7

[†] Ratio of shoot N (g) to root N (g).
[‡] Rating of nodules on roots 6.0 being heavily nodulated and 0.0 having no nodules.
[§] %Ndfa calculated on biomass using the difference method.
[ʅ] Biomass difference calculated using the biomass (g) of each respective plant part with the non-nodulating R99 used as a reference.
[ʒ] Highest and lowest value in each trait of advanced breeding lines. May not be the same line for each trait.

Table 2.07. Pearson Correlations of traits measured of 79 black and navy bean genotypes grown under N free conditions in the greenhouse in East Lansing, MI in 2011 and 2012†.

	Shoot N	Nodule Rating	%Ndfa	Shoot Biomass	Root Biomass	Total Biomass	Root/Shoot§	Shoot Biomass Diff‡	Root Biomass Diff‡
Nodule Rating	0.32*								
%Ndfa	0.45***	0.53***							
Shoot Biomass	0.98***	0.31*	0.44***						
Root Biomass	0.92***	0.23*	0.43***	0.93***					
Total Biomass	0.98***	0.29***	0.45***	0.99***	0.96***				
Root/Shoot§	-0.30*	-0.53***	-0.69***	-0.31*	-0.08	-0.25*			
Shoot Biomass Diff‡	0.65***	0.56***	0.92***	0.65***	0.64***	0.65***	-0.58***		
Root Biomass Diff‡	0.74***	0.38***	0.54***	0.73***	0.80***	0.80***	-0.07	0.80***	
Total Biomass Diff‡	0.73***	0.51***	0.78***	0.73***	0.74***	0.74***	-0.39**	0.96***	0.93***

† Correlation significant at * $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.0001$

‡ Diff = (biomass of N fixer (g)-biomass non-fixer (g))/biomass of N fixer (g)

§ Root/Shoot=root biomass (g)/shoot biomass (g)

Table 2.08. Pearson correlations for field traits and greenhouse traits measured for 79 black and navy bean genotypes grown in the field under organic production systems and in the greenhouse in East Lansing, MI under N free conditions in 2011 and 2012†.

	Seed Yield					Field Ndfa‡					N Yield				
	Wisner 2011	SVREC§ 2011	Wisner 2012	Caro 2012	Caro 2013	Wisner 2011	SVREC§ 2011	Wisner 2012	Caro 2012	Caro 2013	Wisner 2011	SVREC§ 2011	Wisner 2012	Caro 2012	Caro 2013
Shoot N to Root N§	0.21	0.49*	0.52	0.56	0.92*	0.43*	0.62***	0.76**	0.75*	0.95*	0.33*	0.47*	0.56	0.53	0.93**
Nodule Rating	0.36*	0.51**	0.01	0.04	0.26	0.35*	0.57**	0.24	0.08	0.38*	0.31	0.42**	0.01	0.06	0.23
Root/shoot ratio	-0.42*	-0.65***	-0.33*	-0.19	-0.28	-0.71***	-0.85***	-0.54**	-0.25	-0.38*	-0.49*	-0.64***	-0.34*	-0.18	-0.27
%Ndfa	0.46*	0.67***	0.27	0.30	0.38*	0.70***	0.88***	0.51*	0.42*	0.59***	0.49**	0.61***	0.33*	0.28	0.43*
Shoot Biomass Diff˥	0.45*	0.56**	0.23	0.35*	0.39*	0.60***	0.73***	0.46*	0.43*	0.54**	0.44*	0.48*	0.30*	0.33*	0.42*
Total Biomass Diff˥	0.39*	0.40*	0.18	0.39*	0.40*	0.44*	0.51**	0.39*	0.43*	0.49*	0.35*	0.30	0.26	0.38*	0.43*

† Correlation significant at * $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.0001$.
‡ Calculated using the difference method.
§ Saginaw Valley Research and Extension Center, Frankenmuth, MI.
˥ Biomass difference calculated using the biomass (g) of each respective plant part with the non-nodulating R99 used as a reference.

APPENDIX B

CHAPTER 2 SUPPLEMENTAL TABLES

Table S2.01. Seed yield and N yield of 79 black and navy bean genotypes grown under organic conditions in 2011, 2012, and 2013 in Frankenmuth, Caro, and Wisner, MI.

	Seed Yield					N yield				
	2011		2012		2013	2011		2012		2013
Commercial Checks	Wisner	SVREC†	Wisner	Caro	Caro	Wisner	SVREC†	Wisner	Caro	Caro
					-----kg ha^{-1}-----					
Black Velvet	1773	1272				65	42			
Medalist	1038	1350	2187	1247	1683	36	37	83	42	29
Merlin					1759					
R99	847	421	1151	1077	1192	27	12	45	38	19
Shania			1364	1752	1695			59	59	30
Vista	1706	1197	1735	1335	1873	65	38	68	47	31
Zenith			1409	2331	2474			58	85	40
Zorro	2117	1519	1652	1458	1740	76	44	66	53	29
Experimental lines‡										
B09101	1959	1125				68	35			
B09128	1730	1392				63	40			
B09129	1939	1064				63	31			
B09135	1906	1265				67	40			
B09136	1801	1214				66	35			
B09166	1975	1201				71	36			
B09175	1921	1515	1867	1600		69	47	73	56	
B09188	1457	1356				49	39			
B09197	1908	1212	2114	1415	1675	63	35	77	46	26
B09199	1660	1492	1815	1372		53	40	63	42	
B09201	1328	1197				48	32			
B09204	1692	1204	2262	1052		54	34	86	36	

Table S2.01 (*cont'd*)

B10201	1667	1316				62	44			
B10202	1479	1493				56	46			
B10203	1862	1140				62	32			
B10243			907	877				39	33	
B10246	1654	1216				62	41			
B11302			2402	2438	2036			97	89	35
B11334			1931	1314				77	43	
B11343			1461	1537				59	54	
B11361			2570	1748				94	51	
B11363			1609	1194	1752			61	39	28
B11371					2139					33
B11375			1825	2013				73	63	
B11519[§]			1438	1625	1044			53	56	16
B11536[§]					1511					25
B11545[§]					1811					30
B11552[§]			1116	1090				47	41	
B11582[§]					1919					32
B11588[§]			1809	1445	1673			73	46	25
B11594[§]					1646					28
B11611[§]			1421	1091				56	41	
B11617[§]					2215					36
B12709					1928					32
B12720					2190					33
B12721					1667					29
B12724					2157					32

Table S2.01 (*cont'd*)

N07007	1558	1273				60	41			
N09020	1386	1141	1624	1511	1517	49	33	64	50	26
N09034	1347	1044	1981	1447		49	34	76	52	
N09035	1365	1177				48	26			
N09041	1196	1249				43	37			
N09045	1804	1222				64	40			
N09046	1562	1429				63	43			
N09055	1359	1101				53	39			
N09056	1228	1178				48	48			
N09104	1811	1262				64	37			
N09174	1678	1369				58	42			
N09178	1199	1098				44	37			
N10101	1367	934				57	33			
N10108	1827	1297				67	37			
N10109	1527	1314				56	37			
N11202			1861	1454				72	54	
N11216			2431	1310				90	44	
N11225			1461	1483				61	55	
N11226			1615	2151	1608			66	76	27
N11228			1675	2126	1833			66	74	31
N11230					1568					28
N11232			1879	1632				79	59	
N11256			1669	2052	1679			69	72	29
N11257			1200	2068	1948			55	73	34
N11258			1863	1797				76	63	

Table S2.01 (*cont'd*)

N11277					1791					32
N11283			1983	1850	1911			77	58	33
N11284			2145	1539	1451			78	49	24
N11292			1118	1665				47	59	
N11296					1386					22
N11298			1505	1172	1702			61	40	27
N12442					1732					30
N12453					1806					30
N12466					1738					31
Test Mean	1601	1228	1711	1557	1762	58	37	67	54	29
LSD ($p \leq 0.05$)	313.1	283.0	730.1	747.7	416.3	11.8	11.3	27.5	27.2	6.7
CV (%)	14.0	16.4	26.2	32.7	16.8	14.7	21.4	25.0	34.6	16.3

† Saginaw Valley Research and Extension Center, Frankenmuth, MI.

‡ Advanced breeding lines were rotated through the trial based on performance.

§ Selected genotypes from the Puebla 152/Zorro recombinant inbred line (RIL) population (See Chapter 2).

Table S2.02. Percent N derived from the atmosphere (%Ndfa) and percent N in seed of 79 black and navy bean genotypes grown under organic conditions in 2011, 2012, and 2013 in Frankenmuth, Caro, and Wisner, MI.

	%Ndfa Difference Method					% N in Seed				
	2011		2012		2013	2011		2012		2013
Commercial Checks	Wisner	SVREC[†]	Wisner	Caro	Caro	Wisner	SVREC[†]	Wisner	Caro	Caro
Black Velvet	51.5	65.5				3.68	3.22			
Medalist	52.9	66.8	39.6	26.2	34.3	3.48	2.83	3.77	3.34	3.76
Merlin					37.1					3.64
R99	0.0	0.0	0.0	0.0	0.0	3.20	2.89	3.95	3.58	3.37
Shania			23.5	42.2	44.0	3.80		4.32	3.37	3.80
Vista	57.3	61.8	30.6	32.0	39.2	3.80	3.16	3.95	3.52	3.61
Zenith			20.9	52.4	53.3			4.25	3.66	3.51
Zorro	46.8	71.3	29.6	31.0	35.1	3.60	2.86	4.00	3.67	3.62
Experimental lines[‡]										
B09101	45.3	62.7				3.50	3.07			
B09128	52.2	69.7				3.65	2.95			
B09129	91.0	60.6				3.23	2.90			
B09135	65.1	66.5				3.50	3.22			
B09136	39.4	63.8				3.68	2.87			
B09166	60.0	64.7				3.58	2.93			
B09175	53.5	71.7	48.2	27.1		3.60	3.16	3.82	3.47	
B09188	51.9	68.9				3.33	2.97			
B09197	62.5	65.3	40.4	22.9	35.9	3.28	2.88	3.67	3.18	3.41
B09199	46.3	71.1	25.8	15.0		3.18	2.68	3.46	3.05	
B09201	63.3	61.8				3.63	2.79			
B09204	53.1	62.9	45.7	14.9		3.23	2.79	3.79	3.43	
B10201	47.4	66.1				3.73	3.22			

Table S2.02 (*cont'd*)

B10202	37.5	70.7				3.78	3.19			
B10203	38.6	63.3				3.35	2.85			
B10243			9.8	14.8				4.32	3.77	
B10246	59.0	65.3			46.3	3.75	3.32	4.05	3.63	
B11302			52.8	62.3	33.3					3.69
B11334			41.1	11.6				4.00	3.27	
B11343			31.8	28.9				4.08	3.64	
B11361			48.5	35.6				3.71	2.91	
B11363			21.9	11.6				3.76	3.27	3.44
B11371					44.1					3.37
B11375			38.2	40.2				3.99	3.16	
B11519[§]			24.2	30.4	11.1			3.72	3.54	3.30
B11536[§]					30.3					3.50
B11545[§]					40.5					3.62
B11552[§]			13.3	21.3				4.16	3.81	
B11582[§]					40.8					3.57
B11588[§]			37.2	45.2	23.9			4.08	3.22	3.16
B11594[§]					30.7					3.74
B11611[§]			17.8	15.1				4.06	3.80	
B11617[§]					48.6					3.54
B12709					41.4					3.61
B12720					4301.0					3.23
B12721					35.0					3.76
B12724					41.5					3.23
N07007	60.1	66.6				3.85	3.20			

Table S2.02 (*cont'd*)

N09020	55.9	62.4	37.4	34.6		3.50	2.94	3.91	3.30	3.70
N09034	45.1	58.6	38.3	27.1		3.65	3.31	3.82	3.54	
N09035	58.1	64.3				3.53	2.90			
N09041	51.0	65.9				3.60	3.00			
N09045	46.5	65.7				3.53	3.22			
N09046	33.4	70.7				4.08	3.04			
N09055	64.4	61.3				3.90	3.59			
N09056	54.4	63.0				3.93	3.89			
N09104	32.5	65.8				3.55	2.98			
N09174	50.5	68.9				3.48	3.05			
N09178	56.2	61.2				3.68	3.33			
N10101	60.5	49.9				4.18	3.60			
N10108	57.6	66.1				3.65	2.85			
N10109	48.3	71.6				3.65	2.85			
N11202			31.0	35.0				3.85	3.72	
N11216			46.8	13.3				3.71	3.38	
N11225			23.5	30.9				4.25	3.73	
N11226			29.7	56.9	26.4			4.10	3.61	3.55
N11228			36.8	33.8	40.9			3.94	3.44	3.71
N11230					28.7					3.77
N11232			39.5	28.8				4.19	3.54	
N11256			29.4	47.2	32.9			4.16	3.52	3.76
N11257			26.8	47.7	43.6			4.67	3.57	3.73
N11258					34.8			4.03	3.51	
N11277			36.9	37.3	41.8					3.85

Table S2.02 (*cont'd*)

N11283			39.6	29.9	41.8			3.90	3.11	3.68
N11284			37.7	28.6	19.0			3.63	3.25	3.61
N11292			24.4	42.3				4.15	3.57	
N11296					26.4					3.49
N11298			25.2	30.0	31.9			4.06	3.41	3.51
N12442					37.0					3.79
N12453					37.9					3.59
N12466					38.2					3.80
Test Mean	50.400	63.300	32.600	31.100	35.800	3.6	3.1	4	3.5	3.6
LSD ($p \leq 0.05$)	20.8	9.3	25.0	25.0	15.1	0.20	0.50	ns	0.40	0.28
CV (%)	28.8	10.4	42.9	46.0	28.1	3.9	10.6	8.9	7.9	5.6

† Saginaw Valley Research and Extension Center, Frankenmuth, MI.
‡ Advanced breeding lines were rotated through the trial based on performance.
§ Selected genotypes from the Puebla 152/Zorro recombinant inbred line (RIL) population (See Chapter 2).

Table S2.03. Traits measured on 79 black and navy dry bean genotypes grown under N free conditions in the greenhouse in East Lansing MI.

Commercial Checks	Shoot N /Root N[†]	Nodule Rating[‡]	Shoot Biomass	Root Biomass	Total Biomass	Root/Shoot Biomass	%Ndfa[§]	Shoot Biomass Difference[ɩ]	Root Biomass Difference[ɩ]	Biomass Difference[ɩ]
				---g---						
Black Velvet	2.81	6.0	5.85	1.80	7.65	0.31	97.2	93.2	72.2	88.2
Medalist	2.09	4.5	1.75	0.80	2.55	0.46	89.7	77.1	37.5	64.7
Merlin		3.5	9.45	3.56	13.00	0.38	98.3	95.8	85.9	93.1
R99	0.67	0.0	0.40	0.50	0.90	1.25	0.0	0.0	0.0	0.0
Shania		2.0	2.07	1.56	3.63	0.75	91.9	80.7	67.9	75.2
Vista	2.89	2.5	1.85	0.70	2.55	0.38	90.5	78.4	28.6	64.7
Zenith		4.0	9.44	3.04	12.48	0.32	98.4	95.8	83.6	92.8
Zorro	2.70	3.5	2.50	0.80	3.30	0.32	92.4	84.0	37.5	72.7
Experimental lines[ʒ]										
B09101	2.76	3.0	1.70	0.55	2.25	0.32	89.9	76.5	9.1	60.0
B09128	3.16	2.5	0.75	0.30	1.05	0.40	76.7	46.7		14.3
B09129	2.36	3.5	2.75	0.95	3.70	0.35	92.7	85.5	47.4	75.7
B09135	2.84	4.5	3.55	1.10	4.65	0.31	95.5	88.7	54.5	80.6
B09136	3.49	4.0	1.70	0.65	2.35	0.38	89.9	76.5	23.1	61.7
B09166	2.27	2.5	2.30	0.90	3.20	0.39	91.1	82.6	44.4	71.9
B09175	2.88	5.5	4.25	1.20	5.45	0.28	95.7	90.6	58.3	83.5
B09188	2.48	4.0	3.00	1.10	4.10	0.37	93.4	86.7	54.5	78.0
B09197	2.16	3.8	2.00	0.85	2.85	0.43	91.0	80.0	41.2	68.4
B09199	2.49	3.3	1.67	0.65	2.32	0.39	89.0	76.0	23.1	61.2
B09201	2.27	3.5	2.80	1.10	3.90	0.39	93.5	85.7	54.5	76.9
B09204	2.57	4.0	2.80	1.00	3.80	0.36	94.4	85.7	50.0	76.3
B10201	2.24	3.5	2.40	1.10	3.50	0.46	92.6	83.3	54.5	74.3

Table S2.03(*cont'd*)

B10202	2.48	4.0	2.95	1.15	4.10	0.39	94.0	86.4	56.5	78.0
B10203	2.49	4.0	2.85	0.95	3.80	0.33	93.3	86.0	47.4	76.3
B10246	3.37	4.0	2.50	0.70	3.20	0.28	93.7	84.0	28.6	71.9
B11302		3.0	13.40	3.65	17.05	0.27	98.6	97.0	86.3	94.7
B11334		3.0	6.98	2.52	9.50	0.36	97.8	94.3	80.2	90.5
B11361		3.0	6.20	2.20	8.40	0.35	97.7	93.5	77.3	89.3
B11363		3.0	9.65	3.31	12.96	0.34	98.3	95.9	84.9	93.1
B11371		5.0	9.43	2.85	12.28	0.30	98.4	95.8	82.5	92.7
B11375		2.5	6.91	2.73	9.64	0.39	97.5	94.2	81.7	90.7
B11519[§]		4.5	3.18	1.07	4.25	0.34	92.3	87.4	53.2	78.8
B11536[§]		4.0	3.78	1.32	5.10	0.35	94.1	89.4	62.2	82.4
B11545[§]		4.5	5.10	1.94	7.05	0.38	94.8	92.2	74.3	87.2
B11552[§]		4.5	6.23	1.87	8.09	0.30	96.4	93.6	73.2	88.9
B11582[§]		4.0	3.27	1.26	4.53	0.39	93.3	87.8	60.5	80.1
B11588[§]		4.0	3.90	1.60	5.50	0.41	89.4	89.7	68.7	83.6
B11594[§]		2.5	3.01	1.13	4.14	0.38	92.5	86.7	55.8	78.3
B11611[§]		5.0	4.84	1.98	6.82	0.41	95.7	91.7	74.8	86.8
B11617[§]		3.5	3.05	1.22	4.27	0.40	91.1	86.9	58.9	78.9
B12271		6.0	12.80	3.17	.	0.25	98.7			
B12709		2.0	2.23	1.85	4.08	0.83	91.6	82.1	73.0	77.9
B12720		4.0	10.30	3.13	13.43	0.30	98.4	96.1	84.0	93.3
B12721		3.0	10.36	2.52	12.88	0.24	98.5	96.1	80.2	93.0
B12724		3.0	5.37	2.52	7.89	0.47	97.4	92.6	80.2	88.6
N07007	2.47	2.0	0.75	0.30	1.05	0.40	75.8	46.7		14.3
N09020	2.39	2.5	1.65	0.70	2.35	0.42	90.0	75.8	28.6	61.7

Table S2.03(*cont'd*)

N09034	2.33	3.0	1.65	0.65	2.30	0.39	89.6	75.8	23.1	60.9
N09035	3.24	4.0	2.95	0.80	3.75	0.27	94.7	86.4	37.5	76.0
N09041	3.24	3.5	1.50	0.40	1.90	0.27	87.5	73.3		52.6
N09045	2.16	4.5	2.60	1.00	3.60	0.38	93.2	84.6	50.0	75.0
N09046	3.50	2.5	1.90	0.50	2.40	0.26	88.4	78.9	0.0	62.5
N09055	2.83	3.5	2.20	0.70	2.90	0.32	92.5	81.8	28.6	69.0
N09056	2.66	2.0	1.00	0.35	1.35	0.35	81.6	60.0		33.3
N09104	1.64	4.0	1.05	0.60	1.65	0.57	84.7	61.9	16.7	45.5
N09174	2.47	3.5	2.20	0.85	3.05	0.39	92.3	81.8	41.2	70.5
N09178	2.96	3.8	2.30	0.75	3.05	0.33	93.4	82.6	33.3	70.5
N10101	2.49	3.0	1.75	0.70	2.45	0.40	90.7	77.1	28.6	63.3
N10108	2.24	2.5	2.15	0.80	2.95	0.37	89.1	81.4	37.5	69.5
N10109	3.14	3.0	1.75	0.85	2.60	0.49	92.1	77.1	41.2	65.4
N11202		2.0	6.17	3.68	9.85	0.60	97.7	93.5	86.4	90.9
N11216		4.5	13.13	4.24	17.37	0.32	98.8	97.0	88.2	94.8
N11225		3.0	8.37	3.59	11.95	0.43	98.1	95.2	86.1	92.5
N11226		3.0	4.57	2.36	6.93	0.52	96.7	91.2	78.8	87.0
N11228		5.0	10.96	3.80	14.76	0.35	97.8	96.4	86.8	93.9
N11230		3.5	11.68	3.75	15.43	0.32	98.7	96.6	86.7	94.2
N11232		3.5	11.08	3.25	14.32	0.29	98.6	96.4	84.6	93.7
N11256		3.0	4.46	1.69	6.15	0.38	96.5	91.0	70.4	85.4
N11257		5.0	5.96	2.59	8.55	0.43	97.3	93.3	80.7	89.5
N11258		4.0	6.36	3.37	9.73	0.53	97.7	93.7	85.2	90.8
N11277		3.5	6.88	3.12	9.99	0.45	97.9	94.2	83.9	91.0
N11283		4.0	6.92	2.93	9.85	0.42	97.8	94.2	82.9	90.9

Table S2.03(*cont'd*)

N11284		3.0	8.94	2.83	11.77	0.32	97.9	95.5	82.3	92.4
N11292		4.5	10.65	3.43	14.08	0.32	98.6	96.2	85.4	93.6
N11296		4.0	11.60	4.24	15.84	0.37	98.7	96.6	88.2	94.3
N11298		5.0	9.59	4.05	13.63	0.42	98.3	95.8	87.6	93.4
N12442		3.0	4.62	2.46	7.08	0.53	96.3	91.3	79.7	87.3
N12453		4.0	10.47	2.45	12.92	0.23	98.7	96.2	79.6	93.0
N12466		4.0	5.75	2.31	8.06	0.40	97.4	93.0	78.4	88.8
Test Mean	2.59	3.52	4.92	1.78	6.50	0.39	91.6	83.2	53.2	75.1
LSD ($p \leq 0.05$)	n.s.	1.55	2.03	1.49	2.03	n.s.	22.7	22.7	34.5	28.9
CV (%)	30.1	21.9	30.7	36.1	29.2	27.9	14.1	14.7	30.1	22.7

[†] Ratio of shoot N (g) to root N (g).
[‡] Rating of nodules on roots 6.0 being heavily nodulated and 0.0 having no nodules.
[§] %Ndfa calculated on biomass using the difference method.
[ɭ] Biomass difference calculated using the biomass (g) of each respective plant part with the non-nodulating R99 used as a reference.

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LITERATURE CITED

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CHAPTER 3

NITROGEN FIXATION ABILITY OF A PUEBLA 152/ZORRO RIL POPULATION EVALUATED UNDER GREENHOUSE AND FIELD CONDITIONS

Abstract

Variability in symbiotic nitrogen fixation (SNF) can be found within *Phaseolus vulgaris* L. The black bean landrace selection 'Puebla 152' has been identified as having high SNF ability, but it is poorly adapted to cultivation at northern latitudes due to long season and indeterminate type III growth habit. The recombinant inbred line (RIL) population developed by crossing Puebla 152 with the commercial black bean cultivar 'Zorro' was used to investigate the inheritance of enhanced SNF ability. The recombinant inbred line (RIL) population was evaluated in the greenhouse under N free conditions, and under low N conditions in the field in East Lansing (EL), MI and in the field in Isabela, Puerto Rico (PR). Site year averages for percent N derived from the atmosphere (%Ndfa) ranged between 12.7 % up to 66.6 %, although individual RILs ranged up to 90.5 %Ndfa. Traits measured in the greenhouse such as shoot biomass and biomass difference correlated moderately with %Ndfa traits measured in the field.

