

1 Natural variation in teosinte at the domestication locus

2 *teosinte branched1 (tb1)*

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## 1 Abstract

2 The *teosinte branched1* (*tb1*) gene, a repressor of lateral organ growth, is a major QTL  
3 involved in branching differences between maize and its wild progenitor, teosinte.  
4 Previous work has shown that the insertion of a transposable element (*Hopscotch*)  
5 upstream of *tb1* enhances the gene's expression, causing much of the reduction in tillering  
6 observed in domesticated maize. Observations of the maize *tb1* allele in teosinte  
7 individuals, coupled with estimates of an age of insertion of the *Hopscotch* element that  
8 predates domestication, led us to investigate its prevalence and potential role in teosinte.  
9 Results from genotyping across many natural populations suggest that the *Hopscotch*  
10 element is segregating at a higher than expected frequency in a number of populations of  
11 two subspecies of teosinte, *Zea mays* ssp. *parviglumis* and *Zea mays* ssp. *mexicana*.  
12 Analysis of linkage disequilibrium between the *Hopscotch* element and variation in  
13 surrounding regions does not support a hypothesis of recent introgression from maize into  
14 teosinte. Population genetic signatures, however, are consistent with selection on this  
15 locus and suggest the *Hopscotch* insertion at *tb1* may play an ecological role in teosinte.  
16 Finally, two greenhouse experiments with *Zea mays* ssp. *parviglumis* do not suggest *tb1*  
17 controls tillering in natural populations of this subspecies. Our findings suggest that the  
18 role of the *Hopscotch* in tillering in teosinte differs from domesticated maize, and that  
19 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 (e.g. 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

1 lateral branches terminating in a female inflorescence, while teosinte is highly tillered and  
2 bears tassels (male inflorescences) at the end of its lateral branches. The *teosinte*  
3 *branched1* (*tb1*) gene, a repressor of organ growth, was identified as a major QTL  
4 involved in branching (Doebley et al., 1995) and tillering (Doebley and Stec, 1991)  
5 differences between maize and teosinte. A 4.9 kb retrotransposon (*Hopscotch*) insertion  
6 into the upstream control region of *tb1* in maize acts to enhance expression of *tb1*, thus  
7 repressing lateral organ growth (Doebley et al., 1997; Studer et al., 2011). Dating of the  
8 *Hopscotch* retrotransposon suggests that its insertion predates the domestication of  
9 maize, leading to the hypothesis that it was segregating as standing variation in ancient  
10 populations of teosinte and increased to high frequency in maize due to selection during  
11 domestication (Studer et al., 2011). The effects of the *Hopscotch* insertion have been  
12 studied in maize (Studer et al., 2011), and analysis of teosinte alleles at *tb1* has identified  
13 functionally distinct allelic classes (Studer and Doebley, 2012), but little is known about  
14 the role of *tb1* or the *Hopscotch* insertion in natural populations of teosinte.

15 In teosinte and other plants that grow at high population density, individuals detect  
16 competition from neighbors via the ratio of red to far-red light. An increase in far-red  
17 relative to red light accompanies shading and triggers the shade avoidance syndrome: a  
18 suite of physiological and morphological changes such as reduced tillering, increased plant  
19 height and early flowering (Kebrom and Brutnell, 2007). The *tb1* locus appears to play  
20 an important role in the shade avoidance pathway in *Zea mays* and other grasses and  
21 may therefore be crucial to the ecology of teosinte (Kebrom and Brutnell, 2007; Lukens  
22 and Doebley, 1999). In this study we aim to characterize the distribution of the  
23 *Hopscotch* insertion in *parviglumis*, *mexicana*, and landrace maize, and to examine the  
24 phenotypic effects of the insertion in *parviglumis*. We use a combination of PCR  
25 genotyping for the *Hopscotch* element in our full panel and sequencing of two small  
26 regions upstream of *tb1* in a subset of teosinte populations to explore patterns of genetic  
27 variation at this locus. Finally, we test for an association between the *Hopscotch* element

1 and tillering phenotypes in a population of *parviglumis*.

## 2 MATERIALS AND METHODS

### 3 Sampling and genotyping

4 We sampled 1,110 individuals from 350 accessions (247 maize landraces, 17 *mexicana*  
5 populations, and 86 *parviglumis* populations) and assessed the presence or absence of the  
6 *Hopscotch* insertion (Table S1 and Table S2). DNA was extracted from leaf tissue using a  
7 modified CTAB approach (Doyle and Doyle, 1990; Maloof et al., 1984). We designed  
8 primers using PRIMER3 (Rozen and Skaletsky, 2000) implemented in Geneious (Kearse  
9 et al., 2012) to amplify the entire *Hopscotch* element, as well as an internal primer  
10 allowing us to simultaneously check for possible PCR bias between presence and absence  
11 of the *Hopscotch* insertion. Two PCRs were performed for each individual, one with  
12 primers flanking the *Hopscotch* (HopF/HopR) and one with a flanking primer and an  
13 internal primer (HopF/HopIntR). Primer sequences are HopF,  
14 5'-TCGTTGATGCTTTGATGGATGG-3'; HopR, 5'-AACAGTATGATTTTCATGGGACCG-3';  
15 and HopIntR, 5'-CCTCCACCTCTCATGAGATCC-3' (Fig. S1, Fig. S2) *Primers in Fig. S1 should*  
16 *be labeled* . Homozygotes show a single band for absence of the element (~300bp) and two  
17 bands for presence of the element (~5kb and XX *LV, please add the size of the second band* ),  
18 whereas heterozygotes are three-banded (Fig. S2). When only one PCR resolved well, we  
19 scored one allele for the individual. We used Phusion High Fidelity Enzyme (Thermo  
20 Fisher Scientific Inc., Waltham, Massachusetts, USA) and the following conditions for  
21 amplifications: 98°C for 3 min, 30 cycles of 98°C for 15 s, 65°C for 30 s, and 72°C for 3  
22 min 30 s, with a final extension of 72°C for 10 min. PCR products were visualized on a  
23 1% agarose gel and scored for presence/absence of the *Hopscotch* based on band size.

