Natural variation in teosinte at the domestication locus *teosinte branched1* (*tb1*)

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Abstract

The *teosinte branched1* (*tb1*) gene, a repressor of lateral organ growth, is a major QTL involved in branching differences between maize and its wild progenitor, teosinte. Further studies have shown that the insertion of a transposable element (*Hopscotch*) upstream of *tb1* enhances its expression, causing the reduction in branching observed in domesticated maize. Observations of the maize *tb1* allele in teosinte individuals, coupled with estimates of the age of insertion of the *Hopscotch* element, led us to investigate the prevalence and potential role of *tb1* in teosinte. Results from genotyping across many natural populations suggest that the *Hopscotch* element is segregating at a higher than expected frequency in a number of populations of two subspecies of teosinte, *Zea mays* ssp. *parviglumis* and *Zea mays* ssp. *mexicana*. Analysis of linkage disequilibrium between the *Hopscotch* element and variation in surrounding regions does not support a hypothesis of recent introgression from maize into teosinte, and we find no evidence of environmental correlations that might suggest recent selection. Finally, two greenhouse experiments with *Zea mays* ssp. *parviglumis* do not suggest an important role for *tb1* in controlling tillering in natural populations of this subspecies. Our findings suggest that the role of the *Hopscotch* in tillering in teosinte is not as straightforward as is in domesticated maize, and that other loci may play a role in observed variation in this trait.

INTRODUCTION

Domesticated crops and their wild progenitors provide an excellent system in which to study adaptation and genomic changes associated with human-mediated selection (Ross-Ibarra et al., 2007). Perhaps the central focus of the study of domestication has been the identification of genetic variation underlying agronomically important traits such as fruit size and plant architecture (Olsen and Gross, 2010). Additionally, many domesticates show reduced genetic diversity when compared to their wild progenitors, and an understanding of the distribution of diversity in the wild and its phenotypic effects has become increasingly useful to crop improvement (Kovach and McCouch, 2008). But while some effort has been invested into understanding how wild alleles behave in their domesticated relatives (Bai and Lindhout, 2007), very little is known about the role that alleles found most commonly in domesticates play in natural populations of their wild progenitors. (Whitton J, 1997).

Maize (*Zea mays* ssp. *mays*) was domesticated from the teosinte *Zea mays* ssp. *parviglumis* (hereafter, *parviglumis*) roughly 9,000 B.P. in southwest Mexico (Piperno et al., 2009; Matsuoka et al., 2002). Domesticated maize and the teosintes are an attractive system in which to study domestication due to the abundance of genetic tools developed for maize and well-characterized domestication loci (Hufford et al., 2012a; Doebley, 2004; Hufford et al., 2012b). Additionally, large naturally occurring populations of both *Zea mays* ssp. *parviglumis* (the wild progenitor of maize) and *Zea mays* ssp. *mexicana* (highland teosinte; hereafter *mexicana*) can be found throughout Mexico (Wilkes, 1977; Hufford et al., 2013), and genetic diversity of these taxa is estimated to be high (Ross-Ibarra et al., 2009).

Many morphological changes are associated with maize domestication, and understanding the genetic basis of these changes has been a focus of maize research for a number of years (Doebley, 2004). One of the most dramatic changes is found in plant architecture: domesticated maize is characterized by a central stalk with few tillers and

lateral branches terminating in a female inflorescence, while teosinte is highly tillered and bears tassels (male inflorescences) at the end of its lateral branches. The *teosinte branched1* (*tb1*) gene, a repressor of organ growth, was identified as a major QTL involved in domestication branching differences achieved through higher expression of the maize allele than the teosinte allele (Doebley et al., 1995, 1997). Further work showed that the insertion of a 4.9 kb retrotransposon (*Hopscotch*) in the upstream control region of *tb1* caused the increased expression of this gene reduction in branching observed in domesticated maize Studer et al. (2011). The effects of this insertion have been observed in tiller number in maize, but little is known about its role, if any, in natural populations of teosinte (Studer et al., 2011). Dating of this element has suggested that its insertion predates the domestication of maize, leading to the hypothesis that it was segregating as standing variation in ancient populations of teosinte and increased to high frequency in maize due to selection during domestication (Studer et al., 2011). Furthermore, Studer and Doebley (2012) investigated the phenotypic effects of nine teosinte *tb1* alleles in an isogenic maize background and found that the introgressions sort into three distinct phenotypic classes, suggesting that variation at the *tb1* locus may play a functional role in teosinte.

In high-density species such as teosinte, plants can detect impending competition from their neighbors through detection of the ratio of red to far-red light. An increase in far-red relative to red light accompanies shading and triggers physiological and morphological changes such as reduced tillering, increased plant height and early flowering collectively known as the shade avoidance syndrome (Kebrom and Brutnell, 2007). The *tb1* locus appears to play an important role in the shade avoidance pathway in *Zea mays* and other grasses and may therefore be crucial to the ecology of teosinte (Kebrom and Brutnell, 2007; Lukens and Doebley, 1999). In this study we aim to characterize the distribution of the *Hopscotch* insertion in *parviglumis*, *mexicana*, and landrace maize, and to examine the phenotypic effects of the insertion in *parviglumis*. We

use a combination of PCR genotyping for the *Hopscotch* element in our full panel and sequencing of two small regions upstream of *tb1* in a subset of teosinte populations to explore patterns of genetic variation at this locus. Finally, we test for an association between the *Hopscotch* element and tillering phenotypes in a population of *parviglumis*.

MATERIALS AND METHODS

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Sampling and genotyping

We sampled 1,110 individuals from 350 accessions (247 maize landraces, 17 *mexicana* populations, and 86 *parviglumis* populations) and assessed the presence or absence of the *Hopscotch* insertion (Table S1 and Table S2). DNA was extracted from leaf tissue using a modified CTAB approach (Doyle and Doyle, 1990; Maloof et al., 1984). We designed primers using PRIMER3 (Rozen and Skaletsky, 2000) implemented in Geneious (Kearse et al., 2012) to amplify the entire *Hopscotch* element, as well as an internal primer allowing us to simultaneously check for possible PCR bias between presence and absence of the *Hopscotch* insertion. Two PCRs were performed for each individual, one with primers flanking the *Hopscotch* (HopF/HopR) and one with a flanking primer and an internal primer (HopF/HopIntR). Primer sequences are HopF, 5'-TCGTTGATGCTTTGATGGATGG-3'; Hop R, 5'-AACAGTATGATTTCATGGGACCG-3'; and HopIntR, 5'-CCTCCACCTCTCATGAGATCC-3' (Fig. S1, Fig. S2) *Primers in Fig. S1 should be labeled* . Homozygotes show a single band for absence of the element (∼300bp) and two bands for presence of the element (∼5kb and XX *LV, please add the size of the second band*), whereas heterozygotes are three-banded (Fig. S2). When only one PCR resolved well, we scored one allele for the individual. We used Phusion High Fidelity Enzyme (Finnzymes, Inc.) and the following conditions for amplifications: 98°C for 3 min, 30 cycles of 98°C

for 15 s, 65°C for 30 s, and 72°C for 3 min 30 s, with a final extension of 72°C for 10 min. PCR products were visualized on a 1% agarose gel and scored for presence/absence of the *Hopscotch* based on band size.