Introduction

Common or dry bean (*Phaseolus vulgaris* L.) is capable of fixing N through an association with *Rhizobium* spp. but common bean is often considered poor at fixing N (Buttery et al., 1992; Bliss, 1993; Piha and Munns, 1987; Fageria et al., 2014) in comparison to soybean (*Glycine max* (L.) Merr.) and chickpea (*Cicer arietanum* L.). The range in percent N derived from the atmosphere (%Ndfa) dry bean is reported to average 40 %Ndfa ranging up to 73 %Ndfa while soybean averages 68 %Ndfa but can reach as high as 95 %Ndfa in laboratory settings (Herridge et al., 2008). In field studies, common bean averaged 36 %Ndfa compared with crops such as soybean and chickpea reaching 58 %Ndfa and 65 %Ndfa, respectively (Herridge et al., 2008). Piha and Munns (1987) conducted an acetylene reduction assay to determine the activity of the nitrogenase in the nodules of the species. They noted that dry bean evolved more H^+ during fixation than soybean or chickpea representing a reduction in efficiency of SNF for bean. They also compared the size and number of the nodules and discovered that the nodules of dry bean were smaller but more numerous than soybean or chickpea. Piha and Munns (1987) also noted that the period between germination and flowering of dry bean was much shorter, 27 days in their study, compared to chickpea, which averaged 34 days to flowering. In addition, the interval between flowering and physiological maturity was much shorter for chickpea suggesting that the chickpea simply had more time in a vegetative state to establish nodules and fix N before the strong sink strength of the seed for photosynthate competes with the nodules for resources (Piha and Munns, 1987). In an effort to better calculate the contribution of pulse crops to soil N levels, Walley et al. (2007) used published data from the Northern Great Plains area to investigate the contributions of N fixation in pulse crops such as pea (*Pisum sativum* L.), lentil (*Lens culinaris* Medik.), chickpea, dry bean and faba bean (*Vicia fabia* L.). Similar to other findings Walley et al. (2007) determined that dry bean had the lowest average percent nitrogen derived from the atmosphere (%Ndfa) (40 %Ndfa) and also had the highest amount of variability year to year and by location among the pulse crops studied. Faba bean was the highest fixer achieving 84 %Ndfa. Values reported for %Ndfa are also quite variable in dry bean. Pereira et al. (1989) investigated the N fixation ability of a wide range of dry bean genotypes grown in the field in Hancock, Wisconsin. The average %Ndfa was 21.6%, however, the range was from 5.7% for the navy bean ‘Sanilac’ to a 31.6% for the black bean Puebla 152. There was not a strong relationship between %Ndfa and seed yield as some lines that exhibited a higher %Ndfa were not necessarily the best adapted or the highest yielding lines (Pereira et al., 1989). The ability of the genotype to partition fixed N into seed is an important trait that cannot be overlooked as selecting on high %Ndfa may result in genotypes that are not efficient in partitioning.

N fixation in dry bean is a quantitatively inherited trait. St. Clair and Bliss (1991) demonstrated that enhanced SNF ability was heritable in a population of inbred backcross lines (IBLs) developed from Puebla 152 and Sanilac, which was the recurrent parent. The goal was to produce lines with the agronomic characteristics of Sanilac with enhanced SNF ability. Select IBLs were intercrossed to generate F_3 families which were then evaluated for %Ndfa (St. Clair and Bliss, 1991). Several F_3 families did have a higher %Ndfa (51.2 %Ndfa to 60.8 %Ndfa) than Puebla 152 (50.4 %Ndfa) but none of those lines yielded higher than Puebla 152, (St. Clair and Bliss, 1991). It is possible to select progeny with enhanced SNF ability though yield may not be correlated to SNF traits such as %Ndfa (St. Clair and Bliss, 1991; Elizondo Barron et al., 1999; Bliss, 1993; Buttery et al., 1992).

To better understand the importance of partitioning of fixed N into the seed, Wolyn et al. (1991) utilized lines selected from the two IBL populations derived from Puebla 152 and either Sanilac or 'Porrillo Sintetico.' Measurements for %Ndfa were taken at pod fill (R3) and maturity (R9) to track the partitioning of N through development. Those lines with the highest %Ndfa at R3 did not produce the highest %Ndfa at maturity. Line 24-17 had 17.5 %Ndfa at R3, which was the 2nd lowest of the 5 lines studied while at maturity produced the highest value (44.6 %Ndfa; Wolyn et al., 1991). Maturity was positively correlated with N fixation, though some lines, such as line 24-21 matured earlier than Sanilac but had a higher %Ndfa which suggests that it is possible to select lines that were early maturing with enhanced SNF ability than the recurrent parent Sanilac (Wolyn, et al., 1991).

Differences in a genotype's partitioning and discrimination of ^{15}N can result in over or underestimation of %Ndfa. Lazali et al. (2014) looked at six RILs selected from a population developed by crossing BAT477 and DOR364. The individual lines were selected for their tolerance or sensitivity to P deficiency. They found that there were differences across P levels and among the genotypes in their discrimination against ^{15}N (Lazali et al., 2014). Looking at different portions of the plant-roots, shoots, and nodules, they found that a higher proportion of ^{15}N remained in the roots, specifically the nodules, while the proportion of $^{15}N/^{14}N$ was lower in the shoots. Thus, measuring ^{15}N in the shoot might result in an overestimation of %Ndfa. Lazali et al. (2014) did find a significant correlation between ^{15}N in nodules and P sensitivity and N fixed by the plant. In addition to the genotypes discriminating against ^{15}N, different strains of Rhizobia also discriminate differently for ^{15}N. Yoneyama et al. (1986) found that not only did the 10 Rhizobium strains vary in their SNF ability, the amount of ^{15}N in the shoot of the three genotypes ('Himetebou,' 'Daifuku,' and 'Toramame') tested, varied considerably when inoculated by different strains. In the laboratory, it is possible to control the specific strain of Rhizobium, however, in the field nodule occupancy is likely to vary even within the same plant. Yoneyama et al. (1984) noted that the amount of ^{15}N varied by the plant organ with stems and petioles having considerably less ^{15}N than leaves. Studying the kidney bean genotypes 'Shakugosum' and 'Nagauzura,' $\delta^{15}N$ was +9.3 and +8.5 in the nodules, +1.5 and -1.0 for pods, and -0.6 and +2.8 for stems, respectively (Yoneyama et al., 1984). The selection of the plant part in calculations could result in a very different estimation of %Ndfa.

While Puebla 152 is poorly adapted to production at northern latitudes due to late maturity and vigorous indeterminate type III growth habit it has been recognized as a valuable source of enhanced SNF characteristics. The purpose of this study was to generate a RIL population with Puebla 152 to investigate characteristics associated with SNF in a black bean mapping population adapted to production in northern latitudes.

Materials and Methods

Plant Material

A recombinant inbred line (RIL) mapping population consisting of 122 lines was generated and used in the quantitative trait loci (QTL) analysis of traits associated with SNF. The landrace selection Puebla 152 was selected as the donor parent due to its enhanced SNF ability (Chaverra and Graham, 1992). Puebla 152 is a small black seeded genotype belonging to the Mesoamerican gene pool and originates in Puebla, Mexico (St. Clair and Bliss, 1991). Puebla 152 is poorly adapted to production in northern latitudes due to its late maturity and type III growth habit. The commercial cultivar Zorro (Kelly et al., 2009) was selected as the other parent because it is efficient and has a type II growth habit. Zorro is also a small seeded black bean cultivar adapted to production in northern latitudes, and is not known to possess enhanced SNF ability.

In the fall of 2007 Zorro was crossed with Puebla 152 in the Plant Research Greenhouses at Michigan State University in EL, MI. Seed was harvested and planted in the greenhouse in February 2008 to grow out F2 plants. F2 seed was space planted in the field in June 2009 at the Saginaw Valley Research and Extension Center (SVREC) in Frankenmuth, MI. A single pod was harvested from each of 150 plants in September 2009. A single seed from each pod was planted in the greenhouse in September 2009 to begin two generations of single seed decent (SSD). Seed from each plant was harvested separately in May 2010 resulting in F_5 seed which was carried forward as a line. Seed of 122 $F_{4:5}$ lines was increased in June 2010 in the field at SVREC. Selfed seed of $F_{4:5}$ lines was used for all further evaluations in the field and greenhouse.

Field Trials

For field trials in 2011, 2012, and 2013 122 RIL lines, parents, and five commercial genotypes were planted. The checks included the parents, Zorro and Puebla 152, and 'Medalist' (a navy bean cultivar) and 'PR0443-151' (a black bean from PR that performed under low fertility conditions). Additional checks, 'Verano' (a mid-sized white bean cultivar from PR; Beaver et al., 2008), TARS-LFR1 (small red bean developed in PR; Porch et al., 2014), and PR1147-6 (black seeded breeding line developed in PR) were included in EL in 2012 and 2013. In addition the non-nodulating genotype 'R99' (Park and Buttery, 2006) was included as a reference to percent nitrogen derived from the atmosphere (%Ndfa).

Field plots for the SNF population consisted of four- 6 m rows spaced 50 cm apart. The outer two rows were planted to an erect, non-vining commercial navy bean cultivar "Indi" to limit border effects. All lines in the RIL population are black, so the utilization of the white seeded border facilitated removal of border seed mixed with the sample. The center two rows of each plot were the yield rows where the experimental lines were planted.

The plot was located on the old Soils Farm located on the campus of Michigan State University, East Lansing, MI. The field was selected because of the low soil nitrogen. Soil type was a Capac Loam and Riddles-Hillsdale Sandy Loam (USDA-NRCS, 2013). The same field was utilized for this study in 2011, 2012, and 2013. Corn was the crop grown on the field in 2010. Soil test results are given in Table 3.01.

Prior to planting, pre-emergence herbicides Sonalan (Syngenta Crop Protection LLC,), Eptam 7E Selective Herbicide (S-ethyl dipropylthiocarbamate) (Gowan, Yuma, AZ), and Dual (S-Metolachlor) (Dow AgroSciences), were applied at a rate of 69 g ha^{-1} active ingredient, 403 g ha^{-1} active ingredient, and 351 g ha^{-1} active ingredient respectively. A postemergence application of the herbicides Raptor Herbicide (Imazamox) (BASF Corporation, Research Triangle Park, NC) at a rate of 5.7 g ha^{-1} active ingredient and Basagran (Sodium salt of bentazon* (3-(1-methylethyl)-1H-2,1,3-benzothiaddiazin-4 (3H0one 2,2-dioxide) (BASF Corporation, Research Triangle Park, NC) at a rate of 115 g ha^{-1} active ingredient were applied to control weeds prior to flowering of the beans. Potato leaf hoppers (*Empoasca fabae*) was controlled as needed with Warrior (Lambda-cyhalothrin1 [1α(S*),3α(Z)]-(±)-cyano-(3-phenoxyphenyl)methyl-3-(2-chloro-3,3,3-trifluoro-1-propenyl)-2,2-dymethylcyclopropane carboxylate) (Syngenta Crop Protection LLC) at a rate of 12 g ha^{-1} active ingredient. Seed was planted using a four row air planter. No fertilizer was applied to the field at any time to maintain low N conditions.

Seed was treated with rhizobial inoculant consisting of fine buffered peat (American Peat Technology, Aitkin, MN, USA) carrying *Rhizobium tropici* strain CIAT899 which was prepared by culturing the rhizobia in yeast mannitol broth for three d prior to mixing with peat to allow sufficient wetting of the peat. The resulting inoculant was incubated in the dark for 8 to 12 wk at room temperature. Seed was mixed with the peat inoculant and a small amount of water to adhere the peat inoculant to the seed. Treated seed was stored in a cool room until planting.

The study was repeated in Isabela, PR in winter 2012 and 2013. The field site was maintained as a low fertility site. Soil type was an acidic Coto Clay, very fine, kaolinitic, isohyperthermic typic eutrustox. Seed was planted in 3 m rows spaced 60 cm apart with 3 replicates and a single row was planted for each genotype.

Data Collection

Plant stand was measured by randomly placing two 1-m rulers randomly in each plot along the yield rows and counting the number of seedlings falling within the 1 m length. Plant stand was measured at the first trifoliate stage. Days to flower was recorded as the number of days after planting when 50% of the plants in each plot had one open flower. Maturity was determined when 50% or more of the plants had reached physiological maturity, at which time plant height (cm), lodging (1=upright, 5=laying on the ground), and agronomic desirability (1= poor, 6=superior) were recorded. A Minolta SPAD 502 Meter (Konica Minolta, Inc., Tokyo, Japan) was used to measure chlorophyll content at early pod fill. The last completely expanded leaf was selected to measure chlorophyll content.

Harvest

When plants had reached harvest maturity, plots were pulled mechanically and raked into piles. Piles were weighed to measure biomass, then threshed with a Wintersteiger AG plot combine (Winterstieger AG, Austria). Seed was air dried, cleaned with a Clipper Mill (A.T. Ferrell Company, Bluffton, IN, USA) before weighing. Seed moisture was measured with a Dickey-john GAC 2500 moisture meter (Churchill Industries, Minneapolis, MN). Yield was calculated by adjusting values to 18 % moisture content. A biomass sample was collected from each plot, weighed the day of harvest, dried for 7 d in a dryer at 60º C, and weighed again to determine moisture content at harvest. At maturity in Isabela, PR, 2 m of row was hand harvested and thrashed with similar methods to measure seed weight and yield for seed harvested in EL, MI.

Nitrogen Analysis

A 30 g subsample from each plot was placed in an envelope and placed into a dryer at 60º C for 1 wk. Seed and biomass was then ground in a Wiley Mill pass through a 1 mm mesh screen. Seed and biomass samples were then stored at room temperature until sent to the Stable Isotope Facility at UC Davis, Davis CA for 15N analysis. A "PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK)" was used to measure the 15N content in relation to the 15N content in the atmosphere. To determine %Ndfa, the following equation was used (Ramaekers et al., 2013):

$$\%Ndfa = \frac{(\delta\,^{15}N \text{ non fixing reference plant} - \delta\,^{15}N \text{ fixing legume})}{(\delta\,^{15}N \text{ non fixing reference plant} - B)} \times 100$$

With B representing the $\delta\,^{15}N$ of the fixing legume grown under N free conditions in the greenhouse where it relies completely on SNF for its nitrogen requirement. Since each genotype studied was also evaluated under N free conditions in the greenhouse each genotypes respective $\delta\,^{15}N$ was used.

Greenhouse Assay

To study the SNF ability of the RILS at flowering, the same 122 RILs and five commercial checks were grown under greenhouse conditions. Seeds of the genotypes being studied were sterilized by soaking in a 10% bleach solution for 2 min followed by two 2-min rinses with sterile water. Six seeds were planted into each plastic 5.7 L nursery container which had been filled with a 2:1 mix, v:v of perlite to vermiculite which had been autoclaved. Seeds were watered in with tap water. At 3 d after germination, 500 ml YMB culture of *R. tropici* CIAT899 was diluted in 20 L of tap water which had been adjusted to a pH of approximately 6.5 which resulted in a concentration of approximately 10^3 cells per ml. Each nursery container received 250 ml of the final rhizobial dilution. Inoculation was repeated 10 d after planting in the same manner to ensure sufficient population levels of rhizobia to effect symbiosis.

Pots were placed randomly on a greenhouse bench with day length extended to 16 h with HPS lights. Two to three times weekly each pot was watered with 500 ml full strength Broughton and Dilworth N free solution (Broughton and Dilworth, 1970) as needed to avoid drought stress. Plants were thinned to two plants per pot before emergence of the first trifoliate. The analysis was repeated three times. When one or both of the plants had at least one flower open, plant shoots were measured and cut with a razor blade at soil level. The perlite and vermiculite was carefully removed from the roots which were measured for maximum length and scored from nodulation on a scale of 0 to 6, with 0 representing roots without nodules and 6 being roots with a large number of fully developed and functioning nodules. Both root and shoot biomass samples were dried in a dyer at 60º C for 7 d at which time they were ground to pass through a 1mm screen on a Wiley mill. Samples were also sent the U.C. Davis SIF for $\delta^{15}N$ analysis. Biomass difference for shoot, root, and whole plant were calculated using the non-nodulating R99 as a reference as follows:

Biomass Difference = Mass (g) fixer-Mass R99 (g)/Mass (g) fixer

Statistical Analysis

A PROC GLM analysis using SAS 9.4 (SAS Institute Inc. 100 SAS Campus Drive, Cary, NC 27513-2414, USA) to generate an ANOVA showed that there was a significant difference among years and locations. Consequently, each year and location was analyzed separately. PROC CORR was used to generate Pearson Correlations.

Results and Discussion

Mean seed yield of the RILs ranged from a low of 1807 kg ha^{-1} in EL in 2012 to a high of 3026 kg ha^{-1} in 2013 and from 835 kg ha^{-1} in 2012 to 984 kg ha^{-1} in 2013 in PR (Table 3.02). In all years precipitation was below average at EL, and precipitation in 2012 in EL was less than 2011 and 2013 resulting in significant water stress (Table 3.01). Average seed yield follows the same trend as precipitation with the 2012 season producing the lowest yields compared to 2011 and 2013 (Table 3.02). In all years and locations, Zorro yielded more than Puebla 152 except in EL in 2013 suggesting that Zorro is better adapted to both locations. The low yields observed for Puebla 152 resulted from an overall lack of adaptation at both locations and from its inability to mature within the normal growing season. The highest yielding RILs differed between years and location and yielded more than both Zorro and Puebla 152 which suggests that there was considerable transgressive segregation for yield at both locations. Yield ranged from low 97 kg ha^{-1} in PR in 2013 to a high of 4304 kg ha^{-1} in EL in 2011. The non nodulating check R99 outyielded Zorro parent in 2012 in EL. Yields in PR varied each season with overall yields in 2012 lower than 2013 but variability was very high (CV~60%) both years (Table 3.02). R99 yielded significantly below the average both years in PR and was equivalent to the local check Verano indicative of low N levels in the test site. The two lines PR0443-151 and TARS-LFR1 bred for low fertility conditions in PR were the highest yielding lines both years providing supporting evidence for low soil fertility levels in PR.   The test site at Isabella PR was specifically chosen as a low fertility site and beans had been continuously grown on the site for five years prior the 2012, so the soil had become heavily infected with root rot pathogens which introduced added variability to the yield results.

Percent N in seed ranged from 2.8 to 4.6% and from 0.38 to 1.7% in biomass over years and locations (Table 3.03). Values were more consistent and did not follow the highly variable trends observed for seed yield. Among the parents and checks, Puebla 152 tended to have higher N values in the seed (3.6%) and R99 had the lower values (2.8%). The RILs showed a wider range on both extremes than either parent. Comparing years and locations, % N in seed and biomass was not significantly different year to year (data not shown). Since there were year and site differences for yield, %N was presented similarly for consistency. It is notable that the CV for seed N were rather small whereas those for biomass were considerably larger. Those RILs with maturities similar to or later than Puebla 152 had not completely dried down by harvest and thus had a larger biomass at harvest compared to more efficient RILs, which were more similar to the efficient parent Zorro, and produced lower biomass.

Estimation of %Ndfa was accomplished with two different methods, the "difference" method and the "natural abundance" method. Each method results in significantly different estimates (Table 3.04). Each method offers advantages and disadvantages while each can possibly lead to incorrectly estimating the actual N fixed (Peoples et al., 2009). Both the difference method and ^{15}N natural abundance depend on the characteristics of the non-fixing reference plant with regard to the reference's access to the same soil profile as the fixing crop as well as similar pattern in N distribution within the plant. In this study the non-fixing navy bean R99 was utilized as a reference as it is the same species as the Puebla 152 and Zorro parents (Singh et al., 1991). The difference method tends to estimate a higher %Ndfa than natural abundance (%Ndfa-$\delta^{15}N$) and was more consistent year to year in maintaining a similar trend among the checks with Puebla

152 generally fixing a greater portion of N from the atmosphere than Zorro in all but one year, EL 2011. Aside from the difficulties associated with measuring %Ndfa there were RILs with greater %Ndfa than either the Puebla 152 or Zorro parent (Table 3.04). One line (B11519) had a higher %Ndfa than Puebla 152 in all but 1 location/year. Values ranged a high of 68.4% for the highest fixing RIL in P.R. in 2012 down to a low of 0 % in EL in 2012. Given that Puebla 152 is late maturing compared to Zorro (Table 3.06) some RILs are better adapted to production in northern latitudes, while combining the enhanced SNF abilities of Puebla 152. Considering the N in seed (g), however, R99 produces more N in seed than Zorro in 2 of 5 location/years (Table 3.05). Comparing the results from 2011 (a favorable growing season) to 2012 (a dry season) when water stress was lower, Zorro had a greater seed N yield, whereas the relationship was reversed in the dry year (Table 3.05). This inconsistency in performance year to year suggests that other factors are having an effect on N dynamics within the plant and response to stresses such as drought can alter SNF ability. In some situations such as drought in EL in 2012, R99 accumulated more seed N than any other genotype studied resulting in low estimations of %Ndfa using the difference method. Nitrogen derived from the atmosphere was also low using the ^{15}N natural abundance method. Lazali et al. (2014) found that RILs from a ‘BAT477’/’DOR 364’ cross discriminated against ^{15}N under P stress. While not a legume, Robinson et al. (2000) found that in wild barley (*Hordeum spontaneum* C. Koch) discriminated against assimilating ^{15}N from the soil under drought conditions. Perhaps these differences in affinities for ^{15}N depending on genotype and environmental stress contribute to inaccuracies in measuring %Ndfa. Both Lazali et al. (2014) and Robinson et al. (2000) found that not only was ^{15}N preferentially avoided, it’s movement within the plant was limited with a higher proportion of ^{15}N remaining in the roots.

In 2011 and 2012 Zorro had higher seed N yield than Puebla 152, 107.5 kg ha^{-1} and 74.8 kg ha^{-1} compared to 79.4 kg ha^{-1} and 33.9 kg ha^{-1}, respectively (Table 3.05). The opposite trend was seen in 2011 and 2012 for biomass, Puebla 152 yielded 278.4 kg ha^{-1} and 101.4 kg ha^{-1} respectively compared to Zorro which yielded 50.0 kg ha $^{-1}$ and 15.8 kg ha^{-1}, respectively (Table 3.05). The resulting N harvest index (NHI) is substantially lower for Puebla 152, 24.2 % in 2011 and 26.2 % in 2012 and much higher in Zorro at 68.3% and 82.1 % in 2011 and 2012, respectively (Table 3.05). Zorro is much more efficient than Puebla 152 and partitions a greater proportion of N accumulated into the seed, which is highly desirable in a modern dry bean cultivar. Given the total amount N in plant biomass and seed in the same field, Puebla 152 (357.8 kg ha^{-1} in 2011 and 135.3 kg ha^{-1} in 2012) compared to Zorro (157.6 kg ha^{-1} in 2011 and 90.6 kg ha^{-1} in 2012) Puebla 152 likely obtained a significantly higher proportion of N from the atmosphere but is very inefficient compared to Zorro. In 2011 the NHI of RIL B11560 was 84.1% and the second highest B11617 at 77.6 %. In 2012 B11617 had the highest NHI at 85.5 % which was higher than Zorro in each year. Both B11617 and B11560 had slightly below average yield (data not shown). Increasing the amount of fixed N is not useful if that N is not subsequently partitioned into the seed. Genotypes such as Puebla 152 (NHI=26.2%) leave the majority of N fixed in the field within the straw residue. Overall, N yield was lower in PR compared to EL for checks, including those developed in PR (Table 3.05). Average flowering and maturity were earlier in PR than EL, the shorter vegetative phase, combined with less time between flowering and harvest resulted in less time to fix N and could account for some of the differences. In addition, the field in PR had a long history of dry bean production resulting in higher root disease pressure which may have further contributed to lower N yields in PR.

Generally, Puebla 152 had the longest days to flower and maturity of all checks in EL (Table 3.06). In PR, Puebla 152 had similar maturity to Zorro and the other checks. The shorter day length in PR may have helped Puebla 152 to initiate flowering in a manner similar to the day length insensitive checks which resulted in similar maturity (Table 3.06). In EL, however, long days in summer may have contributed to the late maturity of Puebla 152. For both flowering and maturity transgressive segregation was seen as there were RILs that exceeded Puebla 152 as well as RILs which flowered and matured earlier than Zorro (Table 3.06).

In the N-free greenhouse analysis none of the RILs accumulated as much biomass as Puebla 152 (Table 3.07). Puebla 152 also had the highest % N in the shoot, shoot:root ratio, and biomass difference (Table 3.07). Since the only N available to the plant was that present in the seed at planting, in the tap water, or in dust deposited on the potting media it would be expected that the vast majority of the N found in the plant was derived from the atmosphere which resulted in the high average 94.8 %Ndfa. As N is fixed in the nodules it must be mobilized through the plant into the biomass and the amount of biomass accumulated depends on the amount of N available. This circular relationship is further enforced by the fact that photosynthate is necessary to provide the energy for nodule function. Puebla 152 produces a shoot to root ratio of 3.28 compared to Zorro at 2.63 and R99 at 0.9. Perhaps a mechanism employed by dry bean plants is to support root growth to mine N from the soil when N is limiting while resources are only invested in the shoot as N becomes less limiting.

Ideally, greenhouse screening could be a useful tool in selecting genotypes with enhanced SNF ability avoiding the need for expansive and costly field studies. Looking at correlations of greenhouse traits to field traits some greenhouse traits may be helpful in selecting genotypes for improved performance in the field. Interestingly greenhouse traits did not correlate with yield in the field except in EL in 2012 (Table 3.08). Traits moderately and inversely related with yield were: shoot weight ($r=-0.4$, $p\leq0.0001$), shoot N ($r=-0.3$, $p=0.0006$), root weight ($r=-0.335$, $p\leq0.0001$), nodule rating ($r=-0.201$, $p=0.0244$), shoot difference ($r=-0.305$, $p=0.0005$), root difference ($r=-0.323$, $p=0.0002$), and total biomass difference ($r=-0.312$, $p=0.0004$). At this location, 2012 was the driest year and plants were exposed to drought stress for much of the season. All of these traits are measuring the ability of the genotype to accumulate biomass with shoot weight and root weight being positively correlated ($r=0.82$, $p\leq0.0001$) in the greenhouse (Table 3.10). Growth of biomass may not support an increase in yield parameters if the biomass that is accumulated is not translocated into the seed and in fact, harvest index (HI) is positively correlated to yield in the field (Table 3.09). Puebla 152 has a low HI (ranging from 0.07 to 0.27 in EL from 2011 to 2013) compared to the more efficient Zorro (ranging from 0.31 to 0.45 in EL from 2011 to 2013). Under drought conditions a plant must mobilize resources from the biomass into the seed, however, if the biomass is a stronger sink than the seed the result could be lower yield resulting in the negative correlations observed.