## 1 Sequencing

2 In addition to genotyping, we chose a subset of *parviglumis* individuals for sequencing.  
3 We chose twelve individuals from each of four populations from Jalisco state, Mexico  
4 (San Lorenzo, La Mesa, Ejutla A, and Ejutla B). For amplification and sequencing, we  
5 selected two regions approximately 600bp in size from within the 5' UTR of *tb1* (Region  
6 1) and from 1,235bp upstream of the start of the *Hopscotch* (66,169bp upstream from the  
7 start of the *tb1* ORF; Region 2). We designed the following primers using PRIMER3  
8 (Rozen and Skaletsky, 2000): for the 5' UTR, 5'-GGATAATGTGCACCAGGTGT-3' and  
9 5'-GCGTGCTAGAGACACYTGTGCT-3'; for the 66kb upstream region,  
10 5'-TGTCCTCGCCGCAACTC-3' and 5'-TGTACGCCCGCCCTCATCA-3' (Fig. S1). We  
11 used Taq polymerase (New England Biolabs Inc., Ipswich, Massachusetts, USA) and the  
12 following thermal cycler conditions to amplify fragments: 94°C for 3 min, 30 cycles of  
13 92°C for 40 s, annealing for 1 min, 72°C for 40 s, and a final 10 min extension at 72°C.  
14 Annealing temperatures for Region 1 and Region 2 were 59.7°C and 58.8°C, respectively.  
15 To clean excess primer and dNTPs we added two units of Exonuclease1 and 2.5 units of  
16 Antarctic Phosphatase to 8.0  $\mu$ L of amplification product. This mix was placed on a  
17 thermal cycler with the following program: 37°C for 30 min, 80°C for 15 min, and a final  
18 cool-down step to 4°C.

19 We cloned cleaned fragments into a TOPO-TA vector (Life Technologies, Grand  
20 Island, New York, USA) using OneShot TOP10 chemically competent *E. coli* cells, with  
21 an extended ligation time of 30 min for a complex target fragment. We plated cells on LB  
22 agar plates containing kanamycin, and screened colonies using vector primers M13  
23 Forward and M13 Reverse under the following conditions: 96°C for 5 min; then 35 cycles  
24 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.  
25 We visualized amplification products for incorporation of our insert on a 1% agarose TAE  
26 gel.

27 Amplification products with successful incorporation of our insert were cleaned using

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

## 11 Genotyping analysis

12 We examined discrepancies between observed and expected genotype frequencies by  
13 calculating Hardy-Weinberg Equilibrium (HWE). To calculate differentiation between  
14 populations ( $F_{ST}$ ) and subspecies ( $F_{CT}$ ) we used HierFstat (Goudet, 2005). These  
15 analyses only included populations in which 8 or more individuals were sampled. To test  
16 the hypothesis that the *Hopscotch* insertion may be adaptive under certain environmental  
17 conditions, we looked for significant associations between the *Hopscotch* frequency and  
18 environmental variables using BayEnv (Coop et al., 2010). BayEnv creates a covariance  
19 matrix of relatedness between populations and then tests a null model that allele  
20 frequencies in populations are determined by the covariance matrix of relatedness alone  
21 against the alternative model that allele frequencies are determined by a combination of  
22 the covariance matrix and an environmental variable, producing a posterior probability  
23 (*i.e.*, Bayes Factor; Coop et al. 2010). We used genotyping and covariance data from  
24 Pyhäjärvi et al. (2013) for BayEnv, with the *Hopscotch* insertion coded as an additional  
25 SNP (Table S3). Environmental data were obtained from [www.worldclim.org](http://www.worldclim.org), the  
26 Harmonized World Soil Database (FAO/IIASA/ISRIC/ISSCAS/JRC, 2012) and

1 [www.harvestchoice.org](http://www.harvestchoice.org) and summarized by principle component analysis following  
2 Pyhäjärvi et al. (2013).

### 3 **Sequence analysis**

4 For population genetic analyses of sequenced Region 1 and sequenced Region 2 we used  
5 the Libsequence package (Thornton, 2003) to calculate pairwise  $F_{ST}$  between populations  
6 and to calculate standard diversity statistics (number of haplotypes, haplotype diversity,  
7 Watterson's estimator  $\hat{\theta}_W$ , pairwise nucleotide diversity  $\hat{\theta}_\pi$ , and Tajima's D). To produce  
8 a visual representation of differentiation between sequences and examine patterns in  
9 sequence clustering by *Hopscotch* genotype we used Phylip  
10 (<http://evolution.genetics.washington.edu/phylip.html>), creating  
11 neighbor-joining trees with bootstrap-supported nodes (100 repetitions). For creation of  
12 trees we also included homologous sequence data from Maize HapMapV2 (Chia et al.,  
13 2012) for teosinte inbred lines (TILs), some of which are known to be homozygous for the  
14 *Hopscotch* insertion (TIL03, TIL17, TIL09), as well as 59 lines of domesticated maize.

