

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. Further  
4 studies have shown that the insertion of a transposable element (*Hopscotch*) upstream of  
5 *tb1* enhances its expression, causing the reduction in branching observed in domesticated  
6 maize. Observations of the maize *tb1* allele in teosinte individuals, coupled with estimates  
7 of the age of insertion of the *Hopscotch* element, led us to investigate the prevalence and  
8 potential role of *tb1* in teosinte. Results from genotyping across many natural  
9 populations suggest that the *Hopscotch* element is segregating at a higher than expected  
10 frequency in a number of populations of two subspecies of teosinte, *Zea mays* ssp.  
11 *parviglumis* and *Zea mays* ssp. *mexicana*. Analysis of linkage disequilibrium between the  
12 *Hopscotch* element and variation in surrounding regions does not support a hypothesis of  
13 recent introgression from maize into teosinte, and we find no evidence of environmental  
14 correlations that might suggest recent selection. Finally, two greenhouse experiments  
15 with *Zea mays* ssp. *parviglumis* do not suggest an important role for *tb1* in controlling  
16 tillering in natural populations of this subspecies. Our findings suggest that the role of  
17 the *Hopscotch* in tillering in teosinte is not as straightforward as is in domesticated  
18 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

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 domestication branching differences achieved through higher expression of the  
5 maize allele than the teosinte allele (Doebley et al., 1995, 1997). Further work showed  
6 that the insertion of a 4.9 kb retrotransposon (*Hopscotch*) in the upstream control region  
7 of *tb1* caused the increased expression of this gene reduction in branching observed in  
8 domesticated maize Studer et al. (2011). The effects of this insertion have been observed  
9 in tiller number in maize, but little is known about its role, if any, in natural populations  
10 of teosinte (Studer et al., 2011). Dating of this element has suggested that its insertion  
11 predates the domestication of maize, leading to the hypothesis that it was segregating as  
12 standing variation in ancient populations of teosinte and increased to high frequency in  
13 maize due to selection during domestication (Studer et al., 2011). Furthermore, Studer  
14 and Doebley (2012) investigated the phenotypic effects of nine teosinte *tb1* alleles in an  
15 isogenic maize background and found that the introgressions sort into three distinct  
16 phenotypic classes, suggesting that variation at the *tb1* locus may play a functional role  
17 in teosinte.

18 In high-density species such as teosinte, plants can detect impending competition  
19 from their neighbors through detection of the ratio of red to far-red light. An increase in  
20 far-red relative to red light accompanies shading and triggers physiological and  
21 morphological changes such as reduced tillering, increased plant height and early  
22 flowering collectively known as the shade avoidance syndrome (Kebrom and Brutnell,  
23 2007). The *tb1* locus appears to play an important role in the shade avoidance pathway  
24 in *Zea mays* and other grasses and may therefore be crucial to the ecology of teosinte  
25 (Kebrom and Brutnell, 2007; Lukens and Doebley, 1999). In this study we aim to  
26 characterize the distribution of the *Hopscotch* insertion in *parviglumis*, *mexicana*, and  
27 landrace maize, and to examine the phenotypic effects of the insertion in *parviglumis*. We

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

## 5 MATERIALS AND METHODS

6 *from AJB formatting instructions: add name, city, spelled-out state (if in USA), and country of*  
7 *manufacturers/suppliers after brand names*

### 8 Sampling and genotyping

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

1 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.  
2 PCR products were visualized on a 1% agarose gel and scored for presence/absence of the  
3 *Hopscotch* based on band size.