Many of the greenhouse traits are moderately correlated with Ndfa calculated using the difference method. Shoot weight in the greenhouse is associated with Ndfa difference in 2 of 3 field seasons ($r=0.27$, $p\leq0.05$ in 2011 and $r=.23$, $p\leq0.01$ in 2013) (Table 3.08).

Looking at the relationship among field traits %N in seed is inversely correlated to seed yield in 2 of 3 years (Table 3.09). One might expect that as the yield increases the concentration of N in the seed might decrease. The same trend is seen in the greenhouse analysis on plants grown without additional N. Shoot weight and %N are inversely correlated ($r=-0.30$, $p<0.0001$) (Table 3.10) suggesting that the N use efficiency is an important component of yield when improving traits for SNF. Root, shoot, and total biomass was inversely correlated with $\delta^{15}N$ (r=-0.33, -0.27, and -0.32, $p \leq 0.0001$, respectively) (Table 3.10). The lower the $\delta^{15}N$, the greater %Ndfa which stands to reason that those plants with the lowest $\delta^{15}N$ are fixing more N and thus able to accumulate more biomass. Similarly, $\delta^{15}N$ was highly negatively correlated, ($r=-0.66$, $p \leq 0.0001$) with %Ndfa difference. This confirms that those genotypes with lower $\delta^{15}N$ fix more N. Traits measured in the greenhouse were also significantly correlated to SNF traits measured in the field in PR (Table 3.11). Greenhouse shoot weight was correlated with %N in seed in both 2012 and 2013 ($r=0.38$, $p \leq 0.0001$ and $r=0.38$, $p \leq 0.05$). Biomass difference in the greenhouse was correlated with %Ndfa calculated using either the natural abundance ($r=0.39$, $p < 0.0001$) or difference method ($r=0.40$, $p<0.0001$) in 2012 and natural abundance ($r=0.33$, $p<0.05$) and difference method ($r=0.18$, $p<0.05$) in 2013 (Table 3.11). The consistency with which biomass difference is correlated makes this trait suited to use as a selection tool when breeding for lines with enhanced SNF. Shoot weight in the greenhouse was positively correlated with all traits measured in the greenhouse (Table 3.10). Shoot weight of genotypes when grown under N-free conditions may be a useful trait to use in selecting plants with improved SNF traits such as %Ndfa in the field and %N in seed (Table 3.08).

Conclusions

There is considerable variability for SNF characteristics within the Puebla 152/Zorro RIL population. Several RILs combined enhanced N fixation ability with plants better adapted to agronomic conditions in northern latitudes. These RILs could be useful in developing lines better able to acquire a larger proportion of their N needs from the atmosphere. Drought may confound evaluation of SNF as %Ndfa appeared to be lower in years with limited precipitation. Traits measured in the greenhouse may be useful to select for field traits, though stress such as drought may confound the results making field evaluation over several season important in developing a better understanding of the traits being studied.

APPENDICES

APPENDIX A

CHAPTER 3 TABLES

Table 3.01. Planting date, precipitation, and soil characteristics of plots in East Lansing, MI, where Puebla 152/Zorro RILs were grown.

Year	Date Planted	Precipitation (mm)†	pH	% N‡
2011	6/13/2011	315	5.3	0.03
2012	6/19/2012	172	6.5	0.067
2013	6/4/2013	298	6.5	0.08
30 year average		361		

† Report generated at Enviro-Weather (http://www.agweather.geo.msu.edu/mawn/) for the months of June through September for the Hancock Turfgrass Research Center, East Lansing, MI.

‡Total Kjeldahl Nitrogen measured by the Soil and Plant Nutrient Lab at Michigan State University, East Lansing, MI.

Table 3.02. Yield of commercial checks and 122 Puebla 152/Zorro RILs grown in East Lansing in 2011 to 2013 and Puerto Rico in 2012 and 2013.

	East Lansing			Puerto Rico	
Commercial Checks	2011	2012	2013	2012	2013
	---kg ha^{-1}---				
Puebla 152	2204	918	2272	447	714
Zorro	3222	1933	2159	1225	811
R99	2913	2736	1755	261	458
Medalist		2194	1719	513	636
PR0443-151	3273	1767	2316	1297	2739
Verano		2197	2008	233	367
TARS-LFR1		2140	2060	1633	2200
PR1147-6		1678	1831	872	1953
RILs†					
Highest yielding	4304	2732	3042	1692	2514
lowest yielding	1076	763	1405	228	97
Test Mean	3026	1807	2223	835	984
LSD ($p \leq 0.05$)	900.6	751.3	462.6	871.3	1225.2
CV%	17.8	24.9	12.9	64.8	58.1

† Highest and lowest value in each trait of advanced breeding lines. May not be the same line for each trait.

Table 3.03. Percent N in the biomass and seed of commercial checks and 122 Puebla 152/Zorro RILs grown in East Lansing in 2011 to 2013 and Puerto Rico in 2012 and 2013.

Commercial Checks	East Lansing, MI					Puerto Rico	
	2011		2012		2013	2012	2013
	Seed	Biomass	Seed	Biomass	Seed	Seed	Seed
	% N						
Puebla 152	3.60	0.81	3.74	1.22	3.70	3.22	3.61
Zorro	3.31	0.54	3.88	0.49	3.67	3.37	3.29
R99	2.82	0.79	3.90	0.93	3.63	2.97	3.52
Medalist			3.80	0.79	3.45	3.06	2.98
PR0443-151	2.89	0.70	3.66	0.74	3.41	3.02	2.98
Verano			3.98	0.95	4.15	3.45	3.67
TARS-LFR1			3.58	0.56	3.81	3.07	3.37
PR1147-6			4.28	0.87	4.19	3.66	3.29
RILs							
Highest	4.41	1.13	4.74	1.71	4.60	3.92	3.91
Lowest	2.45	0.38	2.99	0.48	2.94	2.24	2.94
Test Mean	3.35	0.66	3.86	0.90	3.8	3.30	3.37
LSD ($p \leq 0.05$)	0.60	0.23	0.46	0.42	0.39	0.55	0.44
CV%	10.8	20.9	7.0	27.9	6.3	10.4	8.1

[†] Highest and lowest value in each trait of advanced breeding lines. May not be the same line for each trait.

Table 3.04. Percent N derived from the atmosphere (%Ndfa) calculated using the natural abundance method ($\delta^{15}N$) and the difference method for checks and 122 RILs of the Puebla 152/Zorro RIL population grown in East Lansing, MI in 2011 to 2013 and Isabella, Puerto Rico in 2012 and 2013†.

	East Lansing						Puerto Rico			
	2011		2012		2013		2012		2013	
Commercial Checks	%Ndfa- $\delta^{15}N$	%Ndfa-Difference	%Ndfa- $\delta^{15}N$	%Ndfa-Difference	%Ndfa- $\delta^{15}N$	%Ndfa-Difference	%Ndfa- $\delta^{15}N$	%Ndfa-Difference	%Ndfa- $\delta^{15}N$	%Ndfa-Difference
Puebla 152	19.5	8.2	13	*†	19.1	41.6	45.5	72.1	32.6	37.9
Zorro	22.1	20.8	5.4	8.0	14.0	37.7	44.4	74.9	22.9	49.7
R99	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Medalist			38	28.6	9.2	27.1	25.8	37.2	15.2	29.2
PR0443-151	19.3	5.7	18.8	*†	14.8	37.2	43.9	81.3	5.5	80.1
Verano			*†	22.8	10.5	41.3	48.5	26.7	31.4	*†
TARS-LFR1			24.7	3.5	10.2	36.9	40.8	84.9	22.7	78.1
PR1147-6			33.1	10.6	15.0	36.5	22.2	75.7	10.6	74.7
RILs										
Highest‡	60.6	51.5	58.6	34.47	33.6	61.0	68.4	90.7	55.5	85.7
Lowest	0.1	0	0	0.7	2.6	12.6	11.67	33.2	1.9	1.1
Test Mean	15.3	24.1	12.7	13.0	15.7	40.2	40.9	66.6	23.4	57.3
LSD ($p \leq 0.05$)	20.1	ns	18.5	ns	12.0	13.5	21.6	36.9	21.7	47.5
CV (%)	63.5	55.3	75.2	61.3	42.8	20.6	32.7	31.5	54.3	39.4

† %Ndfa values for some lines were negative and are not included in this table.

‡ Highest and lowest yield in each trait of advanced breeding lines. May not be the same line for each trait.

Table 3.05. Amount of N (kg ha^{-1}) in seed and biomass, and N harvest index (NHI) of 122 Puebla 152/Zorro RILs grown in East Lansing, MI and Puerto Rico in 2011, 2012, and 2013.

	East Lansing 2011				East Lansing 2012				East Lansing 2013	Puerto Rico 2012	Puerto Rico 2013
Commercial Checks	Seed	Biomass	Total	NHI	Seed	Biomass	Total	NHI	Seed		
		-kg N ha^{-1}-		%		- kg N ha^{-1}-		%		- kg N ha^{-1}-	
Puebla 152	79.4	278.4	357.8	24.2	33.9	101.4	135.3	26.2	84.1	14.7	24.8
Zorro	107.5	50.0	157.6	68.3	74.8	15.8	90.6	82.1	79.6	41.5	26.1
R99	82.5	91.1	173.6	47.8	107.5	51.0	158.4	68.6	64.0	7.8	16.1
Medalist					83.8	49.7	133.6	66.5	59.4	15.9	18.8
PR0443-151	95.0	75.6	160.8	55.4	64.6	18.9	83.5	77.3	78.4	38.9	81.9
Verano					88.2	43.7	131.8	65.9	83.0	13.0	13.3
TARS-LFR1					76.6	16.0	92.6	83.1	78.3	50.0	74.1
PR1147-6					71.9	30.7	102.7	70.6	76.7	32.0	64.2
RILs†											
Highest	151.3	251.3	318.0	84.1	104.2	223.6	259.0	85.5	125.0	54.8	86.8
Lowest	44.0	24.5	119.7	15.4	31.2	12.9	62.2	14.4	52.1	7.8	3.6
Test Mean	101.9	93.8	195.7	53.5	69.5	50.6	119.5	61.0	83.7	27.4	32.9
LSD ($p \leq 0.05$)	41.8	49.1	72.8	12.5	31.5	42.0	49.6	16.5	19.1	n.s.	41.4
CV%	24.5	31.2	22.2	14.0	26.9	49.1	24.4	15.9	14.1	64.7	74.3

† Highest and lowest yield in each trait of advanced breeding lines. May not be the same line for each trait.

Table 3.06. Flowering, maturity, and plant height of 122 Puebla 152/Zorro RILs grown in East Lansing, MI and Puerto Rico in 2011, 2012, and 2013.

	Days to Flowering					Days to Maturity				Height	
	East Lansing			Puerto Rico		East Lansing		Puerto Rico		East Lansing	
	---Days---									--cm--	
Checks and Parents	2011	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013
Puebla 152	54	57	45	39	40	109	101	82	88	40	35
Zorro	51	51	42	40	39	98	94	81	88	65	40
R99	41	46	43	39	40	100	94	82	88	60	45
Medalist		47	44	38	42	98	94	82	84	65	50
PR0443-151	47	49	44	40	38	97	93	82	84	55	35
Verano		45	43	39	39	104	95	78	88	55	45
TARS-LFR1		46	42	38	39	96	91	82	87	55	40
PR1147-6		47	43	38	39	98	95	81	84	68	45
RILs†											
Highest	63	60	48	43	43	111	103	90	94	90	73
Lowest	42	45	41	35	35	95	90	81	84	35	25
Test Mean	48	51	44	39	39	101	96	82	87	62	43
LSD ($p \leq 0.05$)	4.1	4.7	2.6	2.6	3.1	6.1	2.1	4.1	n.s.	20.5	14.8
CV%	5.1	4.4	3.1	4.1	4.2	2.7	2.5	3.2	4.7	15.9	17.2

† Highest and lowest yield in each trait of advanced breeding lines. May not be the same line for each trait.

Table 3.07. SNF traits measured in the greenhouse in East Lansing, MI on 122 Puebla 152/Zorro RILs grown in the greenhouse growing in N free conditions.

Commercial Checks	%N in biomass	Shoot Weight	Root Weight	%Ndfa Difference	Shoot/ Root Ratio	Biomass Diff- Shoot†	Biomass Diff- Root†	Biomass Diff- Total†	Nodule Rating	$\delta^{15}N$‡
		(g)								
Puebla 152	2.52	12.38	0.335	98.1	3.28	95.0	81.3	91.6	4.0	-3.480
Zorro	3.06	4.44	0.136	94.4	2.63	83.5	59.8	77.0	4.5	-3.620
R99	1.14	0.58	0.022	0.0	0.90	0.0	0.0	0.0	0.0	0.443
Medalist	3.43	2.94	0.101	91.8	2.30	74.8	45.6	66.1	4.0	-3.290
PR0443-151	2.41	6.42	0.155	97.8	1.81	92.0	74.6	85.6	4.5	-3.420
RILs§										
High	3.47	9.60	0.276	98.0	3.75	94.0	79.2	90.2	6.0	-2.910
Low	2.08	2.76	0.081	88.7	1.79	58.6	35.1	42.6	2.0	-3.850
Test Mean	2.63	5.58	0.144	94.8	2.72	86.5	63.1	80.0	4.6	-3.223
LSD ($p \leq 0.05$)	0.57	2.41	0.075	7.4	0.78	8.0	16.0	11.0	1.9	0.49
CV%	14.2	28.8	34.3	5.1	18.5	6.1	16.1	9.0	20.2	-9.3

† Difference= (biomass of N fixer (g)-biomass non-fixer (g))/biomass of N fixer (g).
‡ B value used in the natural abundance equation.
§ Highest and lowest yield in each trait of advanced breeding lines. May not be the same line for each trait.

Table 3.08. Pearson correlations between traits measured in the field in 2011-2013 and in the greenhouse on the Puebla 152/Zorro RIL population grown in East Lansing, MI †.

	Greenhouse Traits	Field Traits: Yield	%N in seed	Seed N	%Ndfa $\delta^{15}N$	%Ndfa Difference
East Lansing, 2011	%N in Biomass	0.06	0.07	0.08	0.07	0.13
	Shoot Weight	-0.1	0.42***	0.1	0.24*	0.24*
	Shoot N	-0.09	0.39***	0.09	0.23*	0.27*
	Root Weight	0.03	0.39***	0.12	0.35***	0.25*
	Nodule Rating	0.11	0.27*	0.22*	0.16	0.19*
	Shoot Difference‡	0.02	0.13	0.15	0.19*	0.25*
	Root Difference‡	-0.05	0.27*	0.09	0.19*	0.27*
	Total Difference‡	0.016	0.17*	0.16	0.17	0.28*
East Lansing, 2012	%N in Biomass	0.09	0.9	0.05	-0.09	0.15
	Shoot Weight	-0.40***	0.21*	-0.32**	-0.08	0.16
	Shoot N	-0.30**	0.21*	-0.25*	-0.13	0.19*
	Root Weight	-0.34***	0.19*	-0.27*	-0.04	0.21*
	Nodule Rating	-0.20*	0.11	-0.17*	0.17	0.10
	Shoot Difference‡	-0.31**	0.01	-0.26*	0.02	0.14
	Root Difference‡	-0.32**	0.06	-0.29***	-0.07	0.22*
	Total Difference‡	-0.31**	0.18*	-0.25*	-0.03	0.15
East Lansing, 2013	%N in Biomass	0.32**	-0.09	0.27*	0.22*	0.35***
	Shoot Weight	0.11	0.17*	0.19*	0.12	0.23*
	Shoot N	0.25*	0.08	0.28*	0.17*	0.32*
	Root Weight	0.15	0.09	0.18*	0.08	0.24*
	Nodule Rating	0.03	0.16	0.12	0.23*	0.19*
	Shoot Difference‡	0.14	0.12	0.18	0.20*	0.39***
	Root Difference‡	0.14	0.01	0.13	0.09	0.29**
	Total Difference‡	0.12	0.14	0.18*	0.17*	0.36***

† Correlation significant at * $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.0001$

‡ Difference= (biomass of N fixer (g)-biomass non-fixer (g))/biomass of N fixer (g)

Table 3.09. Pearson Correlations between traits measured in the field on the Puebla 152/Zorro RIL population grown in East Lansing, MI†.

Site and Year		Yield	Seed N	Biomass	%N in Seed	%Ndfa Difference	%Ndfa $\delta^{15}N$	Days to Maturity
East Lansing, 2011	Seed N	0.90***						
	Biomass	-0.07	-0.01					
	%N in Seed	0.25***	0.64***	0.12*				
	%Ndfa Difference	0.82***	0.98***	0.06	0.58***			
	%Ndfa $\delta^{15}N$	0.05	0.17*	0.13*	0.33***	0.14*		
	SPAD	0.32***	0.38***	0.18**	0.31***	0.41***	0.27***	
	Desirability	0.16*	0.12*	-0.21***	-0.04	0.08	-0.02	
	Harvest Index	0.50***	0.43***	-0.77***	0.07	0.34***	0.01	
East Lansing, 2012	Seed N	0.95***						
	Biomass	-0.04	-0.03					
	%N in Seed	-0.18**	0.01	-0.12***				
	%Ndfa Difference	0.68***	0.82***	0.01	0.06			
	%Ndfa $\delta^{15}N$	-0.20**	-0.21**	-0.08	-0.06	-0.07		
	SPAD	-0.15*	-0.20*	0.44***	-0.14*	-0.20	-0.01	
	Desirability	0.30***	0.31***	-0.29***	0.02	0.21	0.05	-0.25***
	Harvest Index	0.49***	0.50***	-0.75***	0.05	0.13	0.05	-0.43***
East Lansing, 2013	Seed N	0.86***						
	Biomass	0.10	0.08					
	%N in Seed	-0.16*	0.36***	-0.04				
	%Ndfa Difference	0.82***	0.95***	0.07	0.34***			
	%Ndfa $\delta^{15}N$	0.10	0.20**	-0.09	0.22***	0.21***		
	SPAD	0.06	0.10	0.04	0.09	0.11	0.05	
	Desirability	0.13*	0.15*	0.25*	0.04	0.15*	-0.12	
	Harvest Index	0.20*	0.16*	-0.18*	-0.08	0.17*	0.01	0.02
	Harvest Index	0.25***	0.24***	-0.87***	0.01	0.22***	0.13*	0.07

† Correlation significant at * $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.0001$

Table 3.10. Pearson Correlations between traits measured on the Puebla 152/Zorro RIL population grown under N-free conditions in the greenhouse in East Lansing, MI[†].

	Shoot Weight	Root Weight	Total Biomass	%N in Biomass	$\delta^{15}N$	Shoot N	Ndfa Difference	Shoot /Root Ratio	Shoot Difference[‡]	Root Difference[‡]	Biomass Difference[‡]
Root Weight	0.82***										
Total Biomass	0.99***	0.90***									
%N in Biomass	-0.30***	-0.38***	-0.33***								
$\delta^{15}N$	-0.33***	-0.27***	-0.32***	-0.11*							
Shoot N	0.82***	0.58***	0.78***	0.24***	-.30***						
Ndfa Difference	0.42***	0.38***	0.42***	0.08	-0.66***	0.36***					
Shoot /Root Ratio	0.36***	-0.18***	0.22***	0.12*	-0.25***	0.42***	0.27***				
Shoot Difference[‡]	0.73***	0.79***	0.70***	-0.28***	-0.45***	0.52***	0.48***	0.02			
Root Difference[‡]	0.99***	0.90***	0.79***	-0.33***	-0.32***	0.78***	0.42***	0.22***	0.70***		
Biomass Difference[‡]	0.75***	0.70***	0.77***	-0.25***	-0.58***	0.55***	0.69***	0.29***	0.96***	0.90***	
Nodule Rating	0.17*	0.16*	0.16*	0.1	-0.33***	0.16*	0.44***	0.21**	0.44***	0.20*	0.38***

[†] Correlation significant at * $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.0001$

[‡] Difference= (biomass of N fixer (g)-biomass non-fixer (g))/biomass of N fixer (g)

Table 3.11. Pearson Correlations between traits measured in the field on the Puebla 152/Zorro RIL population grown in Isabela, Puerto Rico and in the greenhouse in East Lansing, MI†.

	2012			2013		
	%N Seed	%Ndfa 15N	%Ndfa Difference	%N Seed	%Ndfa 15N	%Ndfa Difference
Shoot Weight (g)	0.38***	0.25*	0.17*	0.19*	0.23*	0.0001
Root Weight (g)	0.31**	0.26*	0.17*	0.14	0.20*	0.01
Total Biomass (g)	0.38***	0.26*	0.17*	0.17*	0.24*	-0.01
%Ndfa Difference	0.17*	0.39***	0.42***	-0.05	0.24*	0.28*
Shoot Difference	0.27*	0.41***	0.42***	0.06	0.29*	0.23*
Root Difference	0.26*	0.32**	0.28*	0.03	0.28*	0.08
Biomass Difference	0.29**	0.39***	0.40***	0.07	0.33*	0.18*
Nodule Rating	0.32**	0.41***	0.17*	0.07	0.27*	0.08

† Correlation significant at * $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.0001$

APPENDIX B

CHAPTER 3 SUPPLEMENTAL TABLES

Table S3.01. Yield of commercial checks and 122 Puebla 152/Zorro RILs grown in East Lansing in 2011 to 2013 and Puerto Rico in 2012 and 2013.

	Seed Yield				
	East Lansing			Puerto Rico	
Commercial Checks	2011	2012	2013	2012	2013
			-----kg ha^{-1}-----		
Puebla 152	2204	918	2272	447	714
Zorro	3222	1933	2159	1225	811
R99	2913	2736	1755	261	458
Medalist		2194	1719	513	636
PR0443-151	3273	1767	2316	1297	2739
Verano		2197	2008	233	367
TARS-LFR1		2140	2060	1633	2200
PR1147-6		1678	1831	872	1953
RILs					
B11501	2690	1607	1809	856	631
B11502	3834	1594	2024	542	600
B11503	2794	1494	2389	297	1342
B11504	2408	1462	2169	422	1056
B11505	3286	1412	2107	903	850
B11507	3256	1606	2268	395	213
B11508	2947	1530	2512	828	858
B11509	3013	1970	2367	939	1375
B11510	2817	1632	2228	389	436
B11511	2578	2330	2406	1092	683
B11512	2036	1517	2337	431	325
B11513	2654	1668	1809	656	1181
B11514	2896	1777	1857	650	408
B11515	3260	1765	2575	536	347
B11516	3734	1901	2289	567	372
B11517	2136	1347	1989	633	1322
B11518	3138	1272	1680	608	896
B11519	3748	2180	2872	1086	1156
B11520	2981	1535	2280	1025	733
B11521	3466	1187	2771	467	321
B11522	3382	2150	1890	864	1756
B11523	3287	1957	2108	1222	1139

Table S3.01 (*cont'd*)

B11524	2088	885	2990	878	731
B11525	2704	1621	1869	444	631
B11526	3068	1872	2275	556	821
B11527	2668	2147	2371	269	222
B11528	2698	1621	1433	889	1128
B11529	3777	1224	2041	725	475
B11530	3150	2254	2490	861	1183
B11531	2483	2448	2563	1236	454
B11532	2413	1846	2323	708	1022
B11533	3282	1735	2137	1025	2056
B11534	3375	1473	2066	394	739
B11535	2369	1421	1735	720	703
B11536	3761	2630	2745	742	125
B11537	2698	1136	2599	1136	1347
B11539	3158	1253	2382	850	779
B11540	3154	1462	1563	389	383
B11541	1076	763	2550	789	936
B11542	2870	1735	2388	767	1311
B11543	3424	2280	1974	1086	1225
B11544	3098	2237	2250	956	342
B11545	3115	2257	2018	833	2345
B11546	2425	2232	2059	1031	1375
B11547	2962	2115	2415	1197	1103
B11548	2373	1262	2591	961	708
B11549	3144	2353	2252	583	1547
B11550	2793	1505	1522	758	1411
B11551	3322	1724	2794	806	331
B11552	3818	1523	2726	628	600
B11553	2929	1630	2486	892	1044
B11554	3174	1515	2082	739	1183
B11555	3058	2608	2564	983	828
B11556	2820	1924	2190	1022	1458
B11557	2295	2258	1994	742	1567
B11558	2805	1706	1961	264	417
B11559	3189	1711	2286	1450	953
B11560	2941	1792	1816	483	1119
B11561	3663	1908	2317	1083	406
B11562	2283	1210	2412	1525	1242
B11563	3211	2320	1949	1453	1986
B11564	2871	1652	2334	894	542

Table S3.01 (*cont'd*)

B11565	2854	2168	2335	1489	1450
B11566	2644	1874	1848	1006	1297
B11567	4025	2344	2812	775	586
B11568	2374	1981	2301	681	172
B11569	3017	1671	2645	794	97
B11570	3393	2181	2298	1214	728
B11571	3565	1957	2358	1353	1806
B11572	3139	1473	2443	733	211
B11573	2322	1574	2552	1239	1411
B11574	2456	1610	2541	258	850
B11575	2651	1527	2200	1100	1869
B11576	3087	1261	2190	953	596
B11577	2681	2088	2013	1078	1675
B11578	2172	1216	1797	228	106
B11579	3202	1485	2078	844	1072
B11580	3533	2035	2634	350	981
B11581	2966	1752	2389	1478	1042
B11582	3177	2669	2397	742	1064
B11583	3500	1755	2089	1236	1039
B11584	3189	2061	1974	1117	1328
B11585	2675	1158	1840	847	1014
B11586	3829	1546	2716	539	431
B11587	3305	1877	2144	1422	1333
B11588	4105	2291	2412	1692	1603
B11589	3190	1693	2022	1189	986
B11590	2803	1955	2125	986	1531
B11591	2610	1825	1987	625	358
B11592	3167	2085	1976	1070	650
B11593	2895	1831	2413	1681	1286
B11594	3505	1938	2363	1011	767
B11595	2287	2053	2198	364	1356
B11596	3162	1927	2151	1356	986
B11598	2883	1812	2061	314	850
B11599	2696	1554	2146	1086	1364
B11600	3653	1500	1595	531	219
B11601	2233	1776	1405	739	1247
B11602	3741	2454	2568	897	1542
B11603	3657	2123	2313	500	1283
B11604	2941	1712	2166	781	1458
B11605	2706	818	2379	322	853

Table S3.01 (*cont'd*)

B11606	2707	1658	2126	386	1136
B11607	2223	1726	1914	1119	819
B11608	2536	1550	2194	1069	1539
B11609	2973	2157	2438	367	636
B11610	3595	2037	2241	539	761
B11611	4304	2232	2452	678	161
B11612	2538	1741	2394	1425	2514
B11613	3307	1812	2024	319	822
B11614	3429	2161	2025	1100	397
B11615	3891	1861	2276	664	672
B11616	3131	1913	2258	1567	994
B11617	3063	1863	2469	1064	919
B11619	3469	1816	2256	881	1364
B11620	2909	2732	2511	517	1514
B11621	3865	1821	2195	889	886
B11622	3351	1896	2368	789	686
B11623	3088	2146	2505	1161	1225
B11624	2557	1557	3042	486	158
B11625	3055	1874	2157	597	300
B11626	2711	1523	1866	603	467
Test Mean	3026	1807	2223	835	984
LSD (P<0.05)	900.6	751.3	462.6	871.3	1225.2
CV (%)	17.8	24.9	12.9	64.8	58.1

Table S3.02 Percent N in the biomass and seed of commercial checks and 122 Puebla 152/Zorro RILs grown in East Lansing in 2011 to 2013 and Puerto Rico in 2012 and 2013.