Sequencing

In addition to genotyping, we chose a subset of *parviglumis* individuals for sequencing. We chose twelve individuals from each of four populations from Jalisco state, Mexico (San Lorenzo, La Mesa, Ejutla A, and Ejutla B). For amplification and sequencing, we selected two regions approximately 600bp in size from within the 5' UTR of *tb1* (Region 1) and from 1,235bp upstream of the start of the *Hopscotch* (66,169bp upstream from the start of the *tb1* ORF; Region 2). We designed the following primers using PRIMER3 (Rozen and Skaletsky, 2000): for the 5' UTR, 5-'GGATAATGTGCACCAGGTGT-3' and 5'-GCGTGCTAGAGACACYTGTTGCT-3'; for the 50kb upstream region, 5'-TGTCCTCGCCGCAACTC-3' and 5'-TGTACGCCCGCCCCTCATCA-3' (Fig. S1). We used Taq polymerase (New England Biolabs) and the following thermal cycler conditions to amplify fragments: 94°C for 3 min, 30 cycles of 92°C for 40 s, annealing for 1 min, 72°C for 40 s, and a final 10 min extension at 72°C. Annealing temperatures for sequenced region 1 and sequenced region 2 were 59.7°C and 58.8°C, respectively. To clean excess primer and dNTPs we added two units of Exonuclease1 and 2.5 units of Antarctic Phosphatase to 8.0 μL of amplification product. This mix was placed on a thermal cycler with the following program: 37°C for 30 min, 80°C for 15 min, and a final cool-down step to 4°C.

We cloned cleaned fragments into a TOPO-TA vector (Invitrogen, Carlsbad) using OneShot TOP10 chemically competent *E. coli* cells, with an extended ligation time of 30 min for a complex target fragment. We plated cells on LB agar plates containing kanamycin, and screened colonies using vector primers M13 Forward and M13 Reverse under the following conditions: 96°C for 5 min; then 35 cycles at 96°C for 30 s, 53°C for

30 s, 72°C for 2 min; and a final extension at 72°C for 4 min. We visualized amplification products for incorporation of our insert on a 1% agarose TAE gel.

Amplification products with successful incorporation of our insert were cleaned using Exonuclease 1 and Antarctic Phosphatase following the procedures detailed above, and sequenced with vector primers M13 Forward and M13 Reverse using Sanger sequencing at the College of Agriculture and Environmental Sciences (CAES) sequencing center at UC Davis. We aligned and trimmed primer sequences from resulting sequences using the software Geneious (Kearse et al., 2012). Following alignment, we verified singleton SNPs by sequencing an additional one to four colonies from each clone. If the singleton was not present in these additional sequences it was considered an amplification or cloning error, and we replaced the base with the base of the additional sequences. If the singleton appeared in at least one of the additional sequences we considered it a real variant and kept it for further analyses.

Genotyping analysis

We examined discrepancies between observed and expected genotype frequencies by calculating Hardy-Weinberg Equilibrium (HWE). To calculate differentiation between populations (F_{ST}) and subspecies (F_{CT}) we used HierFstat (Goudet, 2005). These analyses only included populations in which 8 or more individuals were sampled. To test the hypothesis that the *Hopscotch* insertion may be adaptive under certain environmental conditions, we looked for significant associations between the *Hopscotch* frequency and environmental variables using BayEnv (Coop et al., 2010). BayEnv creates a covariance matrix of relatedness between populations and then tests a null model that allele frequencies in populations are determined by the covariance matrix of relatedness alone against the alternative model that allele frequencies are determined by a combination of the covariance matrix and an environmental variable, producing a posterior probability (*i.e.,* Bayes Factor; Coop et al. 2010). We used genotyping and covariance data from

Pyhäjärvi et al. (2013) for BayEnv, with the *Hopscotch* insertion coded as an additional SNP (Table S3). Environmental data were obtained from `www.worldclim.org`, the Harmonized World Soil Database (FAO/IIASA/ISRIC/ISSCAS/JRC, 2012) and `www.harvestchoice.org` and summarized by principle component analysis (Pyhäjärvi et al., 2013).

Sequence analysis

For population genetic analyses of sequenced Region 1 and sequenced Region 2 we used the analysis package of Libsequence (Thornton, 2003) to calculate pairwise F_{ST} between populations and to calculate standard diversity statistics (number of haplotypes, haplotype diversity, Watterson's estimator $\hat{\theta}_W$, pairwise nucleotide diversity $\hat{\theta}_\pi$, and Tajima's D). To produce a visual representation of differentiation between sequences and to examine patterns in sequence clustering by *Hopscotch* genotype we used Phylip (`http://evolution.genetics.washington.edu/phylip.html`) to create neighbor-joining trees with bootstrapping (100 repetitions) to examine the support of nodes in our trees. For creation of trees we also included homologous sequence data from teosinte inbred lines (TILs), some of which are known to be homozygous for the *Hopscotch* insertion (TIL03, TIL17, TIL09), as well as 59 lines of domesticated maize and landraces (data from Maize HapMapV2, Chia et al. 2012).

Introgression analysis

In order to assess patterns of linkage disequilibrium (LD) around the *Hopscotch* element in the context of chromosomal patterns of LD we used Tassel (Bradbury et al., 2007) and calculated LD between SNPs across chromosome 1 using previously published data from twelve plants each of the Ejutla A (EjuA), Ejutla B (EjuB), San Lorenzo (SLO), and La Mesa (MSA) populations (Pyhäjärvi et al., 2013). We chose these populations because we had both genotyping data for the *Hopscotch* as well as chromosome-wide SNP data for

chromosome 1. For each population we filtered the initial set of 5,897 SNPs on chromosome 1 to accept only SNPs with a minor allele frequency of at least 0.1, resulting in 1,671, 3,023, 3,122, and 2,167 SNPs for SLO, EjuB, EjuA, and MSA, respectively. We then used Tassel (Bradbury et al., 2007) to calculate linkage disequilibrium (r^2) across chromosome 1 for each population.

We examined evidence of introgression on chromosome 1 in these same four populations (EjuA, EjuB, MSA, SLO) using STRUCTURE (Falush et al., 2003) and the same phased 55K SNP data from (Pyhäjärvi et al., 2013) that we used for LD analysis, combined with the corresponding SNP data from a diverse panel of 282 maize lines (Cook et al., 2012). SNPs were anchored in a modified version of the IBM genetic map (Gerke et al., 2013). We created haplotype blocks using a custom Perl script that grouped SNPs separated by less than 5kb into haplotypes. We ran STRUCTURE at K=2 under the linkage model, performing 3 replicates with an MCMC burn-in of 10,000 steps and 50,000 steps post burn-in.