### 15 **Introgression analysis**

16 In order to assess patterns of linkage disequilibrium (LD) around the *Hopscotch* element  
17 in the context of chromosomal patterns of LD we used Tassel (Bradbury et al., 2007) and  
18 calculated LD between SNPs across chromosome 1 using previously published data from  
19 twelve plants each of the Ejutla A (EjuA), Ejutla B (EjuB), San Lorenzo (SLO), and La  
20 Mesa (MSA) populations (Pyhäjärvi et al., 2013). We chose these populations because we  
21 had both genotyping data for the *Hopscotch* as well as chromosome-wide SNP data for  
22 chromosome 1. For each population we filtered the initial set of 5,897 SNPs on  
23 chromosome 1 to accept only SNPs with a minor allele frequency of at least 0.1, resulting  
24 in 1,671, 3,023, 3,122, and 2,167 SNPs for SLO, EjuB, EjuA, and MSA, respectively. We  
25 then used Tassel (Bradbury et al., 2007) to calculate linkage disequilibrium ( $r^2$ ) across



1 chromosome 1 for each population.

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

## 10 Phenotyping of *parviglumis*

11 To investigate the phenotypic effects of the *Hopscotch* insertion in teosinte, we conducted  
12 an initial phenotyping trial (Phenotyping 1). We germinated 250 seeds of *parviglumis*  
13 collected in Jalisco state, Mexico (population San Lorenzo) (Hufford, 2010) where the  
14 *Hopscotch* is segregating at highest frequency (0.44) in our initial genotyping sample set.  
15 In order to maximize the likelihood of finding the *Hopscotch* in our association  
16 population we selected seeds from sites where genotyped individuals were homozygous or  
17 heterozygous for the insertion. We chose between 10-13 seeds from each of 23 sampling  
18 sites. We treated seeds with fungicide and germinated them in petri dishes with filter  
19 paper. Following germination, 206 successful germinations were planted into one-gallon  
20 pots with potting soil and randomly spaced one foot apart on greenhouse benches. Plants  
21 were watered three times a day by hand and with an automatic drip containing 10-20-10  
22 fertilizer.

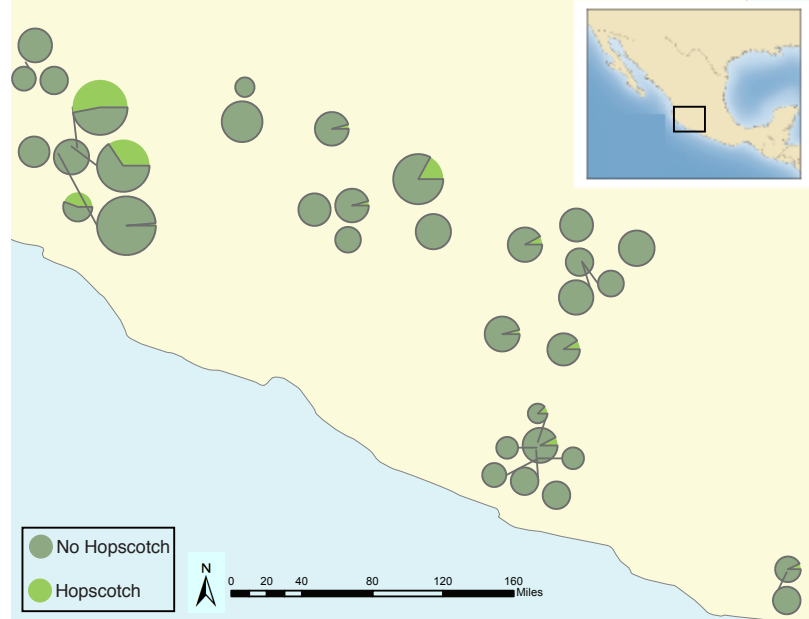
23 Starting on day 15, we measured tillering index as the ratio of the sum of tiller  
24 lengths to the height of the plant (Briggs et al., 2007). Following initial measurements,  
25 we phenotyped plants for tillering index every 5 days through day 40, and then on day 50  
26 and day 60. On day 65 we measured culm diameter between the third and fourth nodes

1 of each plant. Culm diameter is not believed to be correlated with tillering index or  
2 variation at *tb1*. Following phenotyping we extracted DNA from all plants using a  
3 modified SDS extraction protocol (<http://www.ars.usda.gov>). *what is this url?* We  
4 genotyped individuals for the *Hopscotch* insertion following the protocols listed above.  
5 Based on these initial data, we conducted a *post hoc* power analysis using data from day  
6 40 of Phenotyping 1, indicating that a minimum of 71 individuals in each genotypic class  
7 would be needed to detect the observed effect of the *Hopscotch* on tillering index.

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

17 Tillering index data for each genotypic class did not meet the criteria for a repeated  
18 measures ANOVA, so we transformed the data using a Box-Cox transformation ( $\alpha = 0$   
19 *what is the alpha value here?* ; Car Package for R, Fox and Weisberg 2011) to improve the  
20 normality and homogeneity of variance among genotype classes. We analyzed  
21 relationships between genotype and tillering index and tiller number using a repeated  
22 measures ANOVA through a general linear model function implemented in SAS v.9.3  
23 (SAS Institute Inc., Cary, NC, USA). Additionally, in order to compare any association  
24 between *Hopscotch* genotype and tillering and associations at other presumably unrelated  
25 traits, we performed an ANOVA between culm diameter and genotype using the same  
26 general linear model in SAS.