	East Lansing, MI					Puerto Rico	
	2011		2012		2013	2012	2013
Commercial Checks	Seed	Biomass	Seed	Biomass	Seed	Seed	Seed
Puebla 152	3.60	0.81	3.74	1.22	3.70	3.45	3.67
Zorro	3.31	0.54	3.88	0.49	3.67	3.66	3.29
R99	2.82	0.79	3.90	0.93	3.63	3.07	3.37
Medalist			3.80	0.79	3.45	3.67	3.56
PR0443-151	2.89	0.70	3.66	0.74	3.41	3.06	2.98
Verano			3.98	0.95	4.15		
TARS-LFR1			3.58	0.56	3.81		
PR1147-6			4.28	0.87	4.19		
RILs							
B11501	3.21	0.68	3.66	0.82	3.86	3.37	3.47
B11502	3.65	0.74	4.06	0.99	3.93	3.47	3.29
B11503	2.91	0.74	2.99	0.68	3.26	3.04	3.03
B11504	2.83	0.65	3.55	0.84	3.23	3.07	3.29
B11505	3.05	0.42	3.74	0.80	3.77	3.09	3.56
B11507	3.40	0.62	4.01	1.13	3.24	3.03	3.20
B11508	3.57	0.52	3.69	0.76	4.14	3.07	3.05
B11509	3.35	0.55	3.74	0.79	3.85	3.28	3.05
B11510	3.10	0.68	3.75	1.05	3.88	3.18	3.33
B11511	2.98	0.61	3.62	0.78	3.64	2.99	3.21
B11512	3.44	0.45	3.61	1.00	3.68	3.17	3.04
B11513	3.34	0.85	4.11	0.83	3.58	3.21	3.51
B11514	3.26	0.42	4.07	0.88	3.98	2.81	3.20
B11515	3.90	0.46	4.21	1.02	3.63	3.63	3.52
B11516	3.30	0.51	3.65	0.96	3.96	3.26	3.39
B11517	3.16	0.70	4.08	0.99	3.68	3.65	3.75
B11518	3.80	0.85	4.00	1.08	3.96	3.32	3.45
B11519	2.89	0.38	3.41	0.72	4.36	3.13	3.03
B11520	3.32	0.62	3.96	0.90	3.14	2.97	3.29
B11521	3.13	0.57	3.49	1.16	3.63	3.27	3.19
B11522	3.42	0.87	3.73	0.88	3.78	3.24	3.23
B11523	3.58	0.60	3.88	0.73	3.89	3.11	3.36

Table S3.02 (*cont'd*)

B11524	3.61	0.56	4.00	1.40	3.79	3.25	3.67
B11525	3.77	0.60	4.17	0.79	4.60	3.92	3.76
B11526	3.16	0.63	3.94	0.90	3.70	3.24	3.37
B11527	3.48	0.74	4.03	0.66	3.82	3.71	3.36
B11528	3.41	0.70	4.33	0.64	3.92	3.22	3.57
B11529	3.49	0.59	3.66	0.88	3.49	3.16	3.34
B11530	3.53	0.61	3.56	0.67	3.72	3.13	3.38
B11531	2.99	0.59	3.44	0.97	3.45	3.05	2.94
B11532	3.34	0.62	3.46	0.82	3.75	3.11	3.36
B11533	3.74	0.58	4.24	0.65	4.17	3.59	3.52
B11534	3.47	0.80	4.02	0.97	4.03	3.15	3.35
B11535	3.80	0.65	4.25	0.77	4.02	3.45	3.57
B11536	3.06	0.46	3.26	0.76	3.12	3.06	3.05
B11537	2.86	0.64	3.75	1.07	3.62	3.41	3.12
B11539	3.41	0.42	3.70	0.76	3.79	3.63	3.52
B11540	3.33	0.77	4.13	1.27	4.06	3.14	3.40
B11541	4.09	0.80	4.08	1.66	3.80	3.73	3.58
B11542	3.10	0.67	4.01	0.78	3.95	3.38	3.35
B11543	3.10	0.92	3.79	0.97	4.03	3.67	3.56
B11544	3.44	0.77	3.92	0.58	4.07	3.27	3.34
B11545	3.74	0.94	3.95	0.73	3.80	3.41	3.28
B11546	2.73	0.72	3.21	1.12	3.28	3.04	2.97
B11547	3.31	0.42	3.89	0.64	3.86	3.15	3.64
B11548	2.45	0.50	3.30	1.12	2.94	2.91	3.57
B11549	3.34	0.88	3.65	0.90	3.86	3.29	3.22
B11550	3.18	0.90	3.91	1.13	3.68	3.38	3.23
B11551	3.87	0.61	4.02	1.19	4.08	3.74	3.40
B11552	3.38	0.55	3.81	1.11	3.35	3.40	3.33
B11553	3.14	0.64	3.61	1.12	3.85	3.32	3.50
B11554	4.37	0.56	4.35	0.74	4.21	3.51	3.88
B11555	3.66	0.73	4.00	0.77	4.12	3.06	3.23
B11556	3.49	0.83	3.83	0.73	4.21	3.44	3.21
B11557	3.43	0.74	4.31	0.92	3.77	3.38	3.47
B11558	3.60	0.72	4.14	0.87	4.02	3.59	3.24
B11559	3.37	0.82	3.68	1.04	3.93	3.38	3.26
B11560	4.41	0.51	4.27	1.11	4.27	3.74	3.41

Table S3.02 (*cont'd*)

B11561	3.75	0.63	3.87	0.58	4.13	3.38	3.51
B11562	3.04	0.68	3.34	1.13	3.37	2.98	3.23
B11563	3.35	0.78	3.70	1.10	4.03	3.22	3.35
B11564	3.08	0.63	3.98	0.84	3.86	2.24	3.07
B11565	3.60	0.76	3.94	0.68	4.18	3.63	3.64
B11566	3.16	0.65	3.90	0.86	3.36	3.21	3.06
B11567	3.27	0.47	3.77	0.70	3.52	3.26	3.21
B11568	3.31	0.59	3.50	0.90	3.52	3.15	3.32
B11569	3.68	0.74	3.66	0.77	3.66	3.68	3.91
B11570	3.16	0.78	3.80	0.92	3.55	3.57	3.49
B11571	3.70	0.71	3.72	0.71	3.73	3.22	3.03
B11572	3.08	0.71	3.72	1.21	3.55	3.36	3.44
B11573	3.09	0.47	3.90	0.82	3.35	2.97	3.38
B11574	3.30	0.66	3.55	0.79	3.95	3.59	3.44
B11575	3.28	0.78	3.84	0.78	3.69	3.34	3.44
B11576	3.29	0.74	4.15	0.71	3.92	3.44	3.25
B11577	3.22	0.71	3.67	0.81	3.86	3.23	3.75
B11578	3.68	0.92	4.74	1.23	4.41	3.83	3.74
B11579	3.50	0.57	3.88	0.71	4.09	3.69	3.60
B11580	3.54	0.69	3.66	1.09	3.75	3.66	3.49
B11581	3.42	0.49	3.67	1.00	3.49	3.00	3.73
B11582	3.33	0.46	3.64	0.76	4.26	3.30	3.16
B11583	3.73	0.52	3.76	1.16	3.71	3.33	3.04
B11584	3.39	1.13	4.02	1.09	3.79	3.44	3.33
B11585	3.89	0.68	4.16	0.74	4.33	3.42	3.61
B11586	3.69	0.47	4.07	0.83	3.94	3.71	3.56
B11587	3.28	0.58	3.73	0.86	4.28	3.41	3.69
B11588	3.27	0.68	3.74	0.85	3.87	3.35	3.20
B11589	2.91	0.80	3.87	1.01	3.81	3.39	3.18
B11590	3.21	0.81	3.88	0.73	4.00	3.59	3.57
B11591	4.09	0.65	4.56	0.75	3.95	3.57	3.70
B11592	3.29	0.90	3.88	0.98	3.49	3.01	3.70
B11593	3.02	0.51	3.62	0.93	3.54	2.97	3.37
B11594	2.97	0.44	3.82	0.63	3.49	3.25	3.37
B11595	3.01	0.69	3.54	0.73	3.40	3.09	3.23
B11596	3.14	0.65	4.00	0.86	3.40	3.04	3.03
B11598	3.65	0.75	4.40	0.92	3.39	3.22	3.59
B11599	2.65	0.56	3.35	0.95	3.52	2.83	3.10
B11600	3.35	0.70	3.70	0.92	3.73	3.12	3.62
B11601	3.24	0.70	4.19	0.73	3.98	3.36	3.59
B11602	3.19	0.57	3.79	0.59	3.92	3.15	3.34

Table S3.02 (*cont'd*)

B11603	3.63	0.65	3.72	1.31	4.12	3.68	3.28
B11604	3.75	0.68	4.21	0.87	3.60	3.49	3.44
B11605	3.61	0.71	4.02	1.38	4.02	3.72	3.25
B11606	3.50	0.74	4.31	1.08	4.08	3.20	3.50
B11607	3.03	0.61	4.42	0.85	3.77	3.68	3.85
B11608	3.59	0.96	3.99	0.99	3.68	3.38	3.46
B11609	3.19	0.57	3.54	0.77	3.73	3.48	3.16
B11610	3.84	0.73	3.99	0.73	3.59	3.69	3.62
B11611	3.50	0.51	3.83	1.31	4.12	3.66	3.15
B11612	3.40	0.91	4.08	1.03	3.86	3.26	3.44
B11613	3.65	0.70	4.10	0.97	3.45	3.36	3.26
B11614	3.35	0.61	3.65	0.82	3.27	2.94	3.31
B11615	3.15	0.65	3.45	0.98	3.99	3.02	3.02
B11616	3.38	0.73	4.62	0.80	3.90	3.34	3.49
B11617	3.53	0.42	4.09	0.66	3.70	3.38	3.47
B11619	3.14	0.85	3.87	0.99	3.88	3.42	3.32
B11620	3.21	0.52	3.72	0.48	3.56	3.00	3.04
B11621	3.46	0.74	3.69	0.88	3.47	3.18	3.00
B11622	3.04	0.45	3.50	0.70	3.88	2.99	3.50
B11623	3.30	0.42	3.51	0.82	3.29	3.18	3.11
B11624	3.47	0.56	4.11	0.80	3.69	3.07	3.71
B11625	2.91	0.62	4.18	1.00	3.78	3.44	3.02
B11626	3.29	0.79	3.95	1.71	3.90	3.33	3.38
Test Mean	3.35	0.66	3.86	0.90	3.80	3.30	3.37
LSD ($P \leq 0.05$)	0.60	0.23	0.46	0.42	0.39	0.55	0.44
CV (%)	10.80	20.9	7.0	27.9	6.3	10.4	8.1

Table S3.03. Percent N derived from the atmosphere (%Ndfa) calculated using the natural abundance method ($\delta^{15}N$) and the difference method for checks and 122 RILs of the Puebla 152/Zorro RIL population grown in East Lansing, MI in 2011 to 2013 and Isabella, Puerto Rico in 2012 and 2013[†].

	East Lansing						Puerto Rico			
	2011		2012		2013		2012		2013	
Commercial Checks	%Ndfa- $\delta^{15}N$	%Ndfa-Difference	%Ndfa- $\delta^{15}N$	%Ndfa-Difference	%Ndfa- $\delta^{15}N$	%Ndfa-Difference	%Ndfa- $\delta^{15}N$	%Ndfa-Difference	%Ndfa- $\delta^{15}N$	%Ndfa-Difference
Puebla 152	19.5	8.2	13.0		19.1	41.6	45.5	72.1	32.6	37.9
Zorro	22.1	20.8	5.4	8.0	14.0	37.7	44.4	74.9	22.9	49.7
R99	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Medalist			38.0	28.6	9.2	27.1	25.8	37.2	15.2	29.2
PR0443-151	19.3	5.7	18.8		14.8	37.2	43.9	81.2	5.5	80.1
Verano				22.8	10.5	41.3	48.5	26.7	31.4	
TARS-LFR1			24.7	3.5	10.2	36.9	40.8	84.9	22.7	78.1
PR1147-6			33.1	10.6	15.0	36.5	22.2	75.7	10.6	74.7
RILs										
B11501	9.4	27.9	15.0		15.3	30.2	22.7	64.6	18.4	41.5
B11502	18.7	40.8	7.6	3.0	16.7	36.8	43.5	41.2	42.5	50.9
B11503	14.7	9.2	12.3		9.0	37.3	35.2	33.9	6.8	50.5
B11504	9.9	9.3	15.8		10.3	30.2	30.5	35.2	17.3	50.9
B11505	6.4	30.9	12.3		13.5	38.5	39.2	77.5	16.8	63.3
B11507	9.1	22.9	15.6	13.9	19.9	33.4	43.4	33.3	21.3	23.9
B11508	9.3	20.3	12.7		9.5	52.9	29.2	59.0	25.8	65.2
B11509	9.7	16.7	18.3		16.4	46.1	48.5	71.5	12.1	72.7
B11510		18.2	14.1		16.0	42.8	62.6	43.4	35.0	64.8
B11511		14.6	28.3	3.1	17.0	44.4	43.7	73.0	35.2	66.7
B11512	7.7	0.0	8.9		19.2	43.4	48.0	63.3	24.4	51.6
B11513		26.7	11.1		21.1	24.6	27.5	83.5	20.6	68.0
B11514	9.9	25.9	5.1	2.4	19.1	33.4	37.3	55.7	21.4	66.5

Table S3.03 (*cont'd*)

B11515	27.9	35.0	13.0	5.2	23.1	47.9	53.0	36.6	39.3	26.0
B11516	7.0	31.1	12.1		25.5	46.1	49.2	56.6	34.6	25.1
B11517	12.1	20.5	7.7		15.4	33.6	39.7	62.0	8.2	67.5
B11518	24.6	27.2	14.8		7.6	24.0	43.9	74.3	33.4	80.4
B11519	25.9	51.5	13.9	2.3	32.8	61.0	36.2	72.7	13.9	67.9
B11520	12.4	15.8	12.3		8.5	32.0	11.7	71.2	16.2	68.2
B11521	1.9	23.2	9.3		14.2	51.2	53.9	67.0	29.8	51.7
B11522	13.5	27.4	4.7	4.4	10.4	31.5	40.8	68.5	26.3	71.6
B11523	19.6	25.4	3.5	5.0	4.6	40.7	38.7	67.6	23.0	45.2
B11524	41.4	21.7	9.3		20.6	57.0	40.3	72.1	18.6	41.3
B11525	11.0	18.6	8.1	6.0	22.2	42.2	35.4	57.9	13.8	42.9
B11526	12.3	42.0	9.3		7.2	41.9	30.0	54.2	19.1	39.3
B11527	22.5	25.1	9.5	24.1	13.7	46.4	53.1	64.0	39.9	39.9
B11528	0.1	23.6	13.2		5.5	24.5	34.3	74.7	16.8	75.8
B11529	24.1	35.7	13.9		23.9	30.1	47.4	56.9	36.2	26.3
B11530	15.1	23.5	9.6	2.4	28.2	46.8	36.5	71.8	31.9	43.7
B11531		13.2		11.9	8.9	41.8	38.9	75.3	20.1	
B11532	7.0	22.7	14.4		23.2	44.1	40.3	57.5	17.0	69.8
B11533	20.5	29.0	9.7	22.2	19.4	43.5	36.8	71.8	24.5	71.3
B11534	1.6	28.8	4.1		12.4	41.0	49.5	65.3	28.2	71.6
B11535	31.6	7.7	13.3		16.2	29.7	40.9	68.2	16.9	44.1
B11536	23.3	26.7	7.6	12.9	12.5	42.7	37.7	56.8	32.9	
B11537	5.1	4.7	12.9		8.8	46.8	30.2	75.1	17.6	75.5
B11539	17.4	21.5	20.1		9.9	46.1	50.0	69.0	29.5	24.7
B11540	7.9	20.8	11.9		16.8	23.3	39.0	46.4	18.2	45.4
B11541	33.8	0.0	4.8		21.5	49.4	46.6	76.9	23.5	71.1

Table S3.03 (*cont'd*)

B11542	13.9	7.0	9.7	9.6	26.2	46.2	36.6	70.6	16.3	76.5
B11543	18.4	18.7	5.5	18.8	17.5	38.2	36.7	74.4	29.8	52.6
B11544	6.8	29.0	18.0	18.6	12.2	46.7	46.7	61.2	23.0	
B11545	18.0	26.7	10.3	28.0	10.5	33.3	45.4	64.8	22.0	79.4
B11546	7.6	6.1	5.7	27.6	24.9	27.9	36.1	75.9	3.8	58.2
B11547	26.9	25.2	23.4	15.5	24.4	47.8	55.7	80.5	25.3	60.6
B11548	6.3	0.0	32.1		7.2	35.6	37.0	67.6	9.8	79.9
B11549	15.5	20.6	2.2	34.7	9.2	43.9	45.9	62.8	22.8	47.8
B11550	2.2	20.1	10.5		12.4	12.6	44.4	64.8	17.7	60.8
B11551	34.5	33.9	8.7		18.7	56.1	60.2	71.7	47.4	56.0
B11552	8.2	35.3	8.4		15.3	46.7	32.9	62.4	28.3	60.9
B11553	7.7	17.9	12.3		21.1	46.9	41.5	56.4	35.1	70.6
B11554	39.2	40.1	19.2	11.6	24.9	44.5	52.6	59.4	22.2	61.7
B11555	22.0	35.0	8.6	21.7	24.5	53.8	42.5	69.8	29.8	57.3
B11556	9.4	14.8	13.5	15.0	13.9	47.3	45.2	78.3	16.0	77.4
B11557	9.7	2.1	2.6	13.6	18.5	33.4	30.1	61.3	13.3	60.8
B11558	0.2	16.7	12.3		22.3	38.0	46.3	35.1	18.1	63.1
B11559	10.5	19.0	10.6		20.9	44.7	41.2	83.7	37.2	85.6
B11560	31.6	36.4	9.7	12.3	22.4	37.1	42.3	51.1	14.5	46.6
B11561	18.5	38.2	13.3	22.2	16.2	48.9	37.7	70.8	31.4	27.6
B11562	17.4	0.0	11.7		21.6	39.5	28.9	83.4	14.5	57.5
B11563	9.5	22.9	17.5	10.5	17.6	36.7	33.7	81.2	15.1	76.5
B11564	13.4	11.6	15.2	24.2	13.3	44.9	55.1	40.7	41.6	33.3
B11565	13.7	31.0	7.9	4.2	17.2	50.1	39.4	86.3	13.2	58.0
B11566	6.6	15.3	7.8	8.7	2.6	20.4	25.2	75.0	20.5	76.1

Table S3.03 (*cont'd*)

B11567	21.6	37.1	32.3	16.1	11.9	50.4	38.4	46.4	30.6	28.0
B11568	2.8	10.4	3.6	3.4	17.9	38.8	38.6	59.8	29.1	
B11569	34.6	25.2	27.5	5.7	20.8	49.0	34.0	72.6	34.0	
B11570	5.7	22.3	4.8	11.8	26.8	40.2	41.7	82.0	20.3	27.0
B11571	26.4	36.8	14.9	20.9	21.9	44.5	45.0	83.9	11.4	67.8
B11572	17.1	28.2	13.0		21.2	43.3	28.5	59.7	27.8	
B11573	9.3	13.4	17.4		21.3	41.8	43.2	80.5	10.9	62.7
B11574	9.0	9.1	14.8		22.6	49.0	43.1	79.3	35.0	38.0
B11575	60.6	4.4	7.6		12.1	40.2	41.5	79.6	9.8	73.0
B11576	5.3	25.5	21.1		16.3	43.5	55.7	90.7	40.1	68.0
B11577	16.6	13.2	19.2	9.2	8.0	36.8	34.2	77.7	8.7	72.0
B11578		7.0			13.6	38.8	57.6	62.8	44.2	
B11579	14.5	40.3	30.1		10.7	42.4	38.4	76.7	8.9	69.7
B11580	21.4	33.3	6.3	3.1	21.5	50.4	37.3	56.2	22.4	66.4
B11581	14.0	18.0	15.5		14.6	41.3	33.2	82.7	9.3	74.8
B11582	11.5	20.9	14.0	25.3	19.0	49.9	50.5	61.0	28.1	58.7
B11583	13.9	34.4	11.2	14.1	8.3	37.3	45.0	82.0	27.7	71.9
B11584	17.6	21.7	11.2	20.7	11.2	34.8	58.6	76.7	55.5	75.3
B11585	21.7	19.4	7.7		20.2	38.0	47.6	56.8	24.9	50.9
B11586	6.1	40.5	20.4	10.4	17.0	54.2	53.5	62.3	38.8	50.5
B11587	0.2	32.4	14.8	16.0	20.5	47.0	46.4	84.6	25.4	66.4
B11588	14.6	38.4	16.9	23.8	19.4	47.6	51.2	83.7	20.8	66.0
B11589	1.8	10.7	7.5		12.2	36.9	34.3	77.2	31.9	60.3
B11590	20.1	23.0	10.1	3.6	17.4	42.8	47.1	70.0	17.6	70.8
B11591	19.4	19.9	8.7	12.7	12.2	37.2	28.0	67.8	31.7	

Table S3.03 (*cont'd*)

B11592	3.1	19.2	7.4	26.5	13.8	28.0	36.2	73.9	19.1	40.6
B11593	5.0	20.3	15.0		10.0	42.9	39.6	81.8	45.5	63.5
B11594	2.8	28.2	14.2	6.6	12.7	41.2	39.3	89.0	21.1	85.7
B11595	7.8	10.0	8.4	0.8	10.5	33.9	31.8	40.6	11.5	58.1
B11596		27.4	2.6	4.2	5.3	33.0	28.0	77.1	20.9	63.4
B11598	4.2	34.5	8.6	19.6	13.6	42.6	35.9	40.2	12.7	55.4
B11599	11.8	9.7	2.5		8.2	32.1	34.2	75.9	18.8	72.3
B11600	12.5	32.4	10.9		9.3	12.8	50.1	49.6	25.8	1.1
B11601	4.3	19.5	6.2		4.6	15.4	39.5	76.4	14.1	61.9
B11602	19.0	29.6	19.6	34.6	25.8	52.3	45.2	57.6	14.8	54.0
B11603	28.8	36.5	17.5	4.7	33.6	46.3	60.2	57.0	25.0	68.3
B11604	3.9	23.2	7.5	4.7	20.5	42.8	32.6	66.5	16.0	63.2
B11605	30.6	15.4	4.4		27.3	40.3	55.4	61.1	13.1	42.8
B11606	7.9	20.5	12.4	0.7	15.9	42.3	45.6	40.8	15.1	48.9
B11607	5.0	0.0	30.2		3.5	37.8	42.0	81.3	20.6	85.5
B11608	16.0	9.0	9.6		13.6	41.1	27.5	76.9	14.2	61.1
B11609	13.9	48.4	58.6	4.7	5.1	45.4	56.7	45.4	34.1	24.8
B11610	3.8	38.0	15.3	6.0	10.1	41.6	23.4	66.3	14.1	36.9
B11611	14.7	44.2	19.8	13.6	32.0	40.1	68.4	69.4	51.1	
B11612	12.7	25.0	7.0	17.0	10.5	50.8	49.9	81.9	34.1	80.8
B11613	21.2	31.4	7.7		16.7	37.1	59.1	71.8	23.4	62.8
B11614	33.3	26.5	10.4	11.3	10.7	30.0	35.0	75.9	29.2	7.9
B11615	20.9	32.0	20.4	19.8	7.6	33.5	39.2	66.3	10.1	51.0
B11616	8.6	21.6	11.2	10.3	14.4	45.7	40.2	86.0	26.6	66.0
B11617	18.9	22.9	9.7	22.8	20.8	49.4	36.0	79.5	16.8	77.7
B11619	16.3	24.0	6.3		16.5	41.2	34.4	71.1	20.4	57.1

Table S3.03 (*cont'd*)

B11620	24.3	11.4	8.3	27.2	18.8	42.3	20.2	55.3	10.7	75.1
B11621	18.1	36.1	12.4		10.5	42.2	54.0	61.4	48.1	45.0
B11622	5.4	27.4	7.9		13.5	42.0	35.7	73.2	1.9	28.2
B11623	6.2	16.9	13.2	14.8	18.6	44.1	44.4	80.0	20.4	50.0
B11624	16.8	18.6	8.0		22.2	58.1	54.4	40.0	38.5	
B11625		19.0	3.4	18.6	9.4	30.5	39.9	72.6	28.5	43.0
B11626	.	32.1	0.0		8.1	39.9	33.1	81.7	18.6	69.0
Test Mean	15.3	24.1	12.73	13.0	15.7	40.2	40.9	66.6	23.4	57.3
LSD ($P \leq 0.05$)	20.1	N.S.	18.52	N.S.	12.0	13.5	21.6	36.9	21.7	47.5
CV (%)	63.5	55.3	75.2	61.3	42.8	20.6	32.7	31.5	54.3	39.4

[†] %Ndfa values for some lines were negative and are not included in this table.