Phenotyping of *parviglumis*

To investigate the phenotypic effects of the *Hopscotch* insertion in teosinte, we conducted an initial phenotyping trial (Phenotyping 1). We germinated 250 seeds of *parviglumis* collected in Jalisco state, Mexico (population San Lorenzo) (Hufford, 2010) where the *Hopscotch* is segregating at highest frequency (0.44) in our initial genotyping sample set. In order to maximize the likelihood of finding the *Hopscotch* in our association population we selected seeds from sites where genotyped individuals were homozygous or heterozygous for the insertion. We chose between 10-13 seeds from each of 23 sampling sites. We treated seeds with fungicide and germinated them in petri dishes with filter paper. Following germination, 206 successful germinations were then planted into one-gallon pots with potting soil and randomly spaced one foot apart on greenhouse benches. Plants were watered three times a day by hand and with an automatic drip

containing 10-20-10 fertilizer.

Starting on day 15, we measured tillering index as the ratio of the sum of tiller lengths to the height of the plant (Briggs et al., 2007). Following initial measurements, we phenotyped plants for tillering index every 5 days through day 40, and then on day 50 and day 60. On day 65 we measured culm diameter between the third and fourth nodes of each plant. Culm diameter is not believed to be correlated with tillering index or variation at *tb1*. Following phenotyping we extracted DNA from all plants using a modified SDS extraction protocol (`http://www.ars.usda.gov`). We genotyped individuals for the *Hopscotch* insertion following the protocols listed above. Based on these initial data, we conducted a post hoc power analysis using data from day 40 of Phenotyping 1, indicating that a minimum of 71 individuals in each genotypic class are needed to detect the observed effect of the *Hopscotch* on tillering index.

We performed a second phenotyping experiment (Phenotyping 2) in which we germinated 372 seeds of *parviglumis*, choosing equally between sites previously determined to have or not have the *Hopscotch* insertion. Seeds were germinated and planted on day 7 post fruit-case removal into two gallon pots. Plants were watered twice daily, alternating between fertilized and non-fertilized water. We began phenotyping successful germinations (302) for tillering index on day 15 post fruit-case removal, and phenotyped every five days until day 50. At day 50 we measured culm diameter between the third and fourth nodes. We extracted DNA and genotyped plants following the same guidelines as in Phenotyping 1.

Resulting tillering index data for each genotypic class did not meet the criteria for a repeated measures ANOVA, so we transformed the data using a Box-Cox transformation ($\alpha = 0$) *what is the alpha value here?* implemented in the car package in R (Fox and Weisberg, 2011) to improve the normality and homogeneity of variance among genotype classes. We analyzed relationships between genotype and tillering index and tiller number using a repeated measures ANOVA through a general linear model function implemented in SAS

v.9.3 (SAS Institute Inc., Cary, NC, USA). Additionally, in order to compare any association between *Hopscotch* genotype and tillering and associations at other presumably unrelated traits, we performed an ANOVA between culm diameter and genotype using the same general linear model in SAS.

RESULTS

Genotyping

Genotype of the *Hopscotch* insertion was confirmed with two PCRs for 837 individuals. Among the 247 maize landrace accessions genotyped, all but eight were homozygous for the presence of the insertion (Table S1 and Table S2). Within our *parviglumis* and *mexicana* samples we found the *Hopscotch* insertion segregating in 37 and 4 populations, respectively, and at highest frequency in the states of Jalisco, Colima, and Michoacán in central-western Mexico (Fig. 1). We examined Hardy-Weinberg equilibrium in a total of 14 populations (10 *parviglumis* and 4 *mexicana*) with more than 8 individuals sampled per population. Three populations (RIMPA0073, RIMPA0093, and RIMPA0158) show evidence of deviations from expected genotype frequencies under the assumptions of HWE ($p<0.05$). *Jeff mentioned this needs to be fully reported in a supplementary table; alternatively we can just delete the result since we do not interpret or use it later in the manuscript.*

Using our *Hopscotch* genotyping, we calculated differentiation between populations (F_{ST}) and subspecies (F_{CT}) for populations in which we sampled 8 or more alleles. We found that $F_{CT} = 0$ within our dataset and levels of F_{ST} among populations within each subspecies (0.22) *is this an average? this comes from libsequence?* and among all populations (0.23) are similar to those reported genome-wide in previous studies (Pyhäjärvi et al. 2013; Table 1). Although we found large variation in *Hopscotch* allele frequency among our populations, BayEnv analysis did not indicate a correlation between the *Hopscotch* insertion and environmental variables (all Bayes Factors < 1; Table S3).

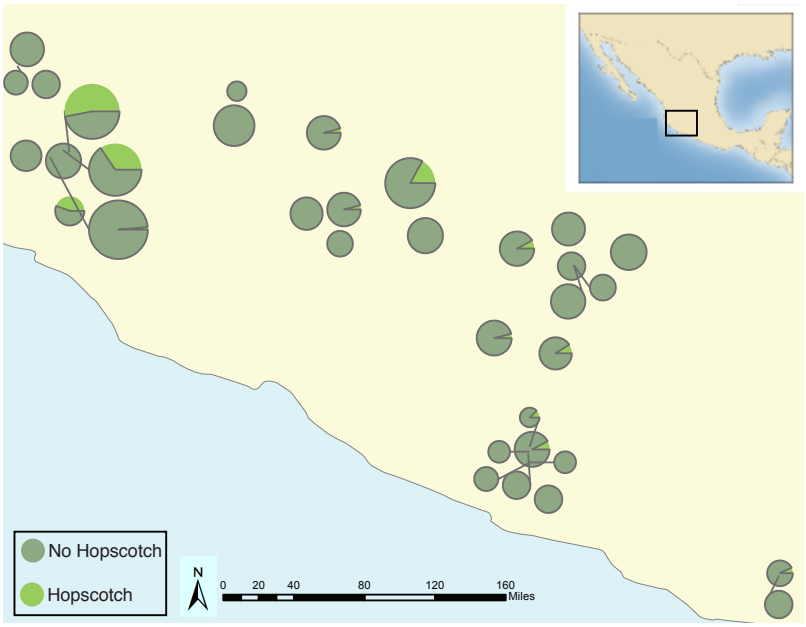

Figure 1: Map showing the frequency of the *Hopscotch* allele in populations of *parviglumis* where we sampled more than 6 individuals. Size of circles reflects number of alleles sampled.

Sequencing

To investigate patterns of sequence diversity and linkage disequilibrium (LD) in the *tb1* region, we sequenced two small (<1kb) regions upstream of the *tb1* ORF in four populations. After alignment and singleton checking we recovered 48 and 40 segregating sites for the 5' UTR region (Region 1) and the 66kb upstream region (Region 2), respectively. For Region 1, Ejutla A has the highest values of haplotype diversity, and θ_π, while Ejutla B and La Mesa have comparable values of these summary statistics, and San Lorenzo has much lower values. Additionally, Tajima's D is strongly negative in the two Ejutla populations and La Mesa, but is less negative in San Lorenzo (Table 2). *need to reference* Hopscotch *frequencies in supplemental table somewhere* For Region 2, haplotype diversity and θ_π, are similar for Ejutla A and Ejutla B, while La Mesa and San Lorenzo have slightly lower values for these statistics (Table 2). Tajima's D is positive in all populations except San Lorenzo, *is the table wrong? MSA is the only negative value in the table* indicating an excess of low frequency variants in this population (Table 2). Pairwise values of F_{ST} within population pairs Ejutla A/Ejutla B and San Lorenzo/La Mesa are 0 for both sequenced

Table 1: Pairwise F_{ST} values from sequence and *Hopscotch* genotyping data

Comparison	**Region 1**	**Region 2**	***Hopscotch***
EjuA & EjuB	0	0	0
EjuA & MSA	0.326	0.328	0.186
EjuA & SLO	0.416	0.258	0.280
EjuB & MSA	0.397	0.365	0.188
EjuB & SLO	0.512	0.290	0.280
MSA & SLO	0.007	0	0.016

regions as well as for the *Hopscotch*, while they are high for other population pairs (Table 1). Neighbor joining trees of our sequence data and data from the teosinte inbred lines (TILs; data from Maize HapMapV2, Chia et al. 2012) do not reveal any clear clustering pattern with respect to population or *Hopscotch* genotype (Figure S3); individuals within our sample that have the *Hopscotch* insertion do not group with the teosinte inbred lines or the lines of domesticated maize that have the *Hopscotch* insertion.