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.



## RESULTS

### 2 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).

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  $F_{CT} = 0$ , and levels of  $F_{ST}$  among populations within each subspecies (0.22) and among all populations (0.23) are these an average of pairwise or is this calculated among all pops? 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

- 1 populations, BayEnv analysis did not indicate a correlation between the *Hopscotch*
- 2 insertion and environmental variables (all Bayes Factors < 1; Table S3).

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

Comparison	Region 1	Region 2	<i>Hopscotch</i>
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

### 3 Sequencing

4 To investigate patterns of sequence diversity and linkage disequilibrium (LD) in the *tb1*  
5 region, we sequenced two small (<1kb) regions upstream of the *tb1* ORF in four  
6 populations. After alignment and singleton checking we recovered 48 and 40 segregating  
7 sites for the 5' UTR region (Region 1) and the 66kb upstream region (Region 2),  
8 respectively. For Region 1, Ejutla A has the highest values of haplotype diversity, and  $\theta_\pi$ ,  
9 while Ejutla B and La Mesa have comparable values of these summary statistics, and San  
10 Lorenzo has much lower values. Additionally, Tajima's D is strongly negative in the two  
11 Ejutla populations and La Mesa, but is less negative in San Lorenzo (Table 2). *need to*  
12 *reference Hopscotch frequencies in supplemental table somewhere* For Region 2, haplotype diversity  
13 and  $\theta_\pi$ , are similar for Ejutla A and Ejutla B, while La Mesa and San Lorenzo have  
14 slightly lower values for these statistics (Table 2). Tajima's D is positive in all  
15 populations except San Lorenzo, *is the table wrong? MSA is the only negative value in the table*  
16 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 regions as well as for the *Hopscotch* [table 1 shows 0.016 for hopscotch, not 0. which is right?](#), 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 domesticated maize that have the *Hopscotch* insertion.

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

Population	# Haplotypes	Hap. Diversity	$\hat{\theta}_{\pi}$	Tajima's D
<i>Region 1 (5' UTR)</i>				
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
<i>Region 2 (66kb upstream)</i>				
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

## 9 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 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*

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	<i>tb1</i> 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 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

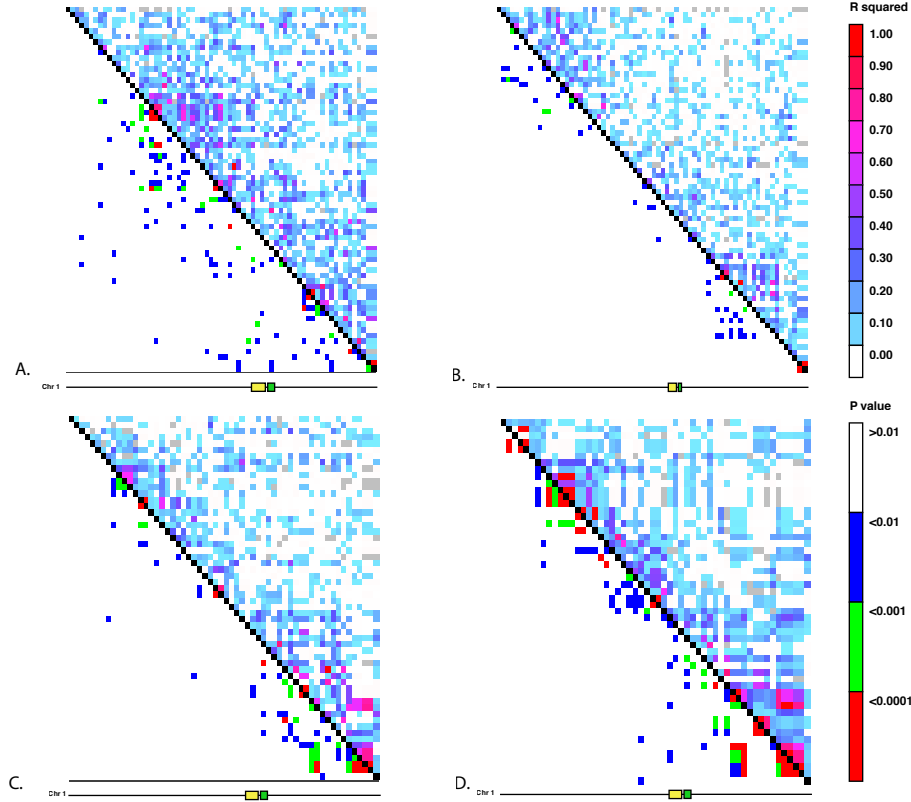


Figure 2: Linkage disequilibrium for SNPs in Mb 261-268 on chromosome 1. The yellow rectangle indicates the location of the *Hopscotch* insertion and the green represents the *tb1* ORF. A) Ejutla A; B) Ejutla B; C) La Mesa; D). San Lorenzo

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

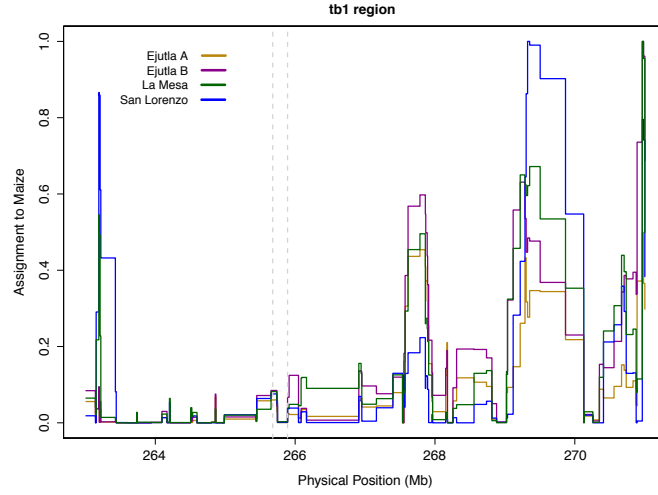


Figure 3: STRUCTURE assignment to maize across a section of chromosome 1. The dotted lines mark the beginning of the sequenced region 50kb upstream (Sequenced region 2) and the end of the *tb1* ORF.