Table S3.04. Amount of N (kg ha^{-1}) in seed and biomass, and N harvest index (NHI) of 122 Puebla 152/Zorro RILs grown in East Lansing, MI and Puerto Rico in 2011, 2012, and 2013.

	East Lansing 2011				East Lansing 2012				East Lansing 2013	Puerto Rico 2012	Puerto Rico 2013
Commercial Checks	Seed	Biomass	Total	NHI	Seed	Biomass	Total	NHI	Seed		
	-kg ha^{-1}-			%	-kg ha^{-1}-			%	-kg ha^{-1}-		
Puebla 152	79.4	278.4	357.8	24.2	33.9	101.4	135.3	26.2	84.1	14.7	24.8
Zorro	107.5	50.0	157.6	68.3	74.8	15.8	90.6	82.1	79.6	41.5	26.1
R99	82.5	91.1	173.6	47.8	107.5	51.0	158.4	68.6	64.0	7.8	16.1
Medalis					83.8	49.7	133.6	66.5	59.4	15.9	18.8
PR0443-151	95.0	75.6	160.8	55.4	64.6	18.9	83.5	77.3	78.4	38.9	81.9
Verano					88.2	43.7	131.8	65.9	83.0		
TARS-LFR1					76.6	16.0	92.6	83.1	78.3	13.0	13.3
PR1147-6					71.9	30.7	102.7	70.6	76.7	50.0	74.1
RILs											
B11501	87.5	104.3	191.7	45.1	58.8	42.0	100.7	58.5	69.7	26.5	22.6
B11502	139.9	85.8	225.6	62.0	64.9	45.5	110.4	58.8	80.2	19.3	19.1
B11503	81.4	102.1	183.4	44.3	44.2	70.2	114.4	40.8	77.7	8.9	41.5
B11504	68.9	142.3	211.2	33.4	51.9	54.7	106.5	49.6	69.6	13.0	34.0
B11505	100.7	44.9	145.6	69.0	53.2	27.5	80.7	65.4	79.3	29.2	31.2
B11507	111.1	71.7	182.8	60.4	63.7	41.1	104.8	59.7	73.6	12.0	6.9
B11508	105.5	104.3	209.8	51.7	56.3	66.8	123.0	45.8	104.2	25.6	27.0
B11509	101.0	94.3	195.3	52.9	73.6	35.4	108.9	67.6	91.3	29.9	41.6
B11510	87.6	86.0	173.7	52.7	60.7	56.6	117.3	51.7	86.0	12.1	13.7
B11511	77.5	74.0	151.5	51.4	84.2	75.2	159.4	54.2	87.6	31.9	21.6
B11512	70.0	172.8	242.8	28.9	54.5	47.2	101.7	56.9	86.0	13.2	9.9
B11513	88.1	106.6	194.7	46.8	68.3	31.6	99.9	68.6	64.8	21.0	42.7
B11514	95.0	38.1	133.1	70.9	71.8	54.8	126.7	58.7	73.7	19.2	11.8

Table S3.04 (*cont'd*)

B11515	126.9	55.9	182.9	69.4	74.4	95.4	169.8	45.4	93.6	19.2	12.0
B11516	123.9	54.8	178.7	69.3	69.6	38.2	107.9	66.4	90.9	18.4	12.8
B11517	67.4	95.6	162.9	39.9	55.0	74.4	129.4	42.6	73.1	21.9	49.1
B11518	120.1	102.5	222.6	53.4	50.8	55.2	106.0	47.7	67.2	22.1	30.6
B11519	111.5	45.3	156.7	70.4	74.2	24.6	98.8	75.5	125.0	34.1	33.7
B11520	99.4	70.0	169.4	59.6	60.7	39.6	100.3	61.5	71.7	29.2	22.3
B11521	108.4	152.4	260.8	41.6	40.2	90.6	130.8	30.4	99.9	16.8	10.9
B11522	116.5	158.3	274.8	42.8	80.1	47.1	127.2	63.5	71.2	26.9	57.0
B11523	119.3	59.7	179.0	66.2	76.1	25.5	101.5	74.9	82.0	38.2	40.4
B11524	76.8	189.0	265.8	29.6	35.4	223.6	259.0	14.4	113.5	28.3	27.0
B11525	102.0	69.9	171.9	59.4	67.9	28.2	96.1	73.4	86.3	16.9	22.9
B11526	98.2	75.3	173.4	55.9	73.9	32.4	104.9	69.2	84.3	17.9	26.8
B11527	93.3	74.8	168.1	54.8	86.1	25.7	111.8	77.2	90.5	10.0	7.0
B11528	92.9	69.4	162.2	56.7	69.8	23.6	93.5	74.5	56.2	28.3	41.6
B11529	132.2	102.0	234.2	57.0	45.2	45.7	90.9	47.5	70.9	24.1	15.6
B11530	111.3	73.0	184.3	60.1	79.3	31.2	110.5	71.8	92.2	27.0	37.9
B11531	74.4	151.9	226.3	35.4	84.8	63.5	138.5	55.5	92.4	37.0	12.8
B11532	80.9	66.5	147.4	58.4	63.7	45.9	109.5	57.8	87.3	21.7	33.8
B11533	124.1	59.7	183.9	67.4	74.6	16.9	91.4	79.6	89.6	36.9	70.5
B11534	117.4	128.0	245.3	47.8	59.6	33.6	93.2	63.5	83.6	11.9	24.9
B11535	89.9	75.4	165.2	54.5	60.4	51.1	111.4	55.1	69.9	24.8	24.8
B11536	114.9	50.5	165.4	69.4	86.2	29.5	115.7	74.0	85.5	22.2	3.6
B11537	77.7	101.0	178.7	44.2	42.6	86.0	128.6	33.8	94.5	39.2	40.5
B11539	108.5	38.2	146.7	73.9	69.2	57.5	101.3	68.0	90.1	30.1	18.3
B11540	105.0	114.3	219.3	48.1	60.7	85.6	146.3	44.7	63.3	12.6	13.1
B11541	44.0	251.3	295.3	15.4	31.2	186.3	217.5	14.8	97.0	31.4	34.5
B11542	88.9	92.5	181.3	49.6	69.8	29.2	99.1	70.9	94.1	26.2	43.1

Table S3.04 (*cont'd*)

B11543	106.8	161.2	268.1	40.6	86.5	53.4	139.9	61.7	79.4	40.1	42.5
B11544	108.3	80.1	188.4	57.2	87.6	21.3	109.0	79.4	91.1	33.0	11.2
B11545	117.8	127.1	244.9	47.6	88.2	31.6	119.8	73.5	76.8	28.9	77.2
B11546	66.3	94.6	160.8	40.9	73.6	89.8	163.4	43.7	67.3	31.5	40.8
B11547	99.3	32.9	132.1	74.1	82.1	16.6	98.7	83.4	93.1	37.8	40.0
B11548	58.6	65.3	123.9	47.2	41.0	62.5	103.6	40.6	75.8	31.9	23.6
B11549	105.4	125.6	231.0	45.8	86.2	38.8	125.0	68.9	86.8	19.3	49.2
B11550	89.5	99.2	188.7	47.8	59.0	49.1	108.2	54.6	56.0	25.0	45.9
B11551	129.3	82.6	211.9	60.9	68.8	148.8	217.6	37.3	114.2	30.4	10.9
B11552	129.6	82.1	211.7	61.0	57.0	85.3	142.3	47.5	91.4	20.6	19.5
B11553	92.2	107.8	200.0	46.1	58.3	65.8	124.1	47.8	96.2	29.5	35.7
B11554	138.7	114.1	252.7	55.1	65.4	34.7	100.0	65.9	87.6	25.3	45.8
B11555	113.2	111.3	224.4	49.9	104.2	41.2	145.4	71.8	106.0	30.0	26.4
B11556	98.0	110.2	208.2	47.1	74.4	24.9	99.3	75.5	92.2	34.4	46.2
B11557	78.4	118.5	196.9	39.9	96.4	68.9	165.3	58.1	75.7	26.0	61.4
B11558	101.7	111.0	212.7	48.2	70.7	35.8	106.5	66.1	78.9	9.2	13.9
B11559	109.6	128.3	237.9	46.1	63.2	77.1	140.3	48.9	89.7	48.5	31.5
B11560	129.7	24.5	154.2	84.1	74.9	42.9	117.8	61.8	77.7	19.3	38.4
B11561	138.0	70.0	208.0	66.5	74.5	17.9	92.4	80.1	95.9	37.9	14.5
B11562	69.4	158.5	227.9	30.6	40.1	116.6	156.7	26.6	81.0	44.4	40.0
B11563	107.5	107.4	214.9	50.4	85.7	115.8	201.4	50.9	78.8	47.4	66.7
B11564	88.8	85.0	173.8	51.0	65.4	39.6	104.9	64.2	90.1	10.6	16.6
B11565	104.3	77.7	181.9	56.0	85.2	26.1	111.3	76.6	97.3	53.7	52.8
B11566	82.5	75.2	157.7	52.0	73.3	41.8	115.1	63.4	61.9	32.3	41.3
B11567	131.5	61.6	193.1	68.2	97.4	30.1	131.2	74.4	99.1	25.0	19.3
B11568	79.6	96.9	176.5	46.1	69.1	105.4	174.5	43.0	81.2	22.1	5.7

Table S3.04 (*cont'd*)

B11569	111.3	99.3	210.7	52.9	61.5	26.5	88.0	70.9	96.7	29.3	3.7
B11570	107.4	99.1	206.6	51.9	83.2	36.9	120.1	69.6	81.6	41.1	25.1
B11571	131.4	99.8	231.2	57.5	73.7	29.6	103.3	69.9	87.5	44.0	55.1
B11572	99.7	126.1	225.8	42.6	54.8	66.8	121.6	49.3	86.5	24.6	7.4
B11573	71.4	61.1	132.5	54.6	59.8	38.2	98.0	61.8	85.7	36.9	47.9
B11574	80.8	115.0	195.9	41.3	57.0	50.8	107.8	52.7	101.3	9.3	30.4
B11575	86.5	90.1	176.7	50.4	57.9	27.0	85.0	69.1	81.1	36.6	64.3
B11576	101.3	81.7	183.1	56.1	52.2	14.1	66.4	78.7	85.9	32.8	19.9
B11577	86.6	68.4	155.0	56.0	75.2	26.6	101.8	73.5	77.6	34.7	59.7
B11578	80.2	133.5	213.6	37.4	58.3	105.7	164.0	36.2	79.3	7.8	4.2
B11579	113.6	46.4	160.0	69.3	55.7	15.1	70.8	78.6	85.0	30.7	37.9
B11580	124.8	85.7	210.5	59.5	73.9	98.5	172.4	43.2	98.7	12.7	34.4
B11581	101.6	68.5	170.1	59.8	63.1	51.6	114.7	56.8	83.3	44.4	38.4
B11582	105.6	47.6	153.2	68.9	97.8	34.0	131.8	74.0	97.1	24.4	35.5
B11583	130.4	70.2	200.6	65.2	66.3	41.2	107.5	60.8	77.4	41.7	32.9
B11584	109.0	209.0	318.0	35.6	82.2	73.9	156.1	52.4	74.7	39.3	46.0
B11585	103.7	73.7	177.5	59.6	49.2	13.0	62.2	74.6	80.0	29.9	36.6
B11586	141.0	58.9	199.9	72.1	62.3	31.4	93.7	65.0	105.9	20.5	15.6
B11587	110.1	67.6	177.7	61.3	70.0	33.3	103.3	67.6	91.6	48.3	48.2
B11588	134.4	77.7	212.2	63.6	84.8	33.1	117.9	69.0	92.8	55.0	50.5
B11589	92.6	117.9	210.5	44.3	65.4	41.4	106.8	61.6	77.1	39.7	31.3
B11590	93.0	96.3	189.3	48.2	75.1	27.1	102.1	73.1	85.0	36.2	55.1
B11591	107.5	74.7	182.2	61.1	83.3	33.0	116.3	73.0	78.1	22.0	12.9
B11592	104.8	127.4	232.2	44.9	81.9	49.5	131.4	62.4	69.2	32.2	24.4
B11593	87.8	59.1	146.9	61.7	66.1	67.0	133.1	51.7	85.3	49.7	44.2
B11594	106.3	46.1	152.4	69.3	73.2	17.6	90.8	80.2	82.5	34.0	27.0

Table S3.04 (*cont'd*)

B11595	69.7	83.5	153.2	45.1	72.5	48.2	120.6	59.9	75.1	11.1	45.7
B11596	102.2	89.3	191.5	52.0	76.9	33.5	110.4	69.5	73.2	41.9	30.0
B11598	107.0	69.4	176.4	61.0	78.2	31.2	109.5	72.2	84.5	10.2	29.7
B11599	71.9	103.6	175.6	41.2	51.9	114.7	166.6	36.5	73.0	31.1	39.5
B11600	122.2	121.1	243.3	50.4	55.2	58.2	113.4	48.3	56.0	16.7	7.9
B11601	70.9	92.3	163.2	42.7	74.2	52.2	126.3	58.9	52.1	24.2	45.4
B11602	118.9	68.7	187.5	64.1	98.3	27.3	125.6	79.1	102.1	29.5	51.4
B11603	133.2	91.0	224.2	59.5	79.0	70.2	146.0	53.1	90.4	18.3	42.1
B11604	111.2	106.4	217.7	50.7	69.4	41.9	111.4	62.5	88.5	27.7	49.9
B11605	97.6	199.6	297.2	33.0	32.8	79.5	101.8	22.2	84.3	11.9	27.5
B11606	95.0	88.2	183.2	51.9	71.5	53.4	124.9	57.1	85.9	12.3	36.1
B11607	67.3	52.3	119.7	57.3	75.7	28.3	104.0	73.1	78.0	40.7	32.0
B11608	90.8	168.0	258.7	35.8	61.2	65.6	126.8	47.6	82.5	35.8	52.9
B11609	98.6	63.1	161.7	59.5	76.5	76.2	106.3	71.7	89.2	13.0	20.3
B11610	139.3	107.6	247.0	55.9	81.0	38.8	119.8	68.7	83.2	19.5	26.8
B11611	151.3	63.1	214.4	70.8	84.9	74.4	159.3	55.9	89.1	25.1	5.0
B11612	88.8	140.7	229.6	37.9	70.4	50.3	120.7	57.2	98.6	47.4	86.8
B11613	120.7	96.2	216.9	55.7	73.5	28.6	102.1	73.0	77.8	10.5	27.0
B11614	115.5	53.6	169.0	68.0	78.9	41.7	120.6	64.3	69.5	30.5	13.2
B11615	123.2	88.3	211.4	58.2	66.3	39.6	105.9	59.5	73.8	20.5	20.9
B11616	105.8	104.8	210.6	50.3	86.1	29.1	115.2	75.9	89.8	52.2	33.9
B11617	107.3	31.1	138.4	77.6	76.4	12.9	89.3	85.5	96.0	36.1	32.9
B11619	109.0	108.2		50.2	70.5	43.7	109.6	60.8	83.2	30.2	46.8
B11620	93.4	57.6	151.0	63.0	101.8	23.7	125.5	81.1	86.5	15.5	48.7

Table S3.04 (*cont'd*)

B11621	135.0	111.3	246.3	54.9	67.3	45.6	112.8	63.4	84.6	29.2	28.7
B11622	103.5	52.4	155.9	67.6	66.3	31.0	97.2	68.3	83.7	25.3	23.7
B11623	102.5	55.1	157.6	64.7	74.5	37.5	112.1	70.9	86.7	36.6	38.3
B11624	90.3	83.8	174.1	54.1	63.9	57.4	121.3	54.4	117.2	13.5	5.9
B11625	89.7	76.9	166.6	53.7	78.7	55.1	133.8	58.3	71.0	20.5	9.5
B11626	90.4	118.7	209.1	42.9	60.2	160.7	220.9	27.2	69.3	19.7	16.4
Test Mean	101.9	93.8	195.7	53.5	69.5	50.6	119.5	61.0	83.7	27.4	32.9
LSD ($P \leq 0.05$)	41.8	49.1	72.8	12.5	31.5	42.0	49.6	16.5	19.1	n.s.	41.4
CV (%)	24.5	31.2	22.2	14.0	26.9	49.1	24.4	15.9	14.1	64.7	74.3

Table S3.05. SNF traits measured in the greenhouse in East Lansing, MI of 122 Puebla 152/Zorro RILs grown in the greenhouse growing in N free conditions.

Commercial Checks	%N in biomass	Shoot Weight	Root Weight	Total Biomass	%Ndfa Difference	Shoot/ Root Ratio	Biomass Diff-Shoot†	Biomass Diff-Root†	Biomass Diff-Total†	Nodule Rating	$\delta^{15}N$‡
			(g)								
Puebla 152	2.52	12.38	3.74	16.1	98.1	3.28	95.0	81.3	91.6	4.0	-3.480
Zorro	3.06	4.44	1.74	6.2	94.4	2.63	83.5	59.8	77.0	4.5	-3.620
R99	1.14	0.58	0.66	1.2	0.0	0.90	0.0	0.0	0.0	0.0	0.443
Medalist	3.43	2.94	1.27	4.2	91.8	2.30	74.8	45.6	66.1	4.0	-3.290
PR0443-151	2.41	6.42	3.54	10.0	97.8	1.81	92.0	74.6	85.6	4.5	-3.420
RILs											
B11501	2.94	5.52	2.26	7.8	96.0	2.46	87.2	66.0	80.9	4.5	-3.615
B11502	2.48	4.95	1.60	7.1	94.6	3.31	85.3	55.8	78.3	5.0	-3.543
B11503	2.91	2.76	1.33	4.0	88.7	2.30	58.6	54.4	42.6	3.0	-3.410
B11504	2.72	4.17	1.67	5.8	94.3	2.87	83.1	66.8	73.4	4.0	-3.005
B11505	2.28	4.89	1.66	6.6	94.8	3.10	87.8	58.1	80.4	4.0	-3.353
B11507	2.79	3.97	1.63	5.6	94.6	2.38	83.8	58.4	76.2	4.0	-3.125
B11508	2.79	8.68	2.99	11.7	95.2	2.87	86.6	68.1	81.3	4.5	-3.573
B11509	2.95	4.68	1.71	6.4	95.5	2.97	86.2	53.3	78.2	5.5	-3.483
B11510	2.65	5.58	2.11	7.7	95.7	2.66	88.2	65.8	82.0	4.5	-3.328
B11512	3.14	4.74	2.05	6.8	96.2	2.34	87.0	67.4	80.8	4.5	-3.425
B11513	2.61	5.87	1.86	7.2	96.1	2.92	89.3	65.3	82.4	5.0	-3.328
B11514	2.67	4.48	1.62	6.1	94.4	2.70	82.4	50.3	73.1	4.0	-3.513
B11515	2.50	6.60	2.09	8.7	96.7	3.33	91.3	66.8	85.6	4.5	-3.368
B11516	3.33	5.80	2.13	7.9	96.5	2.79	87.3	60.3	80.0	6.0	-3.380
B11517	2.54	6.95	2.63	9.6	96.5	2.68	90.2	70.8	84.8	5.5	-3.553

Table S3.05 (*cont'd*)

B11518	2.33	7.34	2.41	9.8	95.7	2.94	89.3	68.6	83.8	5.0	-3.403
B11519	3.43	3.18	1.07	4.2	92.3	2.99	76.6	35.1	66.2	4.0	-3.250
B11520	2.80	6.00	2.18	8.2	96.7	2.77	90.0	68.3	84.0	4.5	-3.108
B11521	2.53	7.51	2.47	10.0	97.2	3.13	92.5	73.3	87.7	5.0	-3.490
B11522	2.85	4.54	1.52	6.1	95.5	3.00	86.6	56.8	78.9	3.5	-3.375
B11523	2.54	6.53	2.56	9.1	95.7	2.59	88.5	67.1	82.6	4.5	-3.543
B11524	2.21	5.54	2.33	7.9	95.4	2.51	89.3	69.3	83.7	5.5	-3.513
B11525	2.11	6.11	2.10	8.2	95.1	2.87	89.0	65.0	82.5	3.8	-3.210
B11526	2.71	5.53	2.59	8.1	95.4	2.15	86.9	69.8	81.2	4.5	-3.510
B11527	2.48	9.36	2.99	12.3	97.5	3.32	93.7	75.2	89.5	6.0	-3.350
B11528	2.42	6.05	2.26	8.3	95.5	2.64	88.1	67.4	82.2	5.5	-3.023
B11529	2.51	6.34	2.39	8.7	96.2	2.75	89.3	68.1	83.5	5.5	-3.230
B11530	2.50	4.35	1.84	6.2	94.0	2.39	84.1	59.7	76.8	4.0	-3.255
B11531	2.68	4.48	1.59	6.1	94.2	2.83	85.4	59.0	78.6	4.5	-3.330
B11532	2.78	4.12	1.75	5.9	93.7	2.40	82.2	53.3	73.6	5.0	-3.490
B11533	2.88	5.97	2.31	8.3	96.4	2.56	88.6	69.5	83.1	5.5	-3.658
B11534	2.42	5.34	1.76	7.1	95.5	3.06	88.3	60.1	81.3	5.0	-3.230
B11535	2.45	6.34	1.89	8.2	95.6	3.35	87.7	55.1	80.3	5.0	-3.038
B11536	3.00	4.52	1.83	6.2	95.7	2.58	86.5	53.7	75.8	3.5	-3.375
B11537	3.08	6.15	1.64	7.8	95.8	3.75	87.7	57.7	81.2	4.5	-3.670
B11539	2.65	5.88	2.64	8.5	96.1	2.27	89.0	72.3	83.9	4.0	-3.250
B11540	2.31	6.36	1.92	8.3	95.1	3.45	88.8	57.2	81.8	5.0	-3.455
B11541	3.08	8.88	3.35	12.2	98.0	2.66	93.4	79.2	89.5	5.5	-3.435
B11542	2.92	5.54	2.11	7.7	95.5	2.67	84.1	56.0	76.6	5.0	-3.270
B11543	2.08	5.50	1.88	7.4	94.8	2.96	88.0	60.6	80.9	5.0	-2.973

Table S3.05 (*cont'd*)