#### 4 Sequencing

5 In addition to genotyping, we chose a subset of *parviglumis* individuals for sequencing.  
6 We chose twelve individuals from each of four populations from Jalisco state, Mexico  
7 (San Lorenzo, La Mesa, Ejutla A, and Ejutla B). For amplification and sequencing, we  
8 selected two regions approximately 600bp in size from within the 5' UTR of *tb1* (Region  
9 1) and from 1,235bp upstream of the start of the *Hopscotch* (66,169bp upstream from the  
10 start of the *tb1* ORF; Region 2). We designed the following primers using PRIMER3  
11 (Rozen and Skaletsky, 2000): for the 5' UTR, 5'-GGATAATGTGCACCAGGTGT-3' and  
12 5'-GCGTGCTAGAGACACYTGTTGCT-3'; for the 50kb upstream region,  
13 5'-TGTCCTCGCCGCAACTC-3' and 5'-TGTACGCCCCGCCCTCATCA-3' (Fig. S1). We  
14 used Taq polymerase (New England Biolabs) and the following thermal cycler conditions  
15 to amplify fragments: 94°C for 3 min, 30 cycles of 92°C for 40 s, annealing for 1 min,  
16 72°C for 40 s, and a final 10 min extension at 72°C. Annealing temperatures for  
17 sequenced region 1 and sequenced region 2 were 59.7°C and 58.8°C, respectively. To  
18 clean excess primer and dNTPs we added two units of Exonuclease1 and 2.5 units of  
19 Antarctic Phosphatase to 8.0  $\mu$ L of amplification product. This mix was placed on a  
20 thermal cycler with the following program: 37°C for 30 min, 80°C for 15 min, and a final  
21 cool-down step to 4°C.

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

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

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

## 14 Genotyping analysis

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

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

## 6 Sequence analysis

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

## 19 Introgression analysis

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



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

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

## 15 **Phenotyping of *parviglumis***

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

1 containing 10-20-10 fertilizer.

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

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

22 Resulting tillering index data for each genotypic class did not meet the criteria for a  
23 repeated measures ANOVA, so we transformed the data using a Box-Cox transformation  
24 ( $\alpha = 0$ ) *what is the alpha value here?* implemented in the car package in R (Fox and Weisberg,  
25 2011) to improve the normality and homogeneity of variance among genotype classes. We  
26 analyzed relationships between genotype and tillering index and tiller number using a  
27 repeated measures ANOVA through a general linear model function implemented in SAS

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

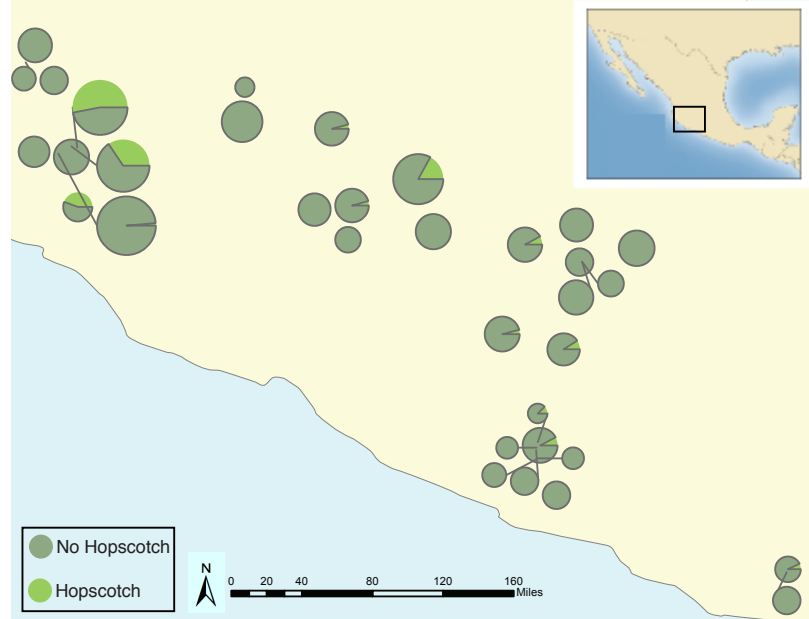
## 5 RESULTS

### 6 Genotyping

7 Genotype of the *Hopscotch* insertion was confirmed with two PCRs for 837 individuals.  
8 Among the 247 maize landrace accessions genotyped, all but eight were homozygous for  
9 the presence of the insertion (Table S1 and Table S2). Within our *parviglumis* and  
10 *mexicana* samples we found the *Hopscotch* insertion segregating in 37 and 4 populations,  
11 respectively, and at highest frequency in the states of Jalisco, Colima, and Michoacán in  
12 central-western Mexico (Fig. 1). We examined Hardy-Weinberg equilibrium in a total of  
13 14 populations (10 *parviglumis* and 4 *mexicana*) with more than 8 individuals sampled  
14 per population. Three populations (RIMPA0073, RIMPA0093, and RIMPA0158) show  
15 evidence of deviations from expected genotype frequencies under the assumptions of  
16 HWE ( $p < 0.05$ ). *Jeff mentioned this needs to be fully reported in a supplementary table; alternatively we can*  
17 *just delete the result since we do not interpret or use it later in the manuscript.*