Evidence of introgression

The highest frequency of the *Hopscotch* insertion in teosinte was found in *parviglumis* sympatric with cultivated maize. Our initial hypothesis was that the high frequency of the *Hopscotch* element in these populations could be attributed to introgression from maize into teosinte. To investigate this possibility we examined overall patterns of linkage disequilibrium across chromosome one and specifically in the *tb1* region. If the *Hopscotch* is found in these populations due to recent introgression we would expect to find large blocks of linked markers near this element. We find no evidence of elevated linkage disequilibrium between the *Hopscotch* and SNPs surrounding the *tb1* region in our resequenced populations (Fig. 2), and r^2 in the *tb1* region does not differ significantly

Table 2: Population genetic statistics from resequenced regions near the *tb1* locus

Population	**# Haplotypes**	**Hap. Diversity**	$\hat{\theta}_\pi$	**Tajima's D**
Region 1(5' UTR)				
EJUA	8	0.859	0.005	-1.650
EJUB	5	0.709	0.004	-1.831
MSA	6	0.682	0.004	-1.755
SLO	3	0.318	0.001	-0.729
Region 2 (66kb upstream)				
EJUA	8	0.894	0.018	0.623
EJUB	8	0.894	0.016	0.295
MSA	3	0.682	0.011	-0.222
SLO	4	0.742	0.014	0.932

between populations with (average r^2 of 0.085) and without (average $r^2 = 0.082$) the *Hopscotch* genotype. In fact, average r^2 is lower in the *tb1* region ($r^2 = 0.056$) than across the rest of chromosome 1 ($r^2 = 0.083$) (3). *LV, please go through and make sure the data entered into all the tables is correct. In Table3, both sequenced regions were labeled as "Region 1". I changed the second to Region 2 but don't know if the data in this column are really from Region 2*

The lack of clustering of *Hopscotch* genotypes in our NJ tree as well as the lack of LD around *tb1* does not support the hypothesis that the *Hopscotch* insertion in these populations of *parviglumis* is the result of recent introgression. However, to further explore this hypothesis we performed a STRUCTURE analysis using Illumina MaizeSNP50 data from four of our *parviglumis* populations (EjuA, EjuB, MSA, and SLO) and the maize 282 diversity panel (Cook et al., 2012; Pyhäjärvi et al., 2013). The linkage model implemented in STRUCTURE can be used to identify ancestry of blocks of linked variants, which would arise as a result of recent admixture between populations. If

Table 3: r^2 values between SNPs on chromosome 1, in the broad *tb1* region, within the 5' UTR of *tb1* (Region 1), and 66kb upstream of *tb1* (Region 2).

Population	**Chr. 1**	***tb1* region**	**Region 1**	**Region 2**
Ejutla A	0.095	0.050	0.747	0.215
Ejutla B	0.069	0.051	0.660	0.186
La Mesa	0.070	0.053	0.914	0.766
San Lorenzo	0.101	0.067	0.912	0.636

the *Hopscotch* insertion is present in populations of *parviglumis* as a result of recent admixture with domesticated maize, we would expect the insertion and linked variants in surrounding sites to be assigned to the "maize" cluster in our STRUCTURE runs, not the "teosinte" cluster. In all runs, assignment to maize in the *tb1* region across all four *parviglumis* populations is low (average 0.017) and much below the chromosome-wide average (0.20; Table 4; Fig. 3).

Table 4: Assignments to maize and teosinte in the *tb1* and chromosome 1 regions from STRUCTURE

	***tb1* region**		**Chr 1**	
Population	**Maize**	**Teosinte**	**Maize**	**Teosinte**
Ejutla A	0.022	0.978	0.203	0.797
Ejutla B	0.019	0.981	0.187	0.813
La Mesa	0.012	0.988	0.193	0.807
San Lorenzo	0.016	0.984	0.205	0.795

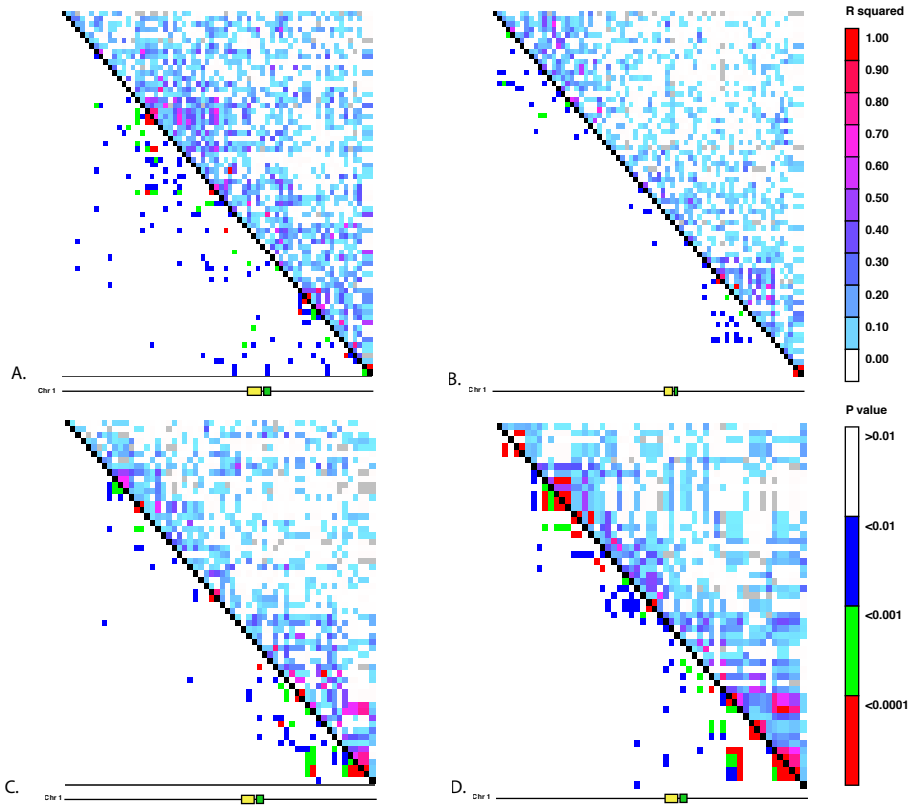

Figure 2: Linkage disequilibrium for SNPs in Mb 261-268 on chromosome 1. The yellow rectangle indicates the location of the *Hopscotch* insertion and the green represents the *tb1* ORF. A) Ejutla A; B) Ejutla B; C) La Mesa; D). San Lorenzo