B11544	3.03	5.52	2.15	7.7	96.0	2.53	87.9	71.1	83.0	4.5	-2.893
B11545	2.54	7.36	3.24	10.6	96.5	2.37	90.4	74.4	85.7	5.5	-3.305
B11546	2.79	3.99	1.68	5.7	91.6	2.53	73.9	70.3	63.7	4.0	-3.347
B11547	2.50	4.73	1.93	6.7	95.9	2.63	87.0	62.6	80.2	4.5	-3.083
B11548	2.41	4.39	1.96	6.3	94.5	2.29	84.5	59.9	77.0	4.0	-3.240
B11549	2.63	4.69	1.79	6.5	95.3	2.64	86.5	60.1	79.3	4.5	-3.218
B11550	2.34	5.29	1.54	6.8	95.2	3.48	87.9	53.6	80.5	6.0	-3.055
B11551	2.85	8.65	3.63	12.3	97.6	2.51	92.6	78.6	88.6	5.0	-3.263
B11552	3.46	6.23	1.87	8.1	96.4	3.33	88.3	62.9	82.4	5.0	-3.010
B11553	2.71	4.84	1.86	6.7	94.3	2.78	84.2	55.0	76.7	5.0	-3.230
B11554	2.49	5.84	3.36	9.2	95.9	1.79	87.5	75.5	83.1	5.5	-3.360
B11555	3.47	4.71	1.55	6.3	96.4	3.02	84.4	55.3	77.2	4.5	-3.250
B11556	2.52	6.04	2.11	8.1	96.2	2.87	89.9	68.3	84.2	4.5	-2.978
B11557	2.64	3.38	1.26	4.6	93.2	2.66	80.9	47.2	71.5	3.5	-3.133
B11558	2.32	5.17	1.68	6.9	94.6	3.03	86.8	58.1	79.4	4.5	-3.245
B11559	2.37	5.53	2.06	7.6	95.1	2.67	87.8	64.5	81.3	6.0	-3.273
B11560	2.63	9.76	3.23	13.0	97.8	3.14	94.0	78.3	90.2	4.5	-3.608
B11561	2.66	5.31	2.04	7.4	95.6	2.87	88.0	58.5	80.7	3.5	-3.265
B11562	2.69	6.53	2.53	9.1	96.9	2.68	91.1	72.0	85.9	4.5	-3.350
B11563	3.05	4.89	2.03	6.9	95.3	2.36	84.3	62.9	77.9	3.5	-3.395
B11564	2.35	4.77	1.97	6.5	95.2	2.52	87.9	69.7	85.8	5.0	-3.855
B11565	2.88	6.39	2.37	8.8	96.9	2.70	90.3	69.5	84.5	4.5	-3.530
B11566	2.98	4.88	1.87	6.7	96.1	2.64	87.2	60.0	79.7	3.5	-3.183
B11567	2.25	7.39	2.53	9.9	96.7	3.03	92.2	73.2	87.4	5.5	-3.355
B11568	2.40	5.38	1.99	7.8	94.8	2.89	87.3	71.7	86.5	4.0	-3.143

Table S3.05 (*cont'd*)

B11569	2.83	5.05	2.03	6.6	95.5	2.45	86.5	58.4	76.7	5.0	-3.333
B11570	2.89	6.33	2.08	8.4	96.8	3.09	90.2	66.4	84.3	4.5	-3.225
B11571	2.81	5.47	2.08	7.5	95.8	2.66	88.2	64.7	81.7	5.0	-3.325
B11572	2.90	5.95	2.51	8.5	96.7	2.45	90.0	71.7	84.7	5.0	-3.135
B11573	2.90	5.66	1.79	7.5	93.4	2.91	80.6	47.4	71.6	3.0	-3.290
B11574	2.50	6.57	2.02	8.6	96.7	3.36	91.2	66.8	85.3	5.5	-3.223
B11575	2.44	5.31	1.80	7.1	95.5	2.93	86.7	58.7	79.3	4.0	-3.380
B11576	2.77	5.74	1.97	7.7	97.0	2.93	89.5	64.2	83.0	5.5	-3.503
B11577	2.26	4.68	1.76	6.4	94.2	2.74	86.1	59.6	78.9	4.0	-3.108
B11578	2.46	4.58	1.84	6.4	93.4	2.40	82.5	58.5	75.3	5.5	-3.228
B11579	2.10	5.65	1.95	7.6	93.7	2.81	84.8	54.4	76.5	4.0	-3.375
B11580	2.96	6.93	2.42	9.3	96.9	2.93	91.3	73.9	87.0	5.0	-2.937
B11581	2.39	4.73	1.78	5.7	94.0	2.26	84.0	49.9	70.9	5.0	-3.040
B11582	3.03	4.03	1.54	5.6	95.0	2.64	84.3	55.1	76.2	5.0	-3.075
B11583	2.40	4.74	1.93	6.7	95.6	2.47	87.8	65.7	81.3	5.5	-2.983
B11584	2.63	4.05	1.97	6.0	93.5	2.00	81.4	65.2	76.2	3.5	-3.108
B11585	2.33	4.89	2.02	6.9	94.5	2.41	86.7	63.0	79.4	4.0	-2.983
B11586	2.64	5.62	2.16	7.8	96.4	2.69	89.2	67.3	83.2	4.0	-3.160
B11587	2.20	6.60	2.24	8.8	95.8	2.99	89.6	67.5	83.8	5.5	-3.173
B11588	2.42	4.46	1.79	6.2	94.4	2.54	86.0	63.1	79.3	4.0	-3.287
B11589	2.38	4.34	1.82	6.8	94.3	2.74	85.7	77.0	89.8	4.0	-2.893
B11590	2.76	6.01	2.70	8.7	95.8	2.34	88.2	71.1	83.4	5.5	-3.417
B11591	3.32	6.32	2.35	8.7	97.3	2.81	90.3	71.8	85.1	4.0	-3.448
B11592	2.84	3.74	1.52	5.3	92.6	2.46	80.7	54.3	73.1	4.0	-2.700
B11593	2.36	4.82	1.89	6.7	93.9	2.44	83.2	62.2	76.7	2.0	-3.515

Table S3.05 (*cont'd*)

B11594	2.96	4.09	1.58	5.7	94.8	2.62	83.6	52.8	75.2	4.5	-3.080
B11595	2.45	4.78	1.74	6.5	94.5	2.75	86.7	63.7	80.6	4.0	-3.150
B11596	2.71	5.31	2.37	7.7	95.6	2.27	87.6	69.4	82.2	3.5	-3.283
B11598	2.88	5.15	1.77	6.9	95.4	2.91	86.2	58.5	79.1	4.0	-3.205
B11599	2.47	5.30	1.88	7.2	95.8	2.91	89.1	63.9	82.5	3.5	-3.438
B11600	2.42	6.23	2.24	8.5	95.6	2.85	88.0	61.8	81.3	6.0	-2.910
B11601	2.15	5.78	1.66	7.4	94.4	3.54	87.8	56.5	81.0	4.5	-3.117
B11602	2.62	5.42	2.04	7.5	95.5	2.64	87.0	64.0	80.5	4.5	-3.123
B11603	2.52	6.39	2.20	8.2	96.4	2.83	89.9	62.4	81.2	5.0	-3.100
B11604	2.76	5.69	2.02	8.2	94.7	3.07	83.8	67.7	83.5	5.0	-3.377
B11605	2.69	6.77	2.62	10.4	96.9	3.10	91.6	70.7	86.3	4.0	-3.318
B11606	2.27	4.63	1.40	5.5	94.5	2.97	86.5	45.0	73.6	3.5	-3.058
B11607	2.40	4.76	1.59	6.4	94.1	2.94	85.3	54.8	77.5	5.0	-3.148
B11608	2.68	6.42	2.17	8.6	96.6	3.01	90.1	65.5	84.0	4.0	-3.218
B11609	2.11	4.96	2.00	7.0	95.5	2.48	86.2	62.4	79.3	5.5	-3.380
B11610	2.83	7.20	2.46	9.7	96.5	2.99	90.1	70.1	85.2	5.5	-3.280
B11611	2.38	5.74	2.04	7.8	96.4	2.82	89.2	66.4	83.3	6.0	-2.970
B11612	2.87	6.37	2.30	8.7	96.4	3.01	89.5	66.3	83.9	4.0	-3.243
B11613	2.51	5.16	2.09	7.3	95.6	2.54	87.3	64.3	80.9	5.0	-3.240
B11614	2.74	4.34	1.73	6.1	95.7	2.58	83.8	54.4	75.3	5.0	-3.195
B11615	2.76	5.56	2.28	7.8	96.3	2.46	88.3	68.1	82.4	6.0	-3.093
B11616	2.91	5.80	2.26	8.1	96.0	2.66	88.9	67.2	83.0	4.0	-3.143
B11617	2.73	2.99	1.28	4.3	92.1	2.36	78.2	40.4	66.6	3.0	-3.057
B11619	2.48	5.19	1.73	6.9	94.6	2.98	86.2	57.1	78.8	4.5	-3.073
B11620	2.81	3.81	1.69	5.5	93.2	2.28	81.0	54.0	72.6	4.0	-3.193

Table S3.05 (*cont'd*)

B11621	3.05	4.87	2.05	6.9	95.6	2.42	85.1	60.9	77.9	3.8	-2.190
B11622	2.54	3.95	1.80	5.8	93.0	2.20	83.3	65.1	77.6	3.0	-3.370
B11623	3.01	5.08	2.09	7.2	96.1	2.42	87.3	66.2	80.8	4.5	-3.128
B11624	2.99	7.46	2.56	10.0	96.8	3.00	90.6	73.6	86.4	5.0	-3.330
B11625	2.86	7.25	2.94	10.1	97.2	2.49	91.3	75.3	86.6	5.0	-3.050
B11626	2.51	6.38	2.66	9.0	97.8	2.41	93.5	77.2	88.6	4.5	-3.090
Test Mean	2.63	5.58	2.09	7.7	94.8	2.72	86.5	63.1	80.0	4.6	-3.223
LSD ($P \leq 0.05$)	0.57	2.41	0.76	3.0	7.4	0.78	8.0	16.0	11.0	1.9	0.490
CV (%)	14.2	28.8	23.6	25.8	5.1	18.5	6.1	16.1	9.0	20.2	-9.3

† Difference= (biomass of N fixer (g)-biomass non-fixer (g))/biomass of N fixer (g).
‡ B value used in the natural abundance equation.

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CHAPTER 4

QTL ANALYSIS OF SYMBIOTIC NITROGEN FIXATION IN THE PUEBLA 152/ZORRO DRY BEAN RIL POPULATION

Abstract

Dry bean (*Phaseolus vulgaris* L) is able, through symbiotic N fixation (SNF) to acquire N from the atmosphere; but dry bean is generally considered a poor N-fixer. Considerable diversity within dry bean germplasm has been identified and several studies have shown that SNF can be enhanced through selection. More recently quantitative trait locus (QTL) analysis and genome wide association studies (GWAS) have been used to identify regions of the genome associated with SNF traits. In the current study a mapping population of 122 recombinant inbred lines (RILs) derived from the Mexican black bean 'Puebla 152' and the black bean cultivar 'Zorro' was genotyped with single-nucleotide polymorphism (SNP) markers developed through the BeanCAP, to construct a genetic map spanning 972 cM and containing 430 SNPs. The population was grown in the field in East Lansing, MI (EL) and Isabela, Puerto Rico (PR) and in the greenhouse (GH) under N free conditions to evaluate for yield, nodule development, biomass growth, agronomic traits, and N fixation. A total of 19 QTL associated with SNF traits were identified on all 11 chromosomes except Pv02 and large clusters of QTL were discovered on Pv01, Pv06, and Pv08. Many of the QTL associated with %Ndfa, N harvest index, and %N in biomass were also associated with candidate genes expressed in the nodules and roots. Candidate genes such as Phvul.006G146400, which is a chitin elicitor receptor kinase that is involved in recognition of rhizobia in the early establishment of the symbiotic relationship. Other candidate genes are transcription factors, such as Phvul.006G034400 that is associated with δ^{15}N, is a MADS-box family gene and is expressed in young and mature green pods. The majority of QTL associated with genes expressed in the root or nodule are derived from Puebla 152 while QTL

associated with genes with enhanced expression in stems and pods are associated with Zorro. This follows a pattern where Puebla 152 has superior SNF, whereas Zorro is highly efficient in partitioning the fixed-N into the seed. The QTL described serve as potential targets for improvement of SNF characteristics in adapted commercial dry bean genotypes.

Introduction

Symbiotic nitrogen fixation (SNF) is a complex trait. Not only must the plant be able to form compatible symbioses with the appropriate rhizobacteria, but it must also form sufficient nodule mass and effectively move fixed N through the plant to the seeds. Nodule number has been shown to vary among dry bean genotypes (Pereira et al., 1993). There was a considerable significant correlation ($r^2=0.64$, $p<0.01$) between nodule number and N fixed in a population of dry beans bred for enhanced N fixation (Pereira et al., 1993). Considerable variation in this trait exists within dry bean germplasm (Wolyn et al., 1991; Pereira et al., 1993; Fageria et al., 2014). Improvement should be possible as dry bean appears to be responsive to selection for improved SNF by selecting directly or indirectly for fixed N (Wolyn et al., 1991; Elizondo Barron et al., 1999).

St. Clair and Bliss (1991) selected four inbred backcross lines from a Puebla 152/Sanilac population which showed superior acetylene reduction assay (ARA) levels. These plants were intercrossed and the F_3 progeny were tested for their ability to fix N. The majority of the 25 resultant progenies were superior N fixers when compared to Sanilac. Several of the lines studied fixed N similar to high N-fixing parent Puebla 152 while having agronomic traits similar to Sanilac which would make them more amenable to direct harvest (St. Clair and Bliss, 1991).

In addition to the ability to fix N, the efficient use of N is important. Fageria et al. (2013) noted variability among the 20 dry bean genotypes for nitrogen use efficiency (NUE). Values ranged from 7.3 mg mg^{-1} seed in genotype 'BRS Valente' for each mg N applied to 21.2 mg mg^{-1} for line CNFP 7624. However, Fageria et al. (2013) did not mention if the potting mix used was sterilized nor if the plants were nodulated, as their N-free treatment yielded nearly as much seed N (43.6 g kg^{-1} for the zero N treatment compared to 46.9 g kg^{-1} in the fertilized treatment) though none was intentionally added. The source of this N is fixation. Thus, traits associated with partitioning likely interact with SNF to achieve enhanced yield.

Several studies have been conducted to map QTL for various SNF traits (Nodari et al., 1993; Tsai et al., 1998). In an attempt to look for QTL involved in the interaction between host and bacteria, Nodari et al. (1993) used an F_3 population from 'BAT93' (Mesoamerican derived genotype with fewer nodules and resistance to common bean blight (CBB)) and 'Jalo EEP558' (Andean selection with high nodule number, susceptible to CBB). Four QTL, which explained a total of 52% of the phenotypic variation for nodule number, were discovered. One locus appeared to have an effect on both nodule number CBB resistance which is not surprising since many stages in the interaction with pathogenic bacteria are similar to interactions with beneficial bacteria. This region, on Pv07 contributed by the BAT93 parent, was associated with CBB resistance but with low nodule number (Nodari et al., 1993). Tsai et al. (1998) used a similar population by crossing the high nodulating dry bean, Jalo EEP558 with the low nodulating BAT93 to investigate nodule number and CBB resistance inheritance under contrasting N conditions. Both parents contributed positive alleles to nodule number and CBB resistance in the F_2 derived F_3 RILs. Given that the low nodulating parent (BAT93) contributed alleles with a positive effect on nodule number and the CBB susceptible parent similarly contributed positive alleles for CBB resistance. Ramaekers et al. (2013) used 85 RILs developed from G2333 x G19839 to investigate characteristics associated with SNF in both the greenhouse under N free conditions and in the field. A total of 204 markers, including SSRs, SNPs, and an isozyme markers were used in the genetic analysis. They measured traits such as leaf chlorophyll content, shoot dry weight, total biomass N, seed yield and total N in seed. Many QTL were discovered for SNF traits, such as SPAD (a measure of chlorophyll, and hence N level in leaves) at different growth stages (R^2= 11.49% to 35.53%), %N in the shoot, root, and plant (R^2= 16.3% to 21.01%), and total N in the shoot, root, and plant (R^2= 14.69% to 20.87%), in the greenhouse. QTL for nodule number (R^2= 17.25% and 16.72%), two QTL for nodule dry weight (R^2= 12.97% and 19.07%), and one QTL for %Ndfa at harvest (R^2=18.79%) were detected in the field. Different QTL were found between the field and greenhouse experiment but overlapped QTL for SPAD QTL on Pv01 and two QTL on Pv07 for SPAD at pod filling were detected in the greenhouse and the field. The QTL reported have low to moderate effect on the phenotype but could prove useful in developing markers for marker assisted selection (MAS).

More recently a genome wide association study (GWAS) found several QTL associated with SNF traits in the Andean Diversity panel of common bean (Kamfwa et al., 2015a). The panel was grown under N free conditions in the greenhouse as well as under low N in the field. QTL for %Ndfa in the shoot in field studies were found on Pv02, Pv03, Pv07, Pv09, Pv10, and Pv11 with an R^2 ranging from 0.11 to 0.22. Chromosomes Pv02, Pv03, Pv07 and Pv09 contained QTL responsible for Ndfa in the greenhouse with R^2 ranging from 0.09 to 0.20 (Kamfwa et al., 2015a). One SNP, ss715648916 on Pv09, was associated with multiple QTL for SNF including Ndfa in the seed, Ndfa in the shoot, %Ndfa in the shoot, %N in the seed, chlorophyll content, shoot biomass, and %N in shoot biomass. The candidate gene associated with this SNP, Phvul.009G136200, is a leucine-rich repeat receptor like kinase (LRR-RLK) and may prove useful as a target for enhancing SNF in dry bean.

It is clear that variation exists in SNF characteristics in dry bean. Many studies have identified regions in the genome that are associated with SNF in Andean populations or in Andean x Middle American populations. The objective of the current study was to investigate the genetic components of SNF in a Middle American black bean RIL population and develop genetic markers for use in MAS to develop genotypes with enhanced SNF. Germplasm with superior SNF characteristics that is adapted to modern conventional and organic agricultural practices is currently not available. Lines which combine improved agronomic traits and superior SNF ability developed in this study will prove useful in increasing %Ndfa in commercial dry bean breeding materials.

Materials and Methods

The phenotyping of the plant material, experimental design and data collection used in the QTL study were previously described in Chapter 3.

Single Nucleotide Polymorphism (SNP) Genotyping

The Puebla 152/Zorro population was genotyped using the SNP array developed by the BeanCAP (www.beancap.org) project. Analysis was conducted at the Soybean Genomics and Improvement USDA Laboratory (USDA–ARS, Beltsville, MD, Agricultural Research Center) following Hyten et al., (2010). The Illumina platform was used following the Infinium HD Assay Ultra Protocol (Illumina Inc.). The Infinium II assay protocol involves making and incubation of amplified DNA, fragmenting the amplified DNA for preparation of the bead chip, and hybridizing the samples to the BARCBean6K_3 BeadChip with 5389 SNPs. The DNA is then extended, stained, and imaged. GenomeStudio Genotyping Module v1.8.4 (Illumina, Inc.) was used to call SNP alleles. Manual adjustments were then made.

Linkage Map Construction

Data was filtered to remove markers with no calls. Also, SNPs with more than 20% missing data and non-informative markers were removed. The remaining 1,116 SNP markers were used for map construction using Joinmap 4 (Van Ooiijen, 2006). Prior to mapping, markers were sorted into their respective linkage groups according to the reference genome (Schmutz et al., 2014). Maximum likelihood was used with a chain length minimum of 20,000 simulations each cycle for a total of 4 cycles with a 5000 simulation burn in. Markers which were 100% identical were eliminated. Nearest neighbor stress and fitness tests were inspected to evaluate convergence with likely positions. Markers with elevated stress values were eliminated. Marker order on each linkage group was verified using the known locations of markers by comparing the completed map to the physical positions in the reference genome (Schmutz et al., 2014) using the fixed orders option in Joinmap 4 to orient the linkage groups.

QTL Analysis

Multiple QTL mapping (MQM) was conducted using MapQTL 5 (Van Ooijen, 2004). The LOD threshold was determined by running a permutation analysis set to 10,000 permutations for each trait. The 95th percentile of permutations for all traits for the genome wide group was selected resulting in an LOD of 3.0. The "Jbrowse" tool at Phytozome 10.2 (Goodstein et al., 2012) was utilized to identify candidate genes in the *Phaseolus vulgaris* genome. Linkage map figures were generated with MapChart 2.3 (Voorrips, 2002). The multiple interval mapping (MIM) option in WinQTLCART 2.5 (Wang et al., 2012) was used to test for QTL x QTL interactions.

Results and Discussion

The resulting genetic map retained 430 SNP markers with a genome size of 972 cM (Figure 4.01). The map approaches the size previously determined by Freyre et al. (1998), which was 1200cM. Hoyos-Villegas et al. (2015) estimated the genome size of the AP630 pinto bean map to be 1499 cM using SNP markers. The genome size of the G2333 x G19839 map was estimated at 1,601 cM using SSRs, SNPs, and an isozyme markers (Ramaekers et al., 2013). The average coverage was one marker for each 2.26 cM which was intermediate to values reported in the AP630 population, with an average distance of 3.6 cM between SNPs (Hoyos-Villegas et al., 2015) and the SEA5/CAL9 population which had an average distance of 0.64 cM between SNPs (Mukeshimana et al., 2014). Chromosomes Pv03 and Pv10 had the lowest number of markers while Pv05 had a disproportionately large number of markers (Supplemental Figure 4.01). The markers remaining prior to map construction were similarly distributed leaving limited coverage of some chromosomes.

A QTL for canopy height, HT1.1, was detected on Pv01 in 2012 and one in 2013 in EL (Table 4.01). While the peak LOD was found at two distinct positions, 49.6 Mb and 48.5 Mb, respectively, they likely refer to the same QTL. A QTL for days to flower, DF1.2, was discovered on Pv01 located near position 48.5 Mb in both years in EL. Mukeshimana et al. (2014), Hoyos-Villegas et al. (2015) and Kamfwa et al. (2015b) also found a similar QTL on Pv01 for days to flower located at position 43.7 Mb. A total of six QTL for yield were discovered, two on Pv01, two on Pv03, and two on Pv11. The same yield QTL, SY1.1, on Pv01 was found in 2012 and 2013 in EL and accounted for 13.0% and 17.1% of the variability, respectively (Table 4.01). The QTL was located at 48.5 Mb. A QTL for yield, SY3.3, on Pv03 were also discovered in 2012 and 2013 in PR (R2=14.6% and 12.1%, respectively) at positions

39.4 Mb and 39.6 Mb and these were attributed to Puebla 152 parent. Hoyos-Villegas et al. (2015), Mukeshimana et al. (2014), and Kamfwa et al. (2015b) found the same QTL for yield in this region in very different genetic populations evaluated in diverse locations. These two loci were located 0.16 Mb apart and likely refer to the same QTL. A QTL for yield was also discovered by Hoyos-Villegas et al. (2015) on Pv03 located at 33.6 Mb with an R^2 of 12.2%. Kamfwa et al. (2015b) also identified a QTL for yield on Pv03 in the Andean diversity panel located at position 38.3 Mb which is intermediate in position from that discovered in the current study and that reported by Hoyos-Villegas et al. (2015). Mukeshimana et al. (2014) identified a QTL for yield in the SEA5/CAL96 population grown in Karama, Rwanda, located at position 4.0 Mb. Two QTL for yield were also discovered on Pv11 in 2011 and 2012 in EL (Table 4.01). These QTL were located 431 kb apart at 47.6 Mb and 48.0 Mb, respectively and likely refer to the same SY11.1 QTL.

Analysis for traits measured in the greenhouse was conducted on the bulk of 4 reps and are presented in Table 4.01. QTL for shoot N, shoot and root weight, total biomass, nodule rating, shoot difference, total biomass difference, and shoot:root ratio were found on Pv01, Pv05, Pv08, and Pv11. Overlapping QTL for shoot N (R^2=22.5%), shoot weight (R^2=13.7%), and total biomass (R^2=12.7%) were detected on Pv01 between 42.2 Mb and 51.3 Mb and all three QTL were contributed by Puebla 152. These traits are related to each other, since total biomass includes shoot biomass, with both depending on N availability so it is not unexpected to have adjacent QTL for each trait. Kamfwa et al. (2015a) also identified a QTL on Pv01 at 48.1Mb for shoot biomass in an Andean bean panel in a similar greenhouse study with an R^2 of 8.0%. This QTL is within 0.35 Mb of the QTL discovered in this study. A QTL, SRR5.1 for shoot:root ratio was located on Pv05 at 39.0 Mb and was contributed by the Zorro parent. Zorro is the adapted, efficient parent which partitions biomass into seed much more efficiently than Puebla 152. A single QTL, RWT8.1for root weight, was found in the greenhouse assay on Pv08 at 9.6 Mb. Five QTL were found on Pv11, and two for shoot difference (R^2=46.2%) and for total biomass difference (R^2=40.9%) colocalized at 4.5 Mb. These QTL explain a high proportion of the variation for these traits and may be useful in investigating harvest index. A QTL for shoot weight (SWT11.1, R^2=13.7%) and another QTL for shoot:root ratio (SRR11.1, R^2=17.3%) colocalized at 5.1 Mb. A single QTL, NoR11.1 for nodule rating (R^2=11.9%) was located at 8.2 Mb on Pv11.