1 average (0.20; Table 4; Fig. 3).

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

	<i>tb1</i> 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



## 1 Phenotyping

2 To assess the contribution of *tb1* to phenotypic variation in tillering in a natural  
3 population, we grew plants from seed sampled from the San Lorenzo population of  
4 *parviglumis*, which had a high mean frequency (0.44) of the *Hopscotch* insertion based on  
5 our initial genotyping. We measured tillering index (TI), the ratio of the sum of tiller  
6 lengths to plant height, for 216 plants (Phenotyping 1) from within the San Lorenzo  
7 population, and genotyped plants for the *Hopscotch* insertion. We found the *Hopscotch*  
8 segregating at a frequency of 0.65 with no significant deviations from expected  
9 frequencies under Hardy-Weinberg equilibrium. After performing a repeated measures  
10 ANOVA between our transformed tillering index data and *Hopscotch* genotype we find a  
11 weak positive correlation between presence of the *Hopscotch* and tillering index on day 40  
12 ( $p=0.0848$ ), a result indicating the *Hopscotch* may actually increase tillering in  
13 *parviglumis* in contrast to its phenotypic effect in maize. We find no correlation between  
14 tillering index and genotype on any other day (4). Additionally we find no significant  
15 correlation between tiller number and *Hopscotch* genotype, or culm diameter and  
16 *Hopscotch* genotype in Phenotyping 1.

17 We performed a second grow-out of *parviglumis* from San Lorenzo (Phenotyping 2)  
18 to assess whether lighting conditions or sample size may have affected our ability to  
19 detect an effect of *tb1*. For the second grow-out we measured tillering index every five  
20 days through day 50 for 302 plants. We found the *Hopscotch* allele segregating at a  
21 frequency of 0.69, *is it in HWE in this pop?* with a 0.6 frequency of *Hopscotch* homozygotes,  
22 and a 0.2 frequency of both heterozygotes and homozygotes for the teosinte allele. We  
23 found similar patterns, with a weak positive correlation between tillering index and  
24 *Hopscotch* genotype at day 40 ( $p<0.0611$ ), with no significant correlation on any day.  
25 Similarly, relationships between *Hopscotch* genotype and tiller number and *Hopscotch*  
26 genotype and culm diameter were not significant.

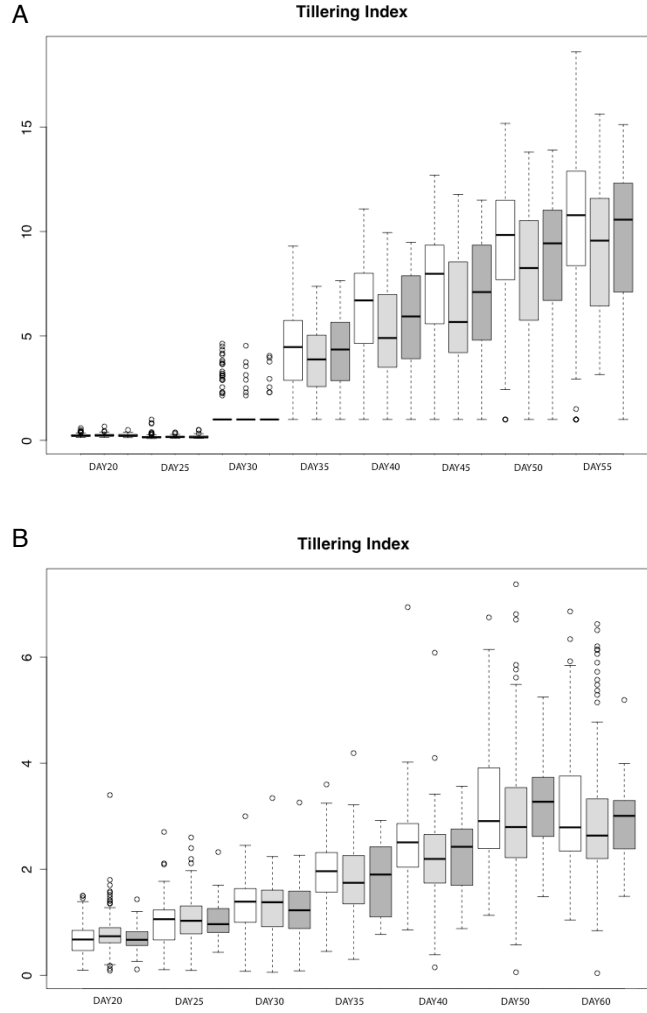


Figure 4: Box-plots showing tillering index in greenhouse grow-outs for Phenotyping 1 (A) and Phenotyping 2 (B). White indicates individuals homozygous for the *Hopscotch*, light grey represents heterozygotes, and dark grey represents homozygotes for the *teosinte* (No *Hopscotch*) allele. Within boxes, 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.](#)

## DISCUSSION

Adaptation occurs due to selection on standing variation or *de novo* mutations.