Phenotyping

To assess the contribution of *tb1* to phenotypic variation in tillering in a natural population, we grew plants from seed sampled from the San Lorenzo population of *parviglumis*, which had a high mean frequency (0.44) of the *Hopscotch* insertion based on our initial genotyping. We measured tillering index (TI), the ratio of the sum of tiller lengths to plant height, for 216 plants (Phenotyping 1) from within the San Lorenzo population, and genotyped plants for the *Hopscotch* insertion. We found the *Hopscotch*

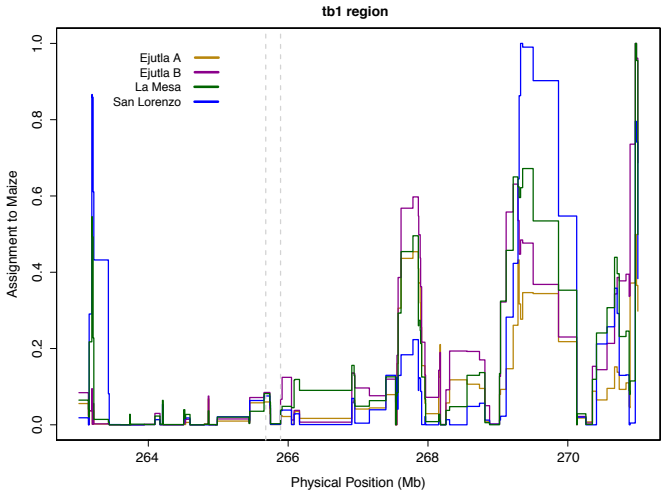

Figure 3: STRUCTURE assignment to maize across a section of chromosome 1. The dotted lines mark the beginning of the sequenced region 50kb upstream (Sequenced region 2) and the end of the *tb1* ORF.

segregating at a frequency of 0.65 with no significant deviations from expected frequencies under Hardy-Weinberg equilibrium. After performing a repeated measures ANOVA between our transformed tillering index data and *Hopscotch* genotype we find a weak positive correlation between presence of the *Hopscotch* and tillering index on day 40 (p=0.0848), a result indicating the *Hopscotch* may actually increase tillering in *parviglumis* in contrast to its phenotypic effect in maize. We find no correlation between tillering index and genotype on any other day (4). Additionally we find no significant correlation between tiller number and *Hopscotch* genotype, or culm diameter and *Hopscotch* genotype in Phenotyping 1.

We performed a second grow-out of *parviglumis* from San Lorenzo (Phenotyping 2) to assess whether lighting conditions or sample size may have affected our ability to detect an effect of *tb1*. For the second grow-out we measured tillering index every five

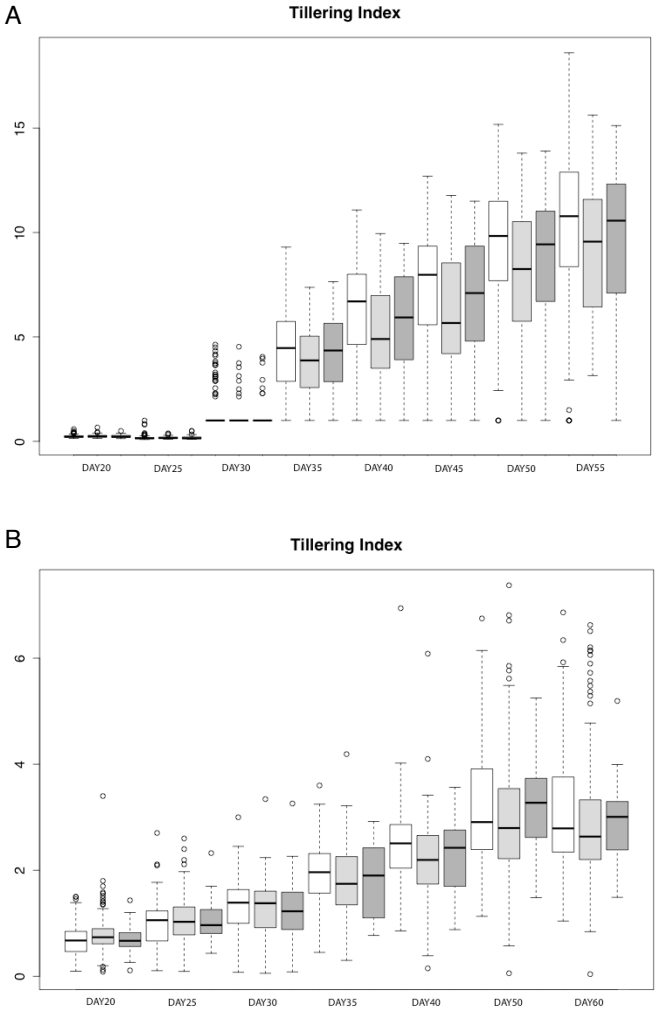

Figure 4: Box-plots showing tillering index in greenhouse grow-outs for Phenotyping 1 (A) and Phenotyping 2 (B). White indicates individuals homozygous for the *Hopscotch*, light grey represents heterozygotes, and dark grey represents homozygotes for the teosinte (No *Hopscotch*) allele. Within boxes, dark black lines represent the median, and the edges of the boxes are the first and third quartiles. *please explain whiskers and dots on figure too.*

days through day 50 for 302 plants. We found the *Hopscotch* allele segregating at a frequency of 0.69, *is it in HWE in this pop?* with a 0.6 frequency of *Hopscotch* homozygotes, and a 0.2 frequency of both heterozygotes and homozygotes for the teosinte allele. We found similar patterns, with a weak positive correlation between tillering index and *Hopscotch* genotype at day 40 ($p<0.0611$), with no significant correlation on any day. Similarly, relationships between *Hopscotch* genotype and tiller number and *Hopscotch* genotype and culm diameter were not significant.

DISCUSSION

Adaptation occurs either due to selection on standing variation or on *de novo* mutations. Adaptation as a result of selection on standing variation has been well-described in a number of systems; for example, selection for lactose tolerance in humans (Plantinga et al., 2012; Tishkoff et al., 2007), variation at the *Eda* locus in three-spined stickleback (Kitano et al., 2008; Colosimo et al., 2005), and pupal diapause in the Apple Maggot fly (Feder et al., 2003). Although the role of standing variation with respect to adaptation has been described in many systems, its importance to domestication is not as well studied.

In maize, alleles at important domestication loci (*RAMOSA1*, Sigmon and Vollbrecht 2010; *barren stalk1*, Gallavotti et al. 2004; and *grassy tillers1*, Whipple et al. 2011) have been shown to have been selected from standing variation, suggesting that diversity already present in teosinte may have played an important role in the domestication of maize. The *teosinte branched1* gene has long been a central focus of research concerning maize domestication, and, while previous studies have suggested that differences in plant architecture between domesticated maize and teosinte are a result of selection on standing variation, little is known about variation at this locus in teosinte (Clark et al., 2006; Studer et al., 2011). Studer et al. (2011) genotyped 90 accessions of teosinte (inbred and outbred), providing the first evidence that the *Hopscotch* insertion is segregating in

teosinte (Studer et al., 2011).