Five QTL for SNF traits were found on Pv01 including $\delta^{15}N$ (D15N1.1), %Ndfa difference (%Ndfa1.1), N harvest index (NHI1.1), and two for seed N (SN1.1) which were discovered in 2011, 2012, and 2013 in the field in EL. The QTL for $\delta^{15}N$ was located at 48.5 Mb with R^2=13.6%. One of six QTL for %Ndfa difference was located at the same position. In 2012 a QTL for N harvest index was found at the same position and is attributed to Zorro and accounts for 11.7% of the variation. Two QTL for seed N were discovered on Pv01, one each year in 2012 and 2013. Kamfwa et al. (2015a) found a QTL for biomass at 48.1 Mb. Ramaekers et al. (2013) also found a large cluster of QTL on Pv01, but the use of different marker systems from the current study limits the ability to make comparisons. Several of the traits associated with this QTL cluster were for %N in the shoot, % N in the plant, total N harvest, and total %Ndfa. Similarly, two QTL for seed N and shoot N were found in this study on Pv01 (Table 4.01, Figure 4.01).

A cluster of six QTL colocalized on Pv08 between positions 9.5 Mb and 12.2 Mb (Table 4.01). A QTL for N harvest index (NHI8.1) was located at 9.5 Mb (R^2=10.3%) and originated from Zorro. Another QTL for seed N (SN8.1) was found at 10.2 Mb (R^2=16.1%) and also originated from Zorro (Table 4.01). Two QTL for seed N (SN8.1) were also discovered, one in 2011 (position 10.2 Mb R^2=15.1%) and 2013 (position 12.2 Mb, R^2=12.7%). While the peak LOD (4.3 and 3.6, respectively) occur at different positions, they are relatively close and are likely the same QTL. A QTL for %Ndfa difference (%Ndfa8.1), R^2=10.6%) was located at 12.2 Mb on Pv08 which originates with the Zorro parent and may be explained by the superior partitioning ability of Zorro compared to Puebla 152.

Four QTL were discovered on Pv11, with two each colocalizing. At position 1.5 Mb %Ndfa difference (NdfaD11.1, R^2=18.8%) was discovered, colocalizing with %N seed (%NS11.1, R^2=10.2%). Seed N and another for %Ndfa difference (%Ndfa11.2) was located at 39.8 Mb. Both QTL were associated with Zorro (Table 4.01). A QTL for root weight (RWT8.1) in the greenhouse was found on Pv08 at position 9.5 Mb and flanked by SNP ss715647419 at 9.1 Mb and SNP ss715648550 at position 12.2 Mb in the RIL population. A similar QTL was found by Ramaekers et al. (2013) in the G2333 x G19839 population at 84.66 cM in their study, which when compared to map positions in the Red Hawk/Stampede population would place it at approximately 54.9 Mb. Based on the cM positions in the Red Hawk/Stampede map is within 30 cM of the QTL discovered in the current study. Use of different markers in this study make direct comparison difficult. Other QTL for SNF traits were found on Pv03 (N yield, NY3.1), Pv04 (N yield, NY4.1), Pv05 (N yield (NY5.1) and %N seed (%NS5.1)), Pv07 (N yield), Pv09 (N yield (NY9l.1) and %Ndfa difference (%Ndfa9.1)), and Pv10 (total N harvest, TN10.1) in the field study in EL.

Six QTL for SNF traits were discovered on Pv06 based on data from the field in EL and PR. A single QTL for %N in biomass (%NB6.1) was discovered which accounted for 12.6% of the variation. This QTL was located near the SNP ss715645785 and was located at position 26.0 Mb

(Table 4.01; Figure 4.01). This QTL colocalized with three other QTL, for seed N (SN6.1), N harvest index (NHI6.1), and %Ndfa difference (%Ndfa6.2) (Figure 4.01). The seed N (SN6.1) QTL was located at the same location and accounted for 13.7% of the variation. The QTL for N harvest index (NHI6.1) was also located at the same location and accounted for 14.1 % of the variation. The fourth QTL in this group accounted for 12.8% of the variation for %Ndfa difference. These traits are related in that they appear to be involved in how N is partitioned within the plant. The second group of QTL on Pv06 contains two QTL, one for %Ndfa (%Ndfa6.1) and one for $\delta^{15}N$ (D15N6.1). The $\delta^{15}N$ QTL was located at position 13.1 Mb and accounted for 10.2% of variation. The QTL for %Ndfa, located at 13.2 Mb, accounted for 13.7 % of variation and was contributed by Zorro (Figure 4.01).

Several QTL for multiple diverse traits such as shoot N, shoot weight (SWT1.1), and total biomass (BM1.1) in the greenhouse and days to flower (DF1.1 and DF1.2), canopy height (HT1.1), lodging (LDG1.2), seed N yield (SN1.1) and yield (SY1.1) in the field are clustered on Pv01 (Table 4.01). This region is gene rich and has several protein kinases such as Phvul.001G222600, Phvul.001G222700, as well as a nodulin transporter gene-Phvul.001G223600.

Several QTL for traits including nodule rating (NoR11.1) in the greenhouse shoot weight (SWT11.1), shoot:root ratio (SRR11.1), in the greenhouse and the yield QTL (SY11.1) from the field are located on Pv11 (Figure 4.02). A nodule Cysteine-rich (NCR) secreted peptide is located at position 8.3 Mb is approximately 51.4 kb from the QTL for nodule rating. This QTL is derived from Puebla 152 and may suggest that this parent has an allele which would be useful in increasing SNF in future dry bean varieties.

Several of the QTL discovered are located at or very near genes transcribed in the roots or nodules and may serve as potential targets for breeding dry bean genotypes with enhanced SNF. A phosphofructokinase gene, Phvul.005G165000 is located at the position of the QTL SRR5.1, on Pv05 for shoot:root ratio (Phytozome 10.2). This gene is involved in metabolism and the expression profile indicates that this gene is highly expressed in the nodules and young pods. The QTL associated with nodule rating found on Pv11 includes the gene Phvul.011G085200.1. This gene is a xyloglucan transglucosylase/hydrolase (XTH) and is highly expressed in the roots (Phytozome 10.2). This class of gene has been implicated in cell wall loosening during fruit growth and ripening (Munoz-Bertomeu et al., 2013). This gene may be important in growth and development of nodules on the root of dry bean.

Another candidate gene, Phvul.006G146400, is a chitin elicitor receptor kinase expressed in the nodules and is found 12 kb from the SNP (ss715645785) (Phytozome 10.2) associated with %N in biomass, %Ndfa difference (%Ndfa6.2), and N harvest index (NHI6.1) on Pv06. The NOD factors produced by rhizobia are chitin-related molecules (Eckardt, 2008) suggesting that this QTL may be involved with the initial interactions between dry bean host and N fixing rhizobia. This allele originates with Puebla 152 suggesting that perhaps this parent has characteristics that allow it to more effectively perceive and respond to rhizobia in the soil. A MADS-box gene, Phvul.006G146600, is located 18.5 kb from the SNP associated with these QTL and is similarly expressed in the roots and nodules. This transcription factor could also be involved in some aspect of regulation of SNF.

Other QTL were associated with genes highly expressed in pods such as the QTL D15N6.1 for $\delta^{15}N$ on Pv06 at position 13.1 Mb. The candidate gene, Phvul.006G034400 belongs to the MADS-box family of genes which are involved in gene regulation. This gene is located at 14.0

Mb. A candidate gene associated with a different QTL for $\delta^{15}N$ (D15N8.1) on Pv08 located at position 12.3 Mb is a MYB family transcription factor (Phvul.008G107000) located at 12.3 Mb and is also expressed in pods and stems and may play a role in partitioning N into seed.

A total of 19 QTL associated with SNF characteristics in a dry bean RIL population were discovered on all chromosomes except Pv02. The number of QTL ranged from a single QTL per chromosome to six QTL on Pv06. Chromosomes Pv01, Pv06, and Pv08 have clusters of SNF QTL and may serve as good targets for improving SNF in dry bean.   Candidate genes associated with these QTL include transcription factors, transferases, and receptors involved in sensing rhizobacteria. The QTL discovered in this study may prove useful for developing future dry bean cultivars with improved SNF.

APPENDICES

APPENDIX A

CHAPTER 4 TABLES AND FIGURES

Table 4.01. Quantitative trait loci (QTL) for biomass, agronomic and SNF traits in the Puebla 152/Zorro RIL population grown in the field in EL and PR in 2011-2013 and in greenhouse under N free conditions in East Lansing, MI.

Trait Name	Year	Location	QTL Name	Chromosome	Position	Indicative Marker	Peak LOD†	$R^{2‡}$	Add§
SNF Traits-Greenhouse					Mb			%	
Shoot N	2012	GH		Pv01	49.1	ss715645906	5.5	22.5	0.0
Nodule Rating	2012	GH	NoR11.1	Pv11	8.2	ss715649670	3.3	11.9	0.4
Shoot Weight	2012	GH	SWT1.1	Pv01	48.5	ss715646586	3.7	13.7	0.6
Shoot Weight	2012	GH	SWT11.1	Pv11	5.1	ss715649142	3.2	13.7	0.8
Root Weight	2012	GH	RWT8.1	Pv08	9.5	ss715649604	2.9	10.5	-0.2
Total Biomass	2012	GH	BM1.1	Pv01	50.5	ss715645248	3.5	12.7	0.7
Shoot:root Ratio	2012	GH	SRR5.1	Pv05	39.0	ss715645320	3.3	13.1	-0.1
Shoot:root Ratio	2012	GH	SRR11.1	Pv11	5.1	ss715649142	4.1	17.3	0.1
Shoot Difference	2012	GH		Pv11	44.6	ss715647551	6.8	46.2	-13.8
Total Biomass Difference	2012	GH		Pv11	44.6	ss715647551	4.8	40.9	-17.2
SNF Traits-Field									
%N in Biomass	2011	EL	%NB6.1	Pv06	26.0	ss715645785	3.6	12.6	14.7
$\delta^{15}N$	2011	EL	D15N1.1	Pv01	48.5	ss715646586	3.8	13.6	-0.3
	2013	EL	D15N6.1	Pv06	13.1	ss715650171	2.9	10.2	0.1
	2011	EL	D15N8.1	Pv08	12.3	ss715648543	2.9	10.3	0.2
%Ndfa-Difference	2013	EL	%Ndfa1.1	Pv01	48.5	ss715646586	3.6	12.7	3.8
	2013	EL	%Ndfa6.2	Pv06	26.0	ss715645785	3.0	12.8	-3.4
	2011	EL	%Ndfa8.1	Pv08	12.2	ss715648550	3.0	10.6	-4.0
	2012	PR	%Ndfa9.1	Pv09	32.8	ss715645632	3.0	10.6	-4.6
	2013	EL	%Ndfa11.1	Pv11	1.5	ss715645474	3.5	18.8	-5.9
	2013	EL	%Ndfa11.2	Pv11	39.8	ss715645776	3.2	18.1	-4.3

Table 4.01 (*cont'd*)

N Yield	2012	PR	NY3.1	Pv03	39.5	ss715646619	3.1	11.1	-4.1
	2012	PR	NY4.1	Pv04	1.7	ss715647821	3.8	13.2	4.3
	2012	PR	NY5.1	Pv05	3.2	ss715648340	4.0	13.9	4.3
	2013	PR	NY7.1	Pv07	0.1	ss715648390	3.4	11.9	6.2
	2012	PR	NY9.1	Pv09	25.2	ss715646059	3.2	12.6	4.4
%Ndfa	2013	EL	%Ndfa6.1	Pv06	13.2	ss715641022	3.9	13.7	-2.6
N Harvest Index	2012	EL	NHI1.1	Pv01	48.5	ss715646586	3.4	11.7	-0.1
	2011	EL	NHI6.1	Pv06	26.0	ss715645785	4.0	14.1	-0.1
	2011	EL	NHI8.1	Pv08	9.5	ss715649604	2.9	10.3	0.0
% N Seed	2012	EL	%NS5.1	Pv05	20.9	ss715642931	3.5	13.3	-0.1
	2011	EL	%NS8.1	Pv08	10.2	ss715649664	4.3	15.1	-0.1
	2013	EL	%NS8.1	Pv08	12.2	ss715648550	3.6	12.7	-0.1
	2012	EL	%NS11.1	Pv11	1.5	ss715645474	2.9	10.2	0.2
Seed N	2012	EL	SN1.1	Pv01	51.3	ss715645302	4.1	16.3	-7.2
	2013	EL	SN1.1	Pv01	48.5	ss715646586	4.6	16.1	6.2
	2013	EL	SN6.1	Pv06	26.0	ss715645758	3.3	13.7	-5.0
	2011	EL	SN8.1	Pv08	10.2	ss715649664	4.7	16.1	-8.9
	2013	EL	SN11.1	Pv11	39.8	ss715645776	3.1	13.0	-5.1
Total N Harvest	2011	EL	TN10.1	Pv10	41.0	ss715645510	3.1	11.0	14.8

Table 4.01 (cont'd)

Agronomic Traits-Field									
Canopy Height	2012	EL	HT1.1	Pv01	49.6	ss715645857	3.5	12.4	-4.7
	2013	EL	HT1.1	Pv01	48.5	ss715646586	3.7	13.0	-3.4
Days to Flower	2011	EL	DF1.2	Pv01	48.5	ss715646586	6.2	24.1	2.1
	2012	EL	DF1.2	Pv01	48.5	ss715646586	7.0	27.2	2.3
Lodging	2012	EL	LDG1.2	Pv01	48.5	ss715646586	6.2	20.8	0.5
	2013	EL	LDG1.2	Pv01	48.5	ss715646586	6.0	20.3	0.5
	2012	EL	LDG2.1	Pv02	2.5	ss715647235	3.1	11.2	0.3
	2013	EL	LDG2.2	Pv02	26.0	ss715650231	6.3	21.1	0.4
Seed Yield	2012	EL	SY1.1	Pv01	48.5	ss715646586	3.2	13.0	-170.8
	2013	EL	SY1.1	Pv01	48.5	ss715646586	4.9	17.1	157.9
	2012	PR	SY3.3	Pv03	39.6	ss715639345	3.2	14.6	-138.8
	2013	PR	SY3.3	Pv03	39.4	ss715646621	3.1	12.1	-192.9
	2011	EL	SY11.1	Pv11	47.6	ss715640198	3.0	10.8	178.5
	2012	EL	SY11.1	Pv11	48.0	ss715648095	3.6	13.5	161.6

† Logarithm of odds.

‡ Percent of the phenotypic variation explained by the QTL.

§ Positive values indicated allele contributed by Puebla 152 parent, negative values indicate allele came from Zorro parent.

ˡ Biomass difference calculated using the biomass (g) of each respective plant part with the non-nodulating R99 used as a reference.

Figure 4.01. Dry bean chromosomes Pv01, Pv03, Pv04, Pv05, Pv06, Pv07, Pv08, Pv09, Pv10, and Pv11 showing QTL for Symbiotic N-fixation (SNF) from the N-free greenhouse analysis and in the field in East Lansing, MI and Isabela, Puerto Rico in 2011 to 2013†.

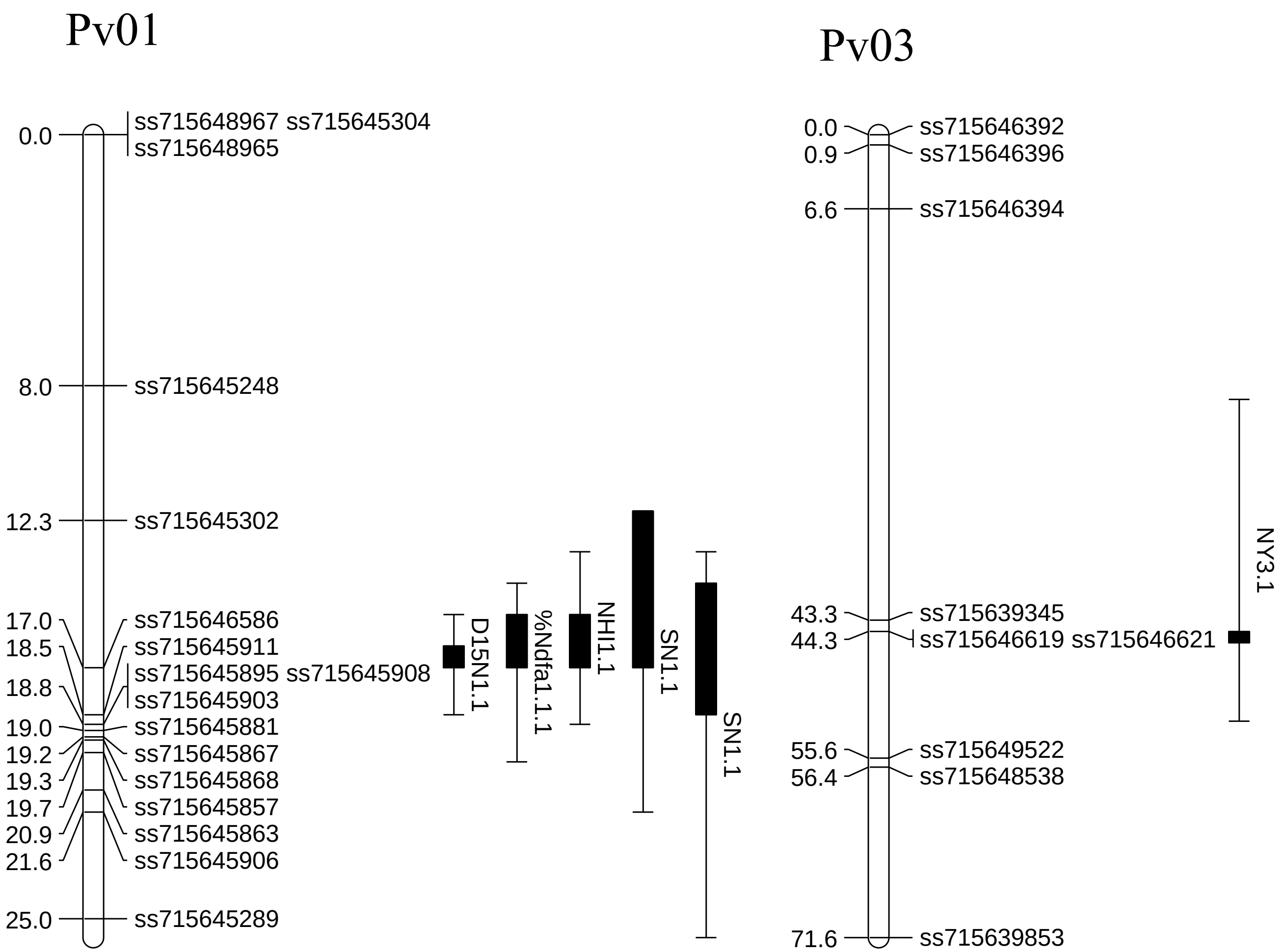


Figure 4.01. (*cont'd*).

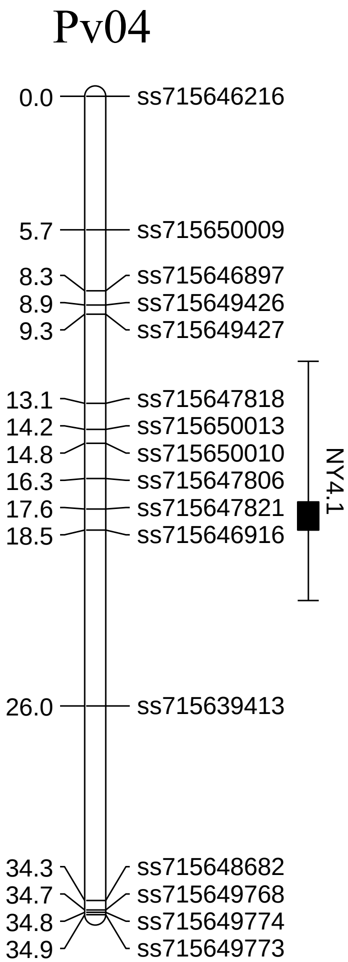


Figure 4.01 (*cont'd*)

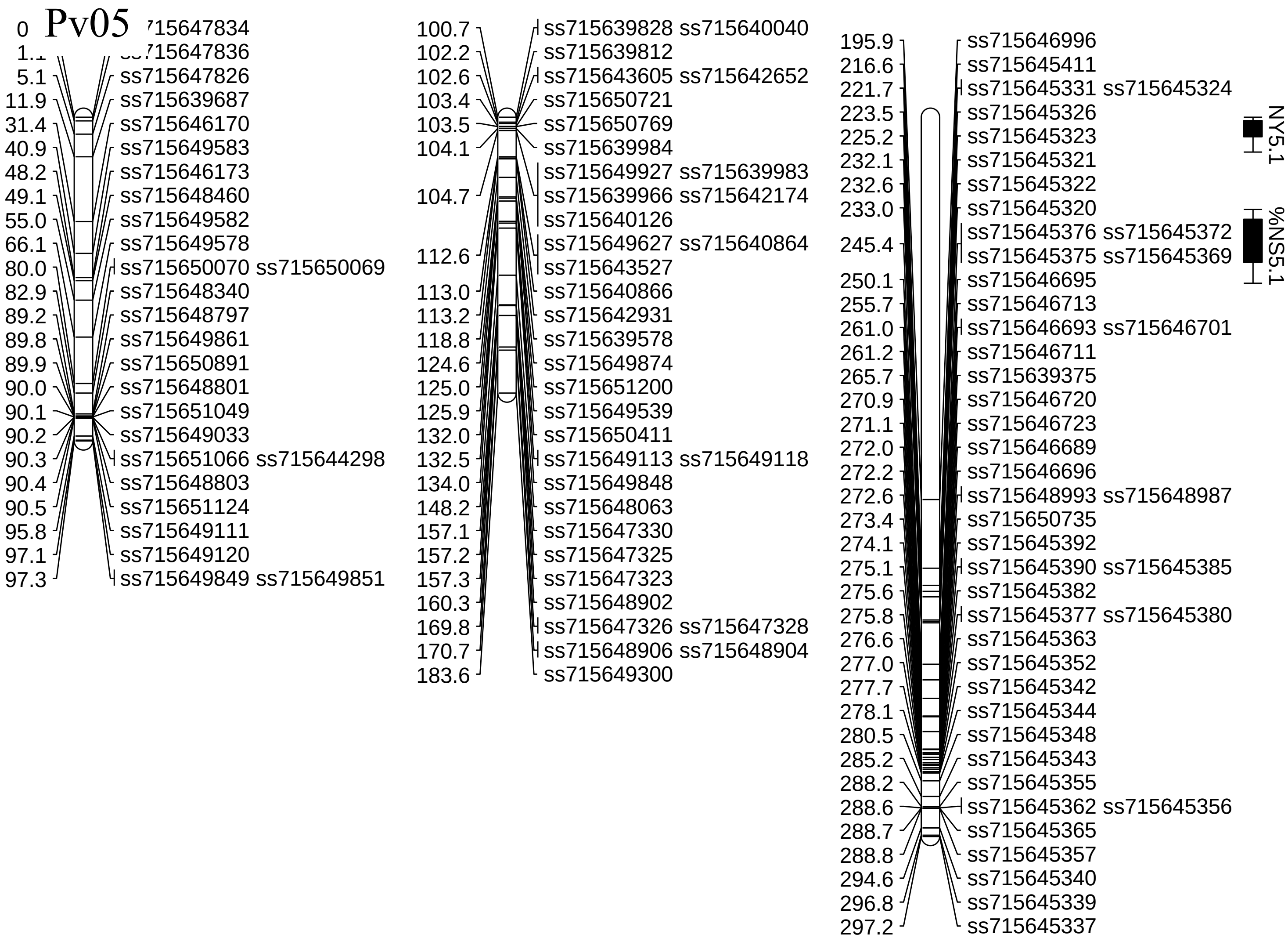


Figure 4.01 (*cont'd*)

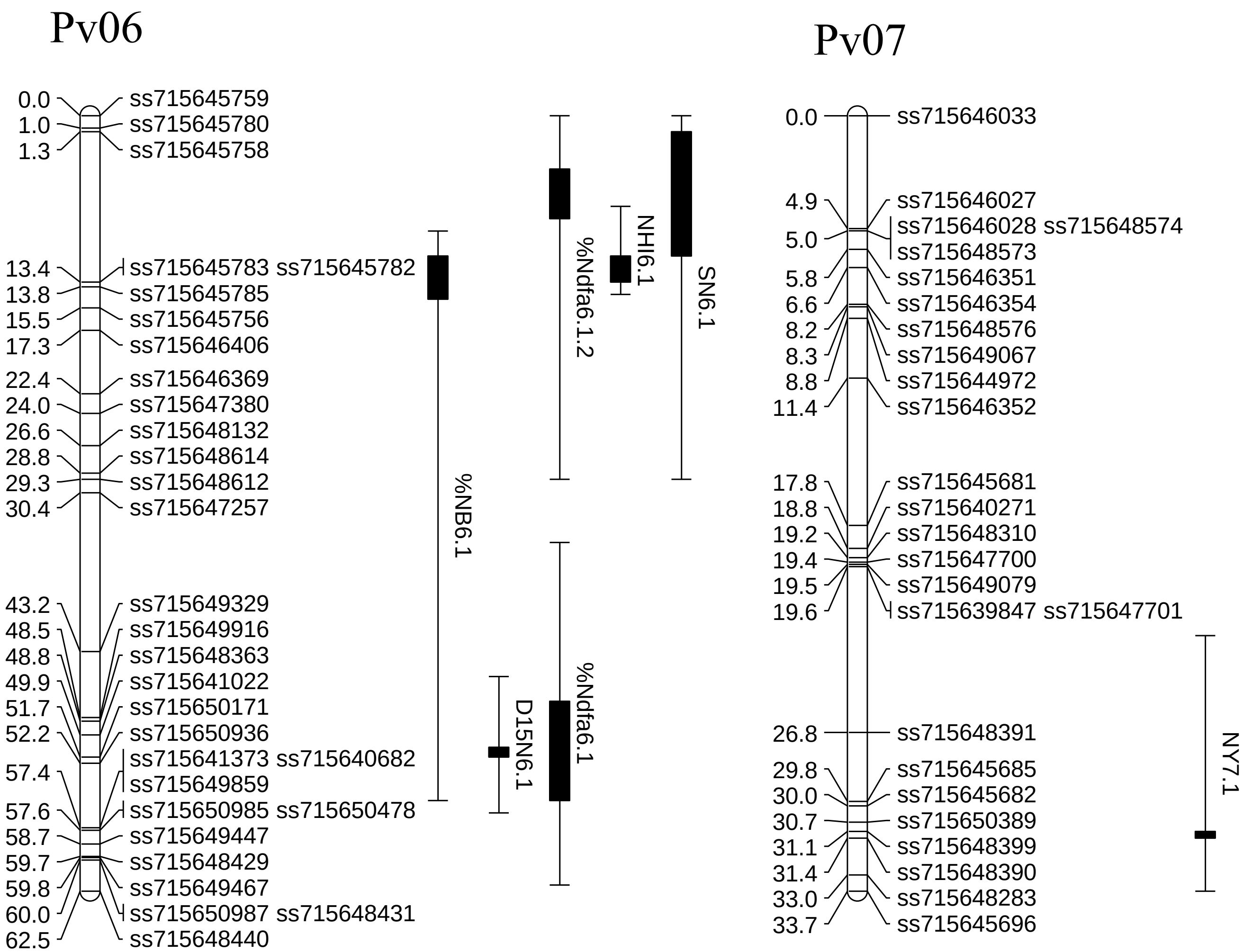


Figure 4.01 (*cont'd*)