Adaptation from 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 adaptive role of standing variation has been described in many systems, its importance in domestication is not as well studied.

In maize, alleles at domestication loci (*RAMOSA1*, Sigmon and Vollbrecht 2010; *barren stalk1*, Gallavotti et al. 2004; and *grassy tillers1*, Whipple et al. 2011) are thought to have been selected from standing variation, suggesting that diversity already present in teosinte may have played an important role in maize domestication. The *teosinte branched1* gene is one of the best characterized domestication loci, and, while previous studies have suggested that differences in plant architecture between maize and teosinte are a result of selection on standing variation at this locus, little is known about natural variation at this locus and its ecological role 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, by widely sampling across 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 identify 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), suggesting a different history of the *tb1*

1 locus in this region than in the Balsas River Basin where maize was domesticated  
2 (Matsuoka et al., 2002). Potential explanations for the high frequency of the *Hopscotch*  
3 element in *parviglumis* from the Jalisco cluster include gene flow from maize, genetic  
4 drift, and natural selection.

5 While gene flow from crops into their wild relatives is well-known, (Ellstrand et al.,  
6 1999; Zhang et al., 2009; Thurber et al., 2010; Baack et al., 2008; Hubner et al., 2012;  
7 Wilkes, 1977; van Heerwaarden et al., 2011; Barrett, 1983), our results are more  
8 consistent with Hufford et al. (2013) who found resistance to introgression from maize  
9 into teosinte. Furthermore, Hufford et al. (2013) showed that domestication loci, such as  
10 *tb1*, are particularly resistant to introgression in both directions of gene flow (i.e., maize  
11 to teosinte and teosinte to maize). We find no evidence of recent introgression in our  
12 analyses. Clustering patterns in our NJ trees do not reflect a pattern expected if maize  
13 alleles at the *tb1* locus had introgressed into populations of teosinte. Moreover, there is  
14 no signature of elevated LD in the *tb1* region relative to the rest of chromosome 1, and  
15 Bayesian assignment to a maize cluster in this region is lower than the chromosome-wide  
16 average (Fig. 3, Table 4). Together, these data point to an explanation other than recent  
17 introgression for the high observed frequency of *Hopscotch* in a subset of our *parviglumis*  
18 populations.

19 Although recent introgression seems unlikely, we cannot rule out ancient introgression  
20 as an explanation for the presence of the *Hopscotch* in these populations. If the  
21 *Hopscotch* allele was introgressed in the distant past, recombination may have broken up  
22 LD, a process that would be consistent with our data. We find this scenario less  
23 plausible, however, as there is no reason why gene flow should have been high in the past  
24 but absent in present-day sympatric populations. In fact, early generation maize-teosinte  
25 hybrids are common in these populations today (MB Hufford, pers. observation), and  
26 genetic data support ongoing gene flow between domesticated maize and both *mexicana*  
27 and *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 this population in the 5' UTR (Region 1) coupled with more positive values of Tajima's D are consistent with these earlier findings suggesting a bottleneck. *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 may have risen to high frequency entirely by chance. This bottleneck, however, does not explain the high frequency of the *Hopscotch* in multiple populations in the Jalisco cluster. Moreover, available information on diversity and population structure among Jaliscoan populations (Hufford, 2010; Pyhäjärvi et al., 2013) is not suggestive of recent colonization or other demographic events that would predict a high frequency of the allele across populations. Finally, 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 a putative 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

1 cupules, and tillering index in domesticated maize have been well documented (Studer  
 2 et al., 2011). Weber et al. (2007) have described significant phenotypic associations  
 3 between markers in and around *tb1* and lateral branch length and female ear length in a  
 4 sample from 74 natural populations of *parviglumis* (Weber et al., 2007); however, these  
 5 data did not include markers from the *Hopscotch* region 66kb upstream of *tb1*. Our study  
 6 is the first to explicitly examine the phenotypic effects of the *Hopscotch* insertion across a  
 7 wide collection of individuals sampled from natural populations of teosinte. We have  
 8 found no significant effect of the *Hopscotch* insertion on tillering index or tiller number, a  
 9 result that is discordant with its clear phenotypic effects in maize. One interpretation of  
 10 this result would be that the *Hopscotch* controls tillering in maize (Studer et al., 2011),  
 11 but tillering in teosinte is affected by variation at other loci. Consistent with this  
 12 interpretation, *tb1* is thought to be part of a complex pathway controlling branching,  
 13 tillering and other phenotypic traits (Kebrom and Brutnell, 2007; Clark et al., 2006). A  
 14 recent study by Studer and Doebley (2012) examined variation across traits in a  
 15 three-taxa allelic series at the *tb1* locus. Studer and Doebley (2012) introgressed nine  
 16 unique teosinte *tb1* segments (one from *Zea diploperennis*, and four each from *mexicana*  
 17 and *parviglumis*) into an inbred maize background and investigated phenotypic effects.  
 18 Phenotypes were shown to cluster by taxon, indicating *tb1* may underlie morphological  
 19 diversification of *Zea*. Additional analysis in Studer and Doebley (2012) suggested  
 20 tillering index was controlled both by *tb1* and loci elsewhere in the genome. Clues to the  
 21 identity of these loci may be found in QTL studies that have identified loci controlling  
 22 branching architecture (*e.g.*, Doebley and Stec 1991, 1993). Many of these loci (*grassy*  
 23 *tillers*, *gt1*; *tassel-replaces-upper-ears1*, *tru1*; *terminal ear1*, *ter1*) have been shown to  
 24 interact with *tb1* (Whipple et al., 2011; Li, 2012), and both *tru1* and *ter1* affect the same  
 25 phenotypic traits as *tb1* (Doebley et al., 1995). *tassel-replaces-upper-ears1* (*tru1*), for  
 26 example, has been shown to act either epistatically or downstream of *tb1*, affecting both  
 27 branching architecture (decreased apical dominance) and tassel phenotypes (shortened

1 tassel and shank length and reduced tassel number; Li 2012). Variation in these  
2 additional loci may have affected tillering in our collections and contributed to the lack of  
3 correlation we see between *Hopscotch* genotype and tillering.