Given that the *Hopscotch* insertion has been estimated to predate the domestication of maize, it is not surprising that it can be found segregating in populations of teosinte. However, in sampling numerous individuals from many teosinte populations our study provides greater insight into the distribution and prevalence of the *Hopscotch* in teosinte. While our findings are consistent with Studer et al. (2011) in that we identified the *Hopscotch* allele segregating in teosinte, we find it at higher frequency than previously suggested (Studer et al., 2011). Many of our populations with high frequency of the *Hopscotch* allele fall in the Jalisco cluster identified by Fukunaga (2005), possibly suggesting a different history of the *tb1* locus in this region than in the Balsas River Basin where maize was domesticated (Matsuoka et al., 2002). Potential explanations for the high frequency of the *Hopscotch* element in *parviglumis* from Jalisco include gene flow from maize, genetic drift, and natural selection.

While gene flow from crops into their wild relatives is well-known, (Ellstrand et al., 1999; Zhang et al., 2009; Thurber et al., 2010; Baack et al., 2008; Hubner et al., 2012; Wilkes, 1977; van Heerwaarden et al., 2011; Barrett, 1983), our results are more consistent with Hufford et al. (2013) who found resistance to introgression from maize into teosinte (Hufford et al., 2013). Furthermore, Hufford et al. (2013) showed that domestication loci, such as *tb1*, are particularly resistant to introgression in both directions of gene flow (i.e., maize to teosinte and teosinte to maize; Hufford et al. 2013). We find no evidence of recent introgression in our analyses. Clustering patterns in our NJ trees do not reflect a pattern expected if maize alleles at the *tb1* locus had introgressed into populations of teosinte. Moreover, analysis of linkage in the *tb1* region does not reveal patterns of high LD relative to the rest of chromosome 1, and assignment to maize in this region in our STRUCTURE analysis is lower than the average across chromosome 1 (Fig. 3, Table 4). Together, these data point to an explanation other than recent introgression for the high observed frequency of *Hopscotch* in some of our *parviglumis* populations.

Although recent introgression seems unlikely, we cannot rule out ancient introgression as an explanation for the presence of the *Hopscotch* in these populations. If the *Hopscotch* allele was introgressed in the distant past, recombination may have broken up LD, a pattern that would be consistent with our data. We find this scenario less plausible, however, as there is no reason why gene flow should have been high in the past but absent in present-day sympatric populations. In fact, early generation maize-teosinte hybrids are easy to find in these populations today (MB Hufford, pers. observation), and genetic data support ongoing gene flow between domesticated maize and both *Zea mays* ssp. *mexicana* and *Zea mays* ssp. *parviglumis* in a number of sympatric populations (Hufford et al., 2013; Ellstrand et al., 2007; van Heerwaarden et al., 2011).

Remaining explanations for differential frequencies of the *Hopscotch* among teosinte populations include both genetic drift and natural selection. Drift may have played a role in the San Lorenzo *parviglumis* population. Previous studies using both SSRs and genome-wide SNP data have found evidence for a population bottleneck in the San Lorenzo population (Hufford, 2010; Pyhäjärvi et al., 2013), and the lower levels of sequence diversity in the 5' UTR region and the more positive values of Tajima's D we present here are consistent with these findings. *deviations from HWE may be consistent too if we see excess of homozygotes. do we?* Such population bottlenecks can exaggerate the effects of genetic drift through which the *Hopscotch* allele at *tb1* could have risen to high frequency entirely by chance. This bottleneck, however, does not explain the high frequency of the *Hopscotch* allele in multiple populations in the Jalisco group. Moreover, available information on diversity and population structure among additional Jaliscan populations (Hufford, 2010; Pyhäjärvi et al., 2013) is not suggestive of recent colonization or other demographic events that might predict a high frequency of the allele across populations. Values of the Tajima's D statistic in the 5' UTR of *tb1* are suggestive of natural selection acting upon the gene in natural population of *parviglumis*. Whereas the genome-wide average of Tajima's D in genic regions of *parviglumis* is 0.45 (Hufford et al., 2012b), the

statistic is quite negative in the 5' UTR of *tb1* (Table 2). This result is consistent with repeated selective sweeps near *tb1* and an important ecological role for the gene in *parviglumis*.

do we know the Hop genotype for sequenced lines? can we separate the sequences into hop/no hop and look for differences? it wasn't until we did this that gt1 stuff really popped out. we should know for some of them, i will check I've added a few sentences on selection. Do we still want to compare sequences with and without Hopscotch*? I agree its a good idea and could end up being really interesting. Perhaps something we could look at after submission and incorporate during revisions?*

Significant effects of the *Hopscotch* insertion on lateral branch length, number of cupules, and tillering index in domesticated maize have been well documented (Clark et al., 2006; Studer et al., 2011). Weber et al. (2007) have described significant phenotypic associations between genic markers in *tb1* and lateral branch length and female ear length within a sample from 74 natural populations of *parviglumis* (Weber et al., 2007); these data did not include markers from the *Hopscotch* region 66kb upstream of *tb1*. Our study is the first to explicitly examine the phenotypic effects of the *Hopscotch* insertion in individuals sampled from natural populations of teosinte. We have found no significant effect of the *Hopscotch* insertion on tillering index or tiller number in our phenotyping experiments, a result that is discordant with its clear phenotypic effects in maize. One interpretation of this result would be that the *Hopscotch* controls tillering in maize (Studer et al., 2011), but tillering in teosinte is affected by variation at other loci. In fact, *tb1* is believed to be a single gene in a complex pathway controlling branching, tillering and other phenotypic traits (Kebrom and Brutnell, 2007).

MBH todo Variation at *tb1* has also been shown to contribute to phenotypes other than tillering (Clark et al., 2006), and a recent study by Studer and Doebley (2012) examined the possibility of an allelic series at the *tb1* locus in teosinte. Studer and Doebley (2012) introgressed 9 separate teosinte segments (one from *Zea diploperennis*, and four from both *Zea mays* ssp. *mexicana* and *Zea mays* ssp. *parviglumis*) spanning

the *tb1* locus into an isogenic maize background and investigated their effects on previously associated phenotypes. They found that plants with teosinte chromosomal segments had greater tillering than their maize isogenic lines, and that different chromosomal segments of *tb1* confer different amounts of tillering, suggesting that there are multiple genetic factors in this region that affect tillering. However, in addition to elucidating variance in tillering among *tb1* teosinte segments, Studer and Doebley (2012) found significant variance among W22 control lines, suggesting that there are other genetic factors aside from alleles at the *tb1* locus that affect tillering in maize. Doebley and Stec (1991, 1993) first attempted to map QTL controlling many of the phenotypic differences between domesticated maize and teosinte, and demonstrated the existence of numerous QTL that contribute to the differences in branching architecture between the two. Many of these loci (*grassy tillers*, *gt1*; *tassel-replaces-upper-ears1*, *tru1*; *terminal ear1*, *ter1*) have been shown to interact with *tb1* (Whipple et al., 2011; Li, 2012), and both *tru1* and *ter1* have been shown to affect the same phenotypic traits as *tb1* (Doebley et al., 1995). *tassel-replaces-upper-ears1* (*tru1*), for example, has been shown to act either epistatically or downstream of *tb1*, affecting both branching architecture (decreased apical dominance) and tassel phenotypes (shortened tassel and shank length and reduced tassel number) (Li, 2012). It seems plausible that variation in some of these other loci could have affected tillering in our greenhouse population, and contributed to the lack of correlation we see between *Hopscotch* genotype and tillering.