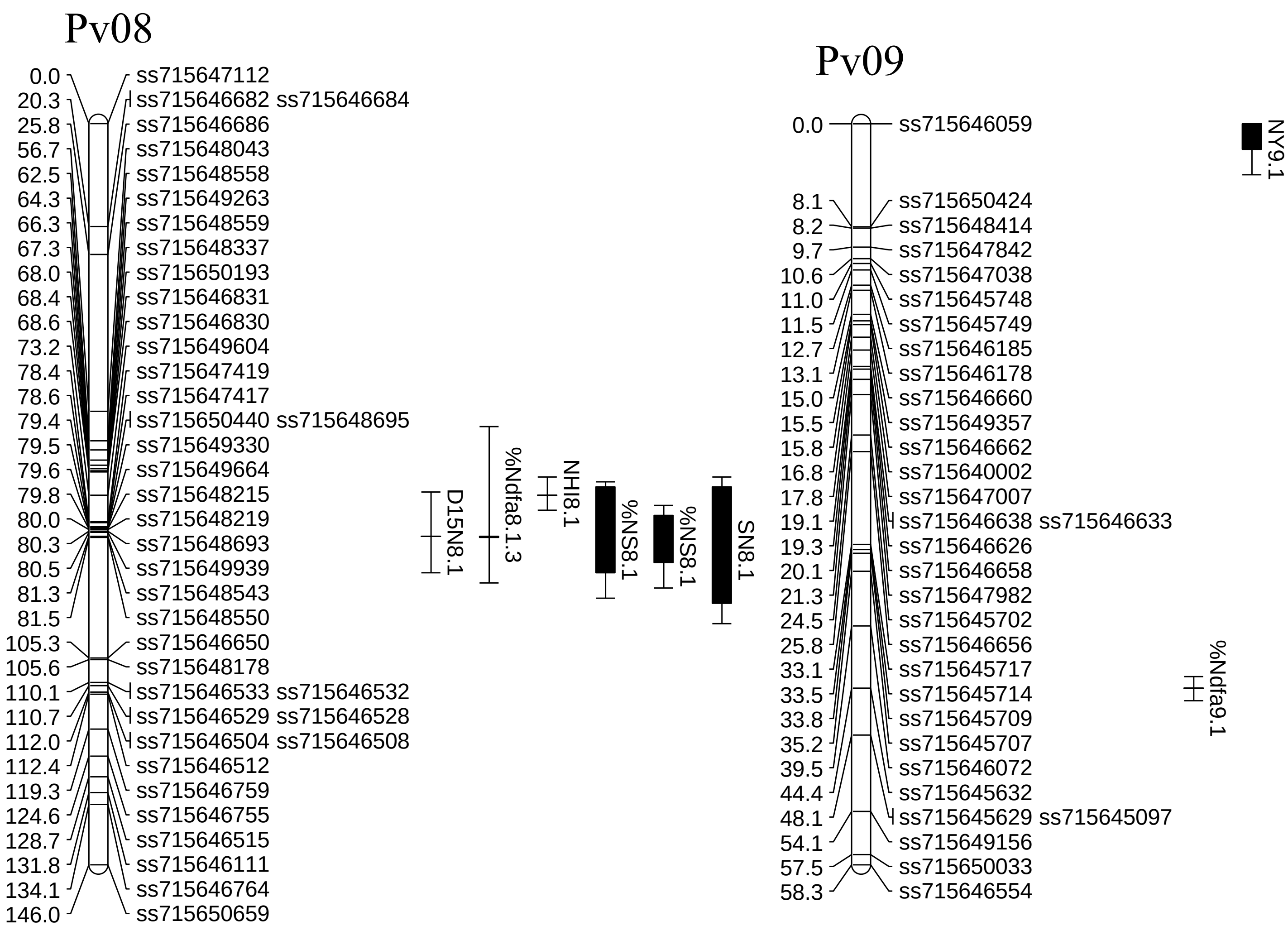
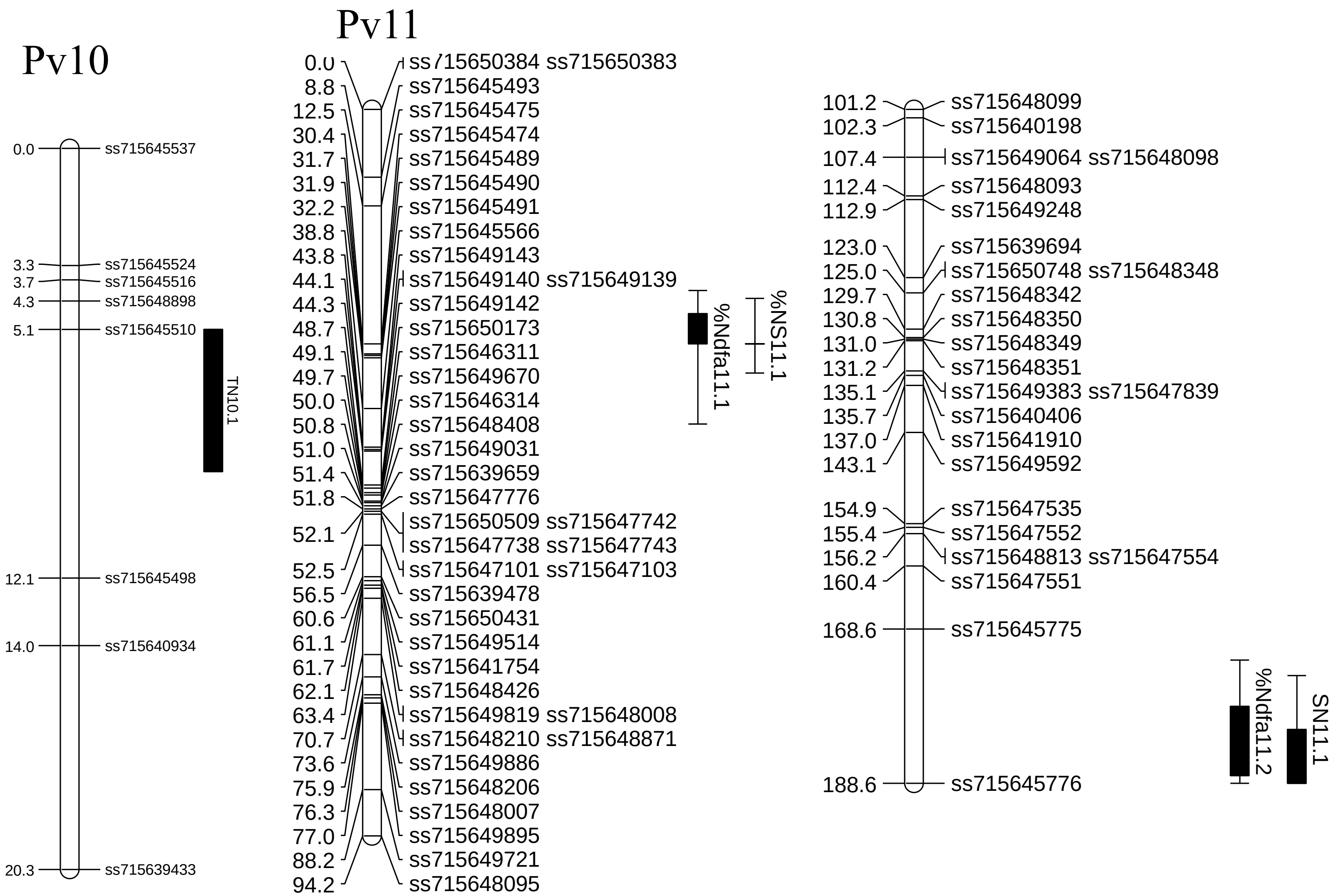


Figure 4.01 (*cont'd*)

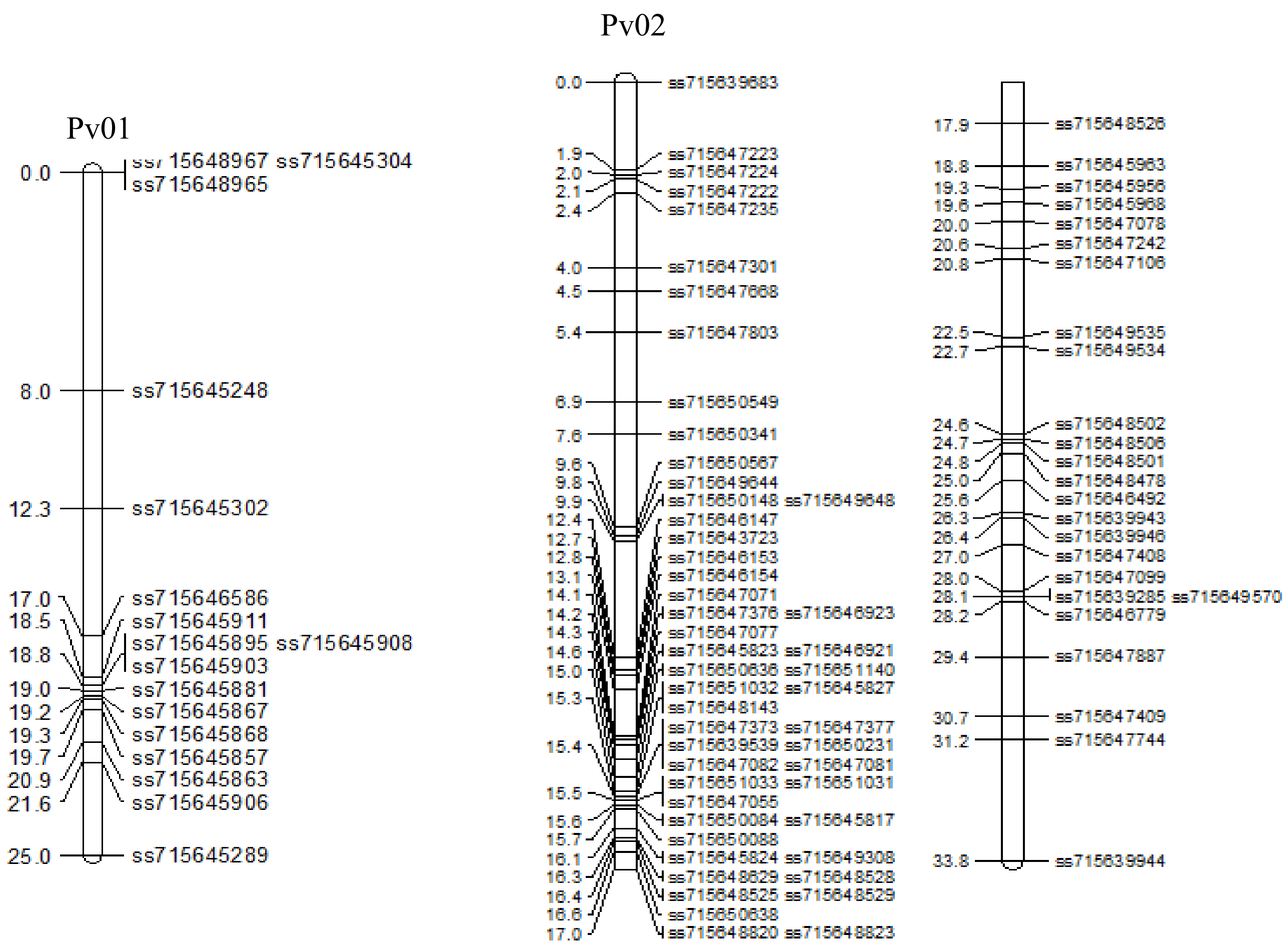


[†] For traits measured in the greenhouse: SHTN=Shoot N, SWT=Shoot Weight, BM=Total Biomass, SRR=Shoot:Root ratio, NoR=Nodule Rating, SD-Shoot Difference, TD=Total Biomass Difference. For traits measured in the field: %NB=Percent N in Biomass, D15N= δ^{15}N, %Ndfa=%Ndfa, NY=N Yield, NHI=Nitrogen Harvest Index, %NS=%N in seed, SN=Seed N, NHI=N Harvest, PN=%NS in Seed.

APPENDIX B

SUPPLEMENTAL FIGURES FOR CHAPTER 4

Figure S4.01. Linkage map of the Puebla 152/Zorro population.



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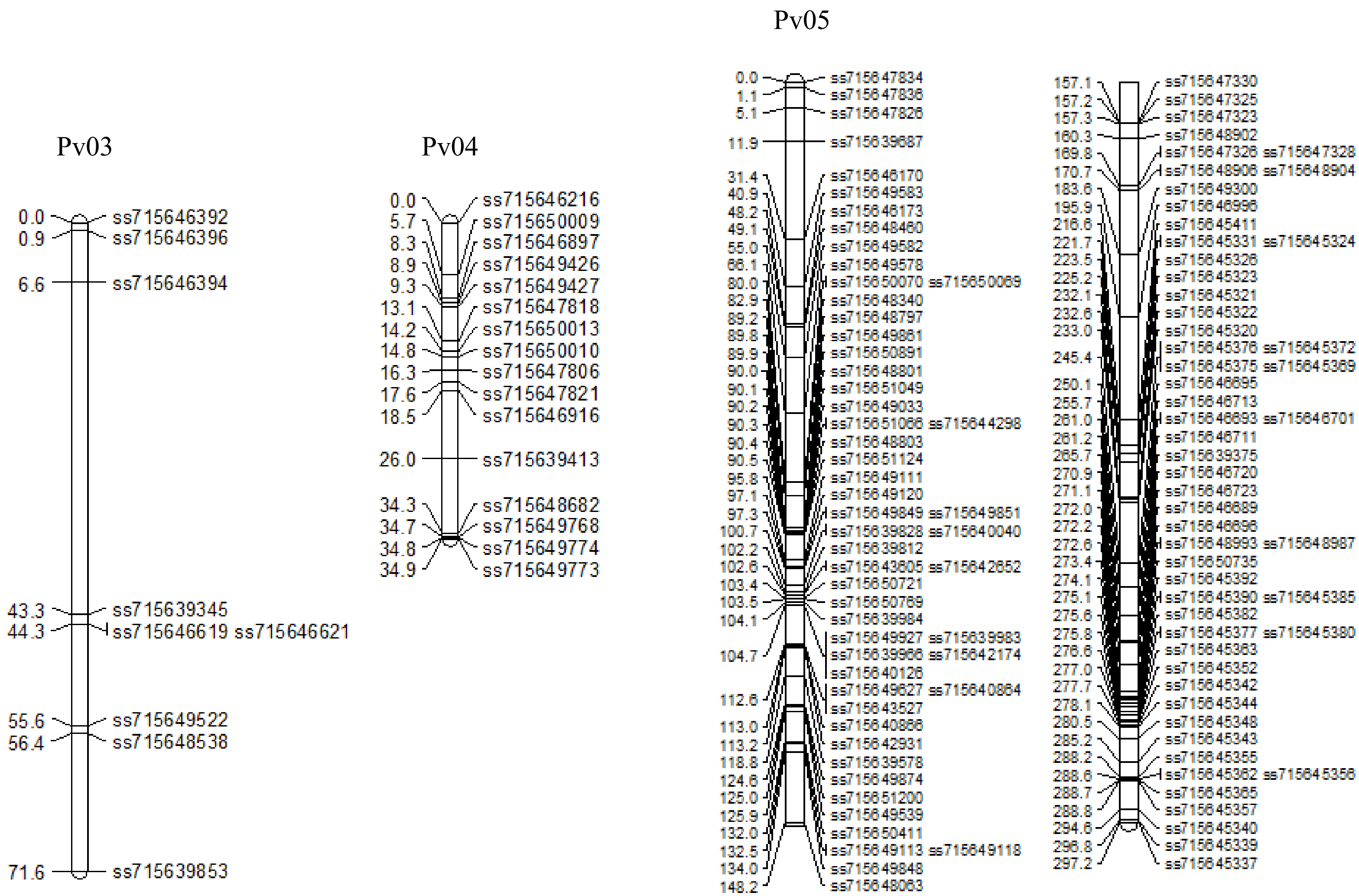


Figure S4.01 (*cont'd*)

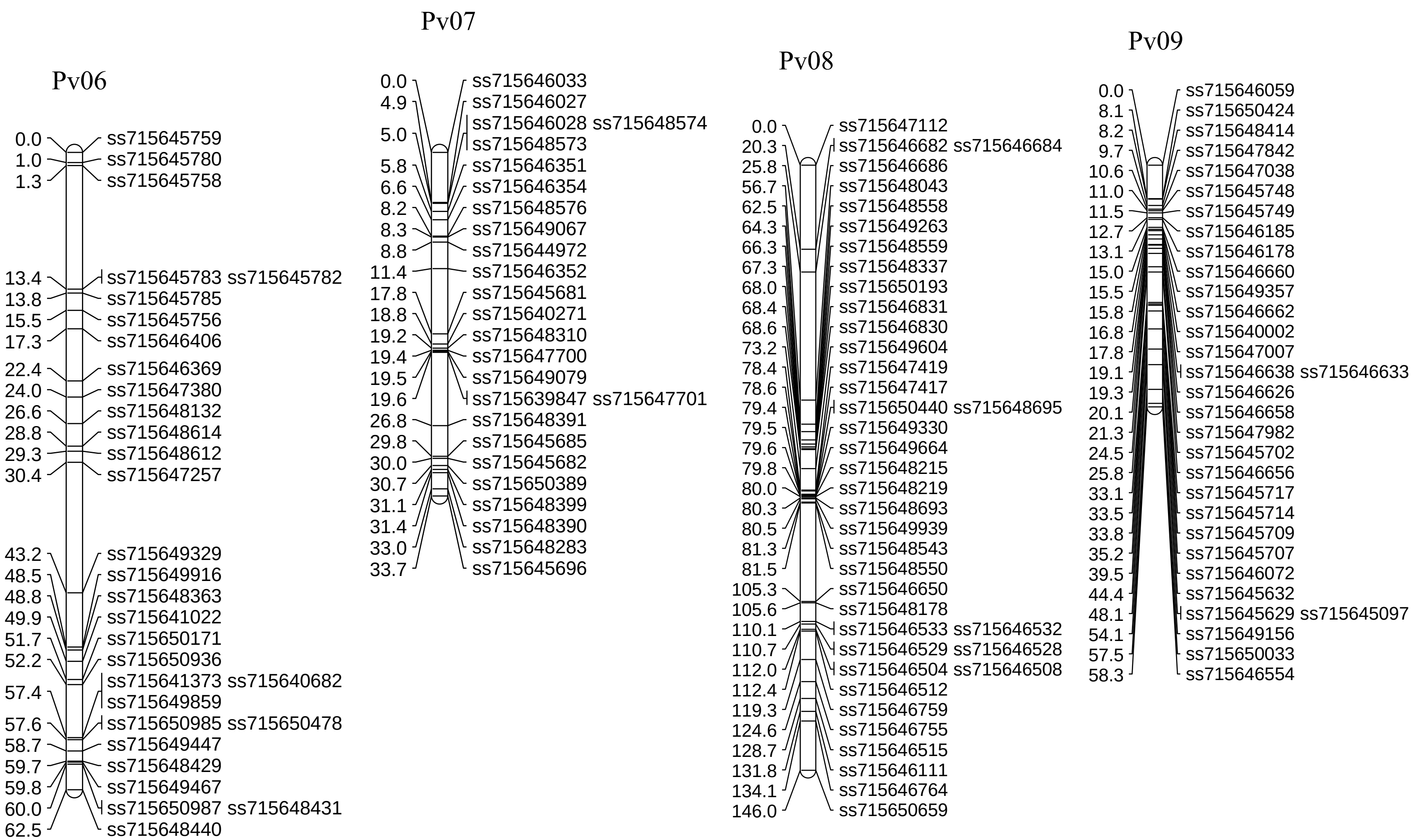
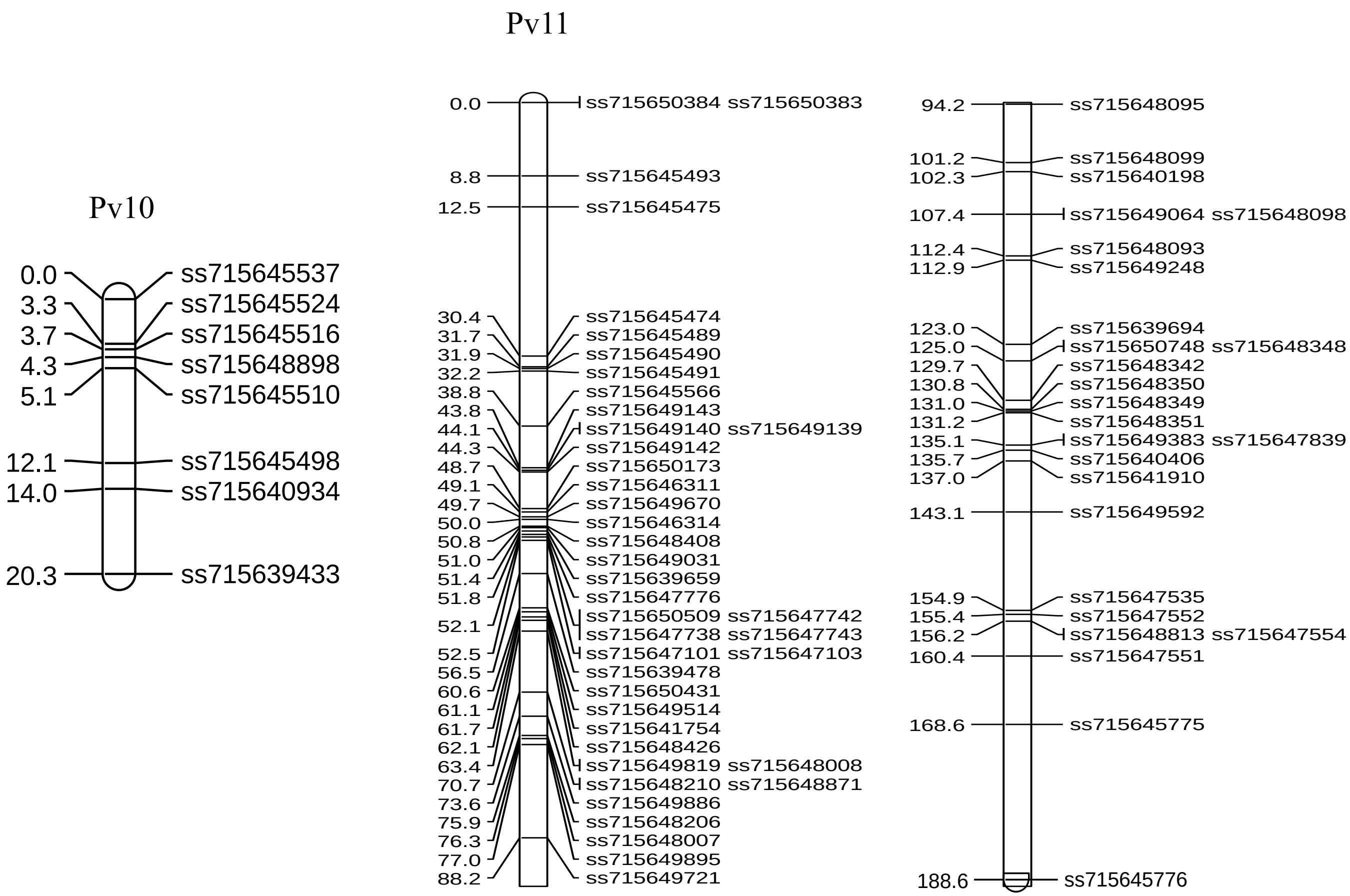


Figure S4.01 (*cont'd*)



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