4 In conclusion, our findings demonstrate that the *Hopscotch* allele is more widespread  
5 in populations of *parviglumis* and *mexicana* than previously thought. Analysis of linkage  
6 using SNPs from across chromosome 1 does not suggest that the *Hopscotch* allele is  
7 present in these populations due to recent introgression; however, it seems unlikely that  
8 the insertion would have drifted to high frequency in multiple populations. We do,  
9 however, find preliminary evidence of selection on the *tb1* locus in *parviglumis*; this  
10 coupled with our observation of high frequency of the *Hopscotch* insertion in a number of  
11 populations suggests that the locus plays an ecological role in teosinte. In contrast to  
12 domesticated maize, the *Hopscotch* insertion in *parviglumis* does not appear to reduce  
13 tillering. Other loci involved in branching architecture may regulate tillering in teosinte.  
14 Future studies should examine expression levels of *tb1* in teosinte with and without the  
15 *Hopscotch* insertion and further characterize the effects of additional loci involved in  
16 branching architecture (e.g. *gt1*, *tru1*, and *ter1*). These data, in conjunction with more  
17 exhaustive phenotyping, should help reveal the ecological significance of the domesticated  
18 *tb1* allele in natural populations of teosinte. *why not Phyb and phya? Are they necessary to*

19 *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*  
20 *some people including phyb etc.*

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

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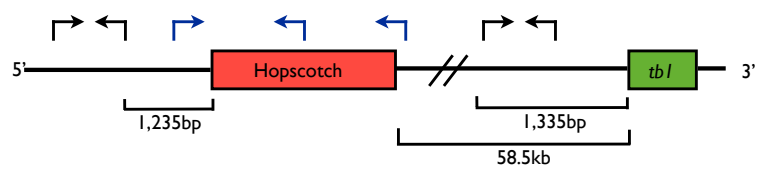
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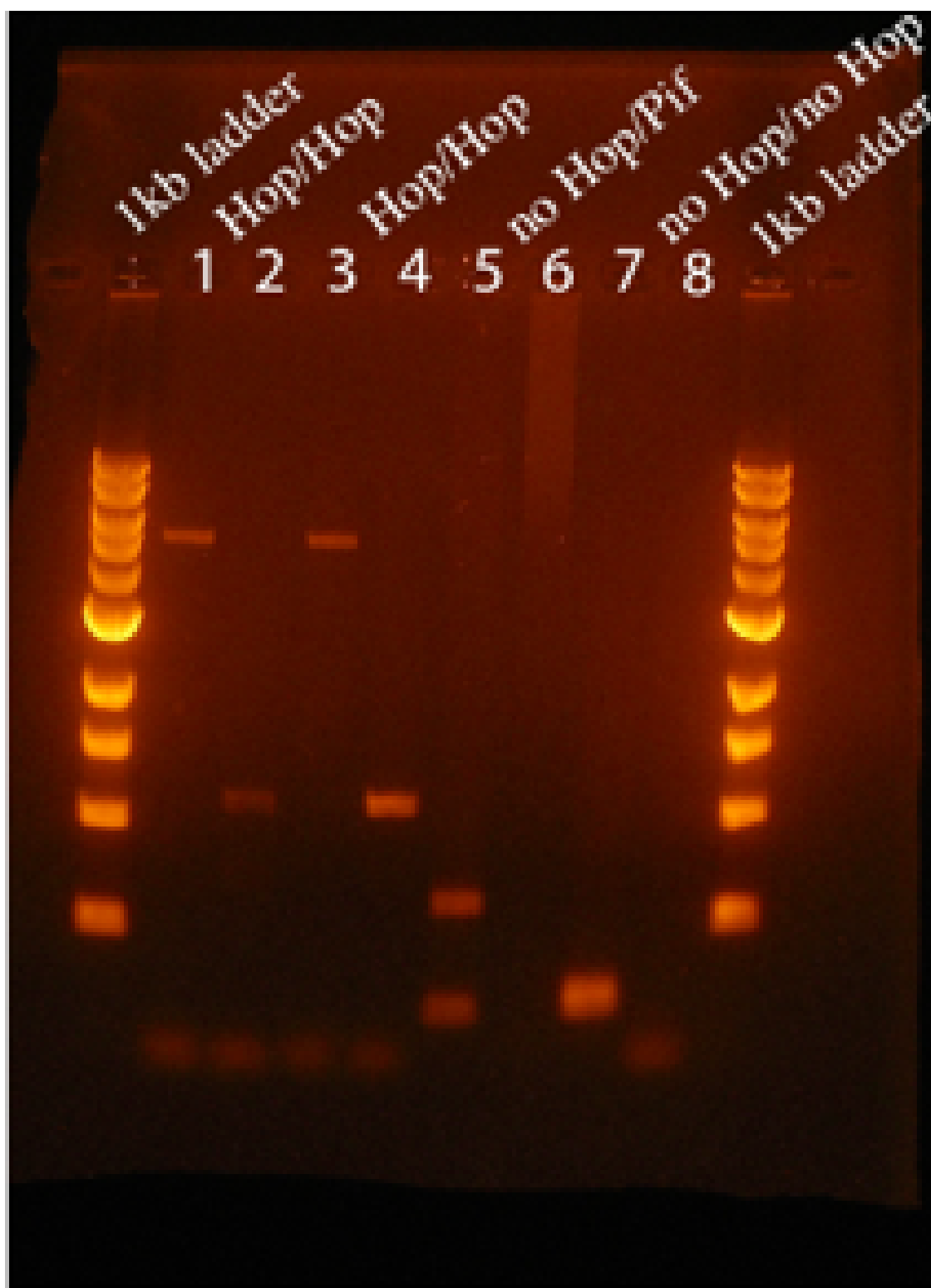
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22 Supplementary Materials