In summary, our findings demonstrate that the *Hopscotch* allele is more widespread in populations of *parviglumis* and *mexicana* than previously thought. Analysis of linkage using SNPs from across chromosome 1 does not suggest that the *Hopscotch* allele is present in these populations due to recent introgression; however, it seems unlikely that it would have drifted to high frequency in multiple populations and there may be another explanation for the high frequency we observe in some of our populations. The *Hopscotch* does not appear to have a strong effect reducing tillering in teosinte as it does in maize,

and other loci involved in branching architecture may play also play roles in the regulation of tillering in teosinte. Finally, although we see no clear evidence of recent strong selection, the high frequency of the *Hopscotch* insertion in a number of populations continues to suggest to us that it plays an ecological role in teosinte. In the future, additional experiments will be needed to examine expression levels of *tb1* and additional loci involved in branching architecture (e.g. *gt1*, *tru1*, and *ter1*) in conjunction with a more exhaustive phenotyping and genotyping assay. *why not Phyb and phya? Are they necessary to include? I'd had them in before in a paragraph but had been voted out I'd ditch gt1 tru1 ter1 and maybe just cite some people including phyb etc.*

please check format of supp figs and tables; some are running off the page. you can use "longtable" to fix that (ask Paul for example). check fig/table references, bibliography, etc. what does "rotation" mean in supp. table 3? it isn't mentioned in methods. please check that all the tables and figs (including supplement) are referenced in the text.

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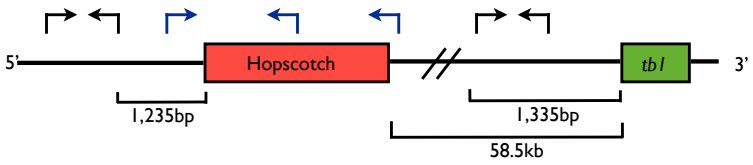
Supplementary Materials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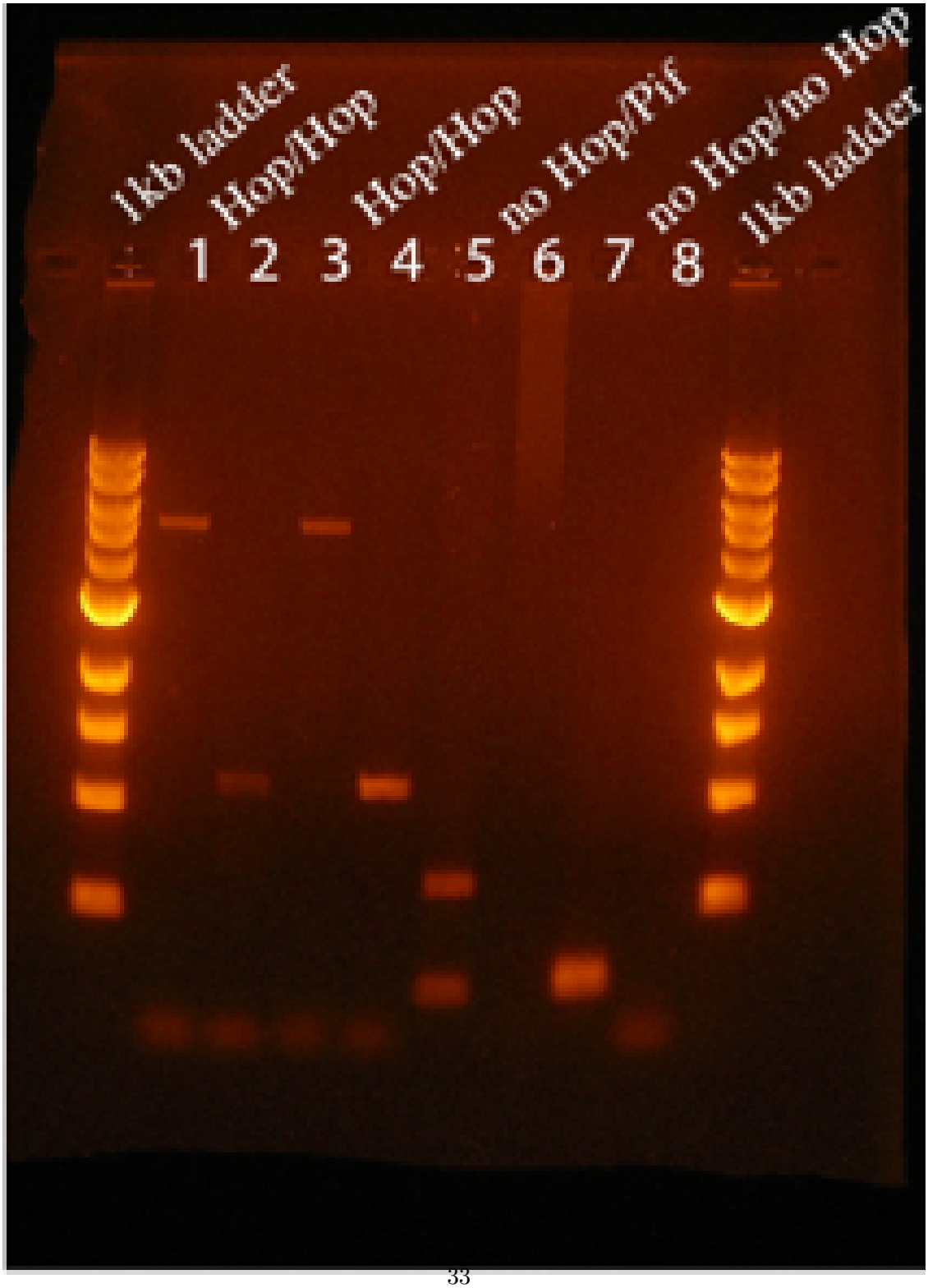
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MSA52
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OH7B
BKN027
BKN033
KY21
ANC
TIL14
CAUMO17
BKN035
BKN023
TIL25
BKN017
MO18W
CML69
CAU178
M16ZW
BKN010
CML52
B97
SLO37
SLO47
EjuA18
EjuB24
EjuB6
EjuA3
HP301
EjuA31
MSA43
EjuB29
EjuA13
TIL07
SLO30
SLO48
MSA1
MSA10
MSA41
SLO7
SLO1
MSA52
MSA50
MSA39
MSA16
MSA12
MSA2
SLO50
SLO49
SLO23
SLO21
SLO20
SLO6
MSA53