18 Using our *Hopscotch* genotyping, we calculated differentiation between populations  
19 ( $F_{ST}$ ) and subspecies ( $F_{CT}$ ) for populations in which we sampled 8 or more alleles. We  
20 found that  $F_{CT} = 0$  within our dataset and levels of  $F_{ST}$  among populations within each  
21 subspecies (0.22) *is this an average? this comes from libsequence?* and among all populations  
22 (0.23) are similar to those reported genome-wide in previous studies (Pyhäjärvi et al.  
23 2013; Table 1). Although we found large variation in *Hopscotch* allele frequency among  
24 our populations, BayEnv analysis did not indicate a correlation between the *Hopscotch*  
25 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.



## 1 Sequencing

2 To investigate patterns of sequence diversity and linkage disequilibrium (LD) in the *tb1*  
3 region, we sequenced two small (<1kb) regions upstream of the *tb1* ORF in four  
4 populations. After alignment and singleton checking we recovered 48 and 40 segregating  
5 sites for the 5' UTR region (Region 1) and the 66kb upstream region (Region 2),  
6 respectively. For Region 1, Ejutla A has the highest values of haplotype diversity, and  $\theta_\pi$ ,  
7 while Ejutla B and La Mesa have comparable values of these summary statistics, and San  
8 Lorenzo has much lower values. Additionally, Tajima's D is strongly negative in the two  
9 Ejutla populations and La Mesa, but is less negative in San Lorenzo (Table 2). *need to*  
10 *reference Hopscotch frequencies in supplemental table somewhere* For Region 2, haplotype diversity and  
11  $\theta_\pi$ , are similar for Ejutla A and Ejutla B, while La Mesa and San Lorenzo have slightly  
12 lower values for these statistics (Table 2). Tajima's D is positive in all populations except  
13 San Lorenzo, *is the table wrong? MSA is the only negative value in the table* indicating an excess of  
14 low frequency variants in this population (Table 2). Pairwise values of  $F_{ST}$  within  
15 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	<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

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.

## 7 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
<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

1 between populations with (average  $r^2$  of 0.085) and without (average  $r^2 = 0.082$ ) the  
 2 *Hopscotch* genotype. In fact, average  $r^2$  is lower in the *tb1* region ( $r^2 = 0.056$ ) than  
 3 across the rest of chromosome 1 ( $r^2 = 0.083$ ) (3). *LV, please go through and make sure the data*  
 4 *entered into all the tables is correct. In Table3, both sequenced regions were labeled as "Region 1". I changed the*  
 5 *second to Region 2 but don't know if the data in this column are really from Region 2*  
 6 The lack of clustering of *Hopscotch* genotypes in our NJ tree as well as the lack of LD  
 7 around *tb1* does not support the hypothesis that the *Hopscotch* insertion in these  
 8 populations of *parviglumis* is the result of recent introgression. However, to further  
 9 explore this hypothesis we performed a STRUCTURE analysis using Illumina  
 10 MaizeSNP50 data from four of our *parviglumis* populations (EjuA, EjuB, MSA, and  
 11 SLO) and the maize 282 diversity panel (Cook et al., 2012; Pyhäjärvi et al., 2013). The  
 12 linkage model implemented in STRUCTURE can be used to identify ancestry of blocks of  
 13 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	<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

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

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

Population	<i>tb1</i> region		Chr 1	
	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