Accession	USDA Accession ID	Locality	Number alleles sampled	<i>Hopscotch</i>
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	34 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	<i>Hopscotch</i> 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	1
RIMMA0149	2	1
RIMMA0152	2	1
RIMMA0153	2	1
RIMMA0154	2	1
RIMMA0155	2	1

PC1		PC2		PC3		PC4		PC5		PC6	
Var	Rot	Var	Rot	Var	Rot	Var	Rot	Var	Rot	Var	Rot
bio1	0.146	bio4	0.244	prec7	0.287	ts_clay	0.41	bio2	0.38	bio10	0.086
tmean11	0.146	bio3	0.241	prec8	0.276	v_mod	0.359	sq4	0.328	x_mod	0.111
tmean12	0.145	bio7	0.241	prec11	0.262	ts_sand	0.329	ts_loam	0.289	sq3	0.207
bio11	0.145	prec6	0.237	bio13	0.247	bio15	0.272	ts_sand	0.266	bio1	0.146
tmax12	0.145	sq7	0.218	prec1	0.246	prec4	0.259	sq7	0.231	v_mod	0.359
tmin5	0.145	prec9	0.217	bio16	0.242	x_mod	0.244	bio18	0.213	prec1	0.287
tmean1	0.145	sq3	0.207	prec12	0.24	prec3	0.226	bio13	0.207	bio1	0.146
tmean2	0.145	prec12	0.207	bio19	0.238	sq3	0.21	prec11	0.183	sq4	0.328
tmin4	0.145	bio12	0.204	bio12	0.231	prec5	0.21	bio7	0.17	sq3	0.207
tmax1	0.145	bio19	0.196	prec2	0.222	prec7	0.19	bio16	0.163	ts_sand	0.329
tmean4	0.145	prec2	0.188	bio18	0.221	sq4	0.186	bio4	0.157	bio1	0.146
tmin11	0.144	prec1	0.185	sq4	0.2	bio3	0.185	bio12	0.156	prec1	0.287
tmax11	0.144	prec10	0.184	prec9	0.18	bio18	0.178	bio3	0.155	tmax1	0.145
tmin12	0.144	bio16	0.183	prec10	0.171	sq7	0.132	prec6	0.154	tmax1	0.145
tmin2	0.144	prec8	0.17	prec5	0.161	bio14	0.116	x_mod	0.152	bio1	0.146
tmean5	0.144	prec5	0.165	prec4	0.154	bio13	0.099	prec9	0.144	tmax1	0.145
tmean10	0.144	bio14	0.158	sq3	0.147	bio16	0.095	prec8	0.143	bio1	0.146
bio6	0.144	bio13	0.151	bio2	0.143	prec8	0.09	v_mod	0.142	ts_loam	0.289
tmax2	0.144	bio17	0.149	bio17	0.129	bio7	0.077	bio15	0.136	ts_clay	0.41
tmean3	0.144	prec3	0.144	ts_loam	0.127	bio4	0.075	prec7	0.112	tmin1	0.143
tmin1	0.143	ts_clay	0.141	v_mod	0.123	bio2	0.074	prec4	0.108	tmin1	0.143
tmin10	0.143	bio2	0.129	prec3	0.113	prec2	0.074	bio14	0.096	prec1	0.287
Altitude	0.143	prec7	0.108	x_mod	0.111	bio19	0.068	tmax7	0.093	tmin1	0.143
bio9	0.143	tmax6	0.107	bio14	0.099	prec12	0.056	tmax8	0.092	tmin1	0.143
tmin3	0.143	x_mod	0.106	bio4	0.07	ts_loam	0.053	prec1	0.091	tmin1	0.143
bio10	0.142	bio15	0.098	tmax3	0.067	tmax12	0.047	prec2	0.086	tmean1	0.145
tmax10	0.142	ts_loam	0.088	ts_clay	0.065	bio17	0.047	tmin11	0.086	tmax1	0.145
tmax3	0.142	tmean6	0.085	bio15	0.056	bio9	0.043	prec5	0.082	tmax1	0.145
tmax4	0.142	tmin7	0.082	tmax2	0.055	tmax8	0.042	bio17	0.082	tmean1	0.145
tmin6	0.142	bio5	0.082	tmean3	0.052	tmax1	0.041	tmin12	0.08	bio1	0.146
tmean9	0.141	tmean7	0.081	ts_sand	0.05	tmax5	0.039	prec3	0.078	tmin1	0.143
tmin9	0.141	prec4	0.08	prec6	0.048	tmax7	0.039	tmax9	0.078	prec1	0.287

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