Accession	USDA Accession ID		Locality	Number alleles sampled	*Hopscotch*
RIHY0009	N/A		N/A	2	
RIMME0006	566673		Durango, Mexico	2	
RIMME0007	566680		Guanajuato, Mexico	2	
RIMME0008	566681		Michoacan, Mexico	2	
RIMME0009	566682		Distrito Federal, Mexico	2	
RIMME0011	566685		Mexico, Mexico	2	
RIMME0014	714151		Breeders line; Puga: 11066	6	
RIMME0017	699874		Ayotlan, Mexico	8	
RIMME0021	N/A		El Porvenir, Mexico	69	0
RIMME0026	N/A		Opopeo, Mexico	42	0
RIMME0028	N/A		Puruandiro, Mexico	28	0
RIMME0029	N/A		Ixtlan, Mexico	35	
RIMME0030	N/A		San Pedro, Mexico	27	
RIMME0031	N/A		Tenango del Aire, Mexico	25	
RIMME0032	N/A		Nabogame, Mexico	24	
RIMME0033	N/A		Puerta Encantada, Mexico	25	
RIMME0034	N/A		Santa Clara, Mexico	23	
RIMME0035	N/A		Xochimilco, Mexico	25	
RIMPA0001	87168		El Salado, Mexico	4	
RIMPA0003	87171		Mazatlan, Mexico	8	
RIMPA0017	87200		N/A	4	
RIMPA0019	87213		El Salado, Mexico	2	
RIMPA0029	87244		N/A	2	
RIMPA0031	87249		N/A	2	
RIMPA0035	87288		Jalisco, Mexico	4	
RIMPA0040	288185		Mexico, Mexico	4	
RIMPA0042	288187		Guerrero, Mexico	4	
RIMPA0043	288188	35	Guerrero, Mexico	4	
RIMPA0045	288193		Guerrero, Mexico	4	
RIMPA0055	714152		Breeders line	2	
RIMPA0056	714153		Breeders line	2	
RIMPA0057	714154		Breeders line	2	
RIMPA0058	N/A		N/A	4	

Accession	Number of alleles sampled		*Hopscotch* Frequency
RIMMA0066	2		1
RIMMA0075	2		1
RIMMA0077	2		1
RIMMA0079	2		1
RIMMA0081	2		1
RIMMA0084	2		1
RIMMA0086	2		1
RIMMA0088	2		1
RIMMA0089	2		1
RIMMA0090	2		1
RIMMA0092	4		1
RIMMA0094	4		1
RIMMA0097	2		1
RIMMA0099	2		1
RIMMA0100	2		1
RIMMA0101	2		1
RIMMA0104	2		1
RIMMA0108	2		1
RIMMA0111	6		1
RIMMA0115	2		1
RIMMA0117	2		1
RIMMA0130	2		1
RIMMA0133	2		1
RIMMA0134	2		1
RIMMA0135	2		1
RIMMA0142	2		0.5
RIMMA0143	4		1
RIMMA0146	4	36	1
RIMMA0149	2		1
RIMMA0152	2		1
RIMMA0153	2		1
RIMMA0154	2		1
RIMMA0155	2		1

PC1		PC2		PC3		PC4		PC5		PC
Var	Rot	Var	Rot	Var	Rot	Var	Rot	Var	Rot	Va
bio1	0.146	bio4	0.244	prec7	0.287	ts_clay	0.41	bio2	0.38	bio
tmean11	0.146	bio3	0.241	prec8	0.276	v_mod	0.359	sq4	0.328	x_mo
tmean12	0.145	bio7	0.241	prec11	0.262	ts_sand	0.329	ts_loam	0.289	sq
bio11	0.145	prec6	0.237	bio13	0.247	bio15	0.272	ts_sand	0.266	bio
tmax12	0.145	sq7	0.218	prec1	0.246	prec4	0.259	sq7	0.231	v_mo
tmin5	0.145	prec9	0.217	bio16	0.242	x_mod	0.244	bio18	0.213	prec1
tmean1	0.145	sq3	0.207	prec12	0.24	prec3	0.226	bio13	0.207	bio1
tmean2	0.145	prec12	0.207	bio19	0.238	sq3	0.21	prec11	0.183	sq
tmin4	0.145	bio12	0.204	bio12	0.231	prec5	0.21	bio7	0.17	sq
tmax1	0.145	bio19	0.196	prec2	0.222	prec7	0.19	bio16	0.163	ts_san
tmean4	0.145	prec2	0.188	bio18	0.221	sq4	0.186	bio4	0.157	bio
tmin11	0.144	prec1	0.185	sq4	0.2	bio3	0.185	bio12	0.156	prec
tmax11	0.144	prec10	0.184	prec9	0.18	bio18	0.178	bio3	0.155	tmax
tmin12	0.144	bio16	0.183	prec10	0.171	sq7	0.132	prec6	0.154	tmax
tmin2	0.144	prec8	0.17	prec5	0.161	bio14	0.116	x_mod	0.152	bio
tmean5	0.144	prec5	0.165	prec4	0.154	bio13	0.099	prec9	0.144	tmax
tmean10	0.144	bio14	0.158	sq3	0.147	bio16	0.095	prec8	0.143	bio
bio6	0.144	bio13	0.151	bio2	0.143	prec8	0.09	v_mod	0.142	ts_loa
tmax2	0.144	bio17	0.149	bio17	0.129	bio7	0.077	bio15	0.136	ts_cla
tmean3	0.144	prec3	0.144	ts_loam	0.127	bio4	0.075	prec7	0.112	tmin
tmin1	0.143	ts_clay	0.141	v_mod	0.123	bio2	0.074	prec4	0.108	tmin
tmin10	0.143	bio2	0.129	prec3	0.113	prec2	0.074	bio14	0.096	prec
Altitude	0.143	prec7	0.108	x_mod	0.111	bio19	0.068	tmax7	0.093	tmin1
bio9	0.143	tmax6	0.107	bio14	0.099	prec12	0.056	tmax8	0.092	tmin1
tmin3	0.143	x_mod	0.106	bio4	0.07	ts_loam	0.053	prec1	0.091	tmin
bio10	0.142	bio15	0.098	tmax3	0.067	tmax12	0.047	prec2	0.086	tmean
tmax10	0.142	ts_loam	0.088	ts_clay	0.065	bio17	0.047	tmin11	0.086	tmax
tmax3	0.142	tmean6	0.085	bio15	0.056	bio9	0.043	prec5	0.082	tmax
tmax4	0.142	tmin7	0.082	tmax2	0.055	tmax8	0.042	bio17	0.082	tmean
tmin6	0.142	bio5	0.082	tmean3	0.052	tmax1	0.041	tmin12	0.08	bio
tmean9	0.141	tmean7	0.081	ts_sand	0.05	tmax5	0.039	prec3	0.078	tmin1
tmin9	0.141	prec4	0.08	prec6	0.048	tmax7	0.039	tmax9	0.078	prec

Ejutla A	4	0.15217	0.11902	0.76191
Ejutla B	5	0.15258	0.14877	0.07412
La Mesa	3	0.12802	0.08926	1.09209
San Lorenzo	3	0.09098	0.08926	0.04845