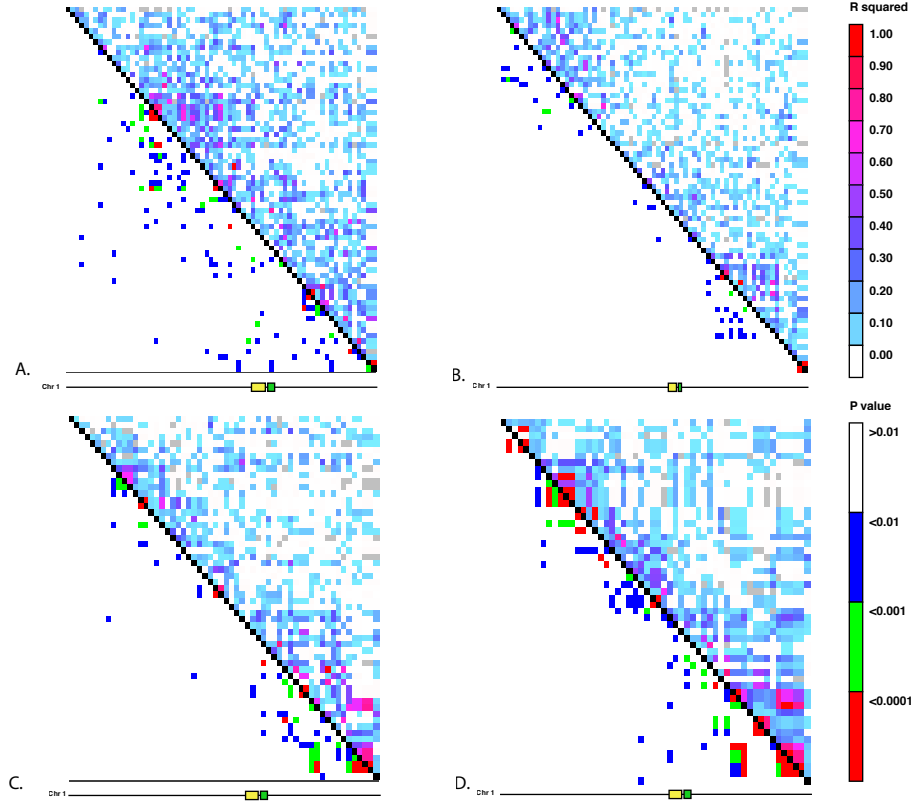


Figure 2: Linkage disequilibrium for SNPs in Mb 261-268 on chromosome 1. The yellow rectangle indicates the location of the *Hopscotch* insertion and the green represents the *tb1* ORF. A) Ejutla A; B) Ejutla B; C) La Mesa; D). San Lorenzo

## 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*



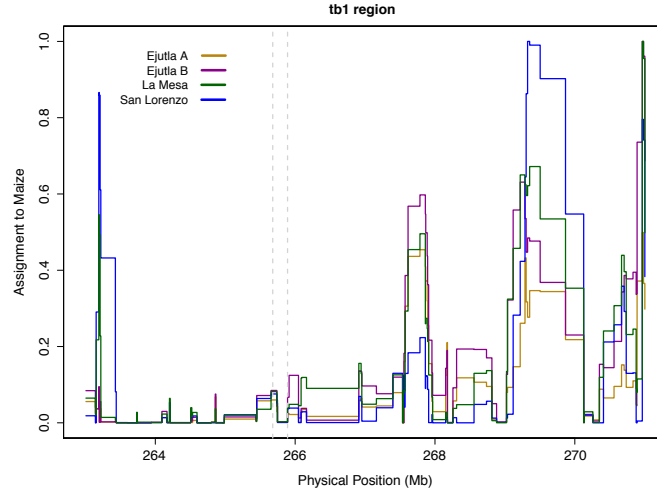


Figure 3: STRUCTURE assignment to maize across a section of chromosome 1. The dotted lines mark the beginning of the sequenced region 50kb upstream (Sequenced region 2) and the end of the *tb1* ORF.

- 1 segregating at a frequency of 0.65 with no significant deviations from expected
- 2 frequencies under Hardy-Weinberg equilibrium. After performing a repeated measures
- 3 ANOVA between our transformed tillering index data and *Hopscotch* genotype we find a
- 4 weak positive correlation between presence of the *Hopscotch* and tillering index on day 40
- 5 ( $p=0.0848$ ), a result indicating the *Hopscotch* may actually increase tillering in
- 6 *parviglumis* in contrast to its phenotypic effect in maize. We find no correlation between
- 7 tillering index and genotype on any other day (4). Additionally we find no significant
- 8 correlation between tiller number and *Hopscotch* genotype, or culm diameter and
- 9 *Hopscotch* genotype in Phenotyping 1.
- 10 We performed a second grow-out of *parviglumis* from San Lorenzo (Phenotyping 2)
- 11 to assess whether lighting conditions or sample size may have affected our ability to
- 12 detect an effect of *tb1*. For the second grow-out we measured tillering index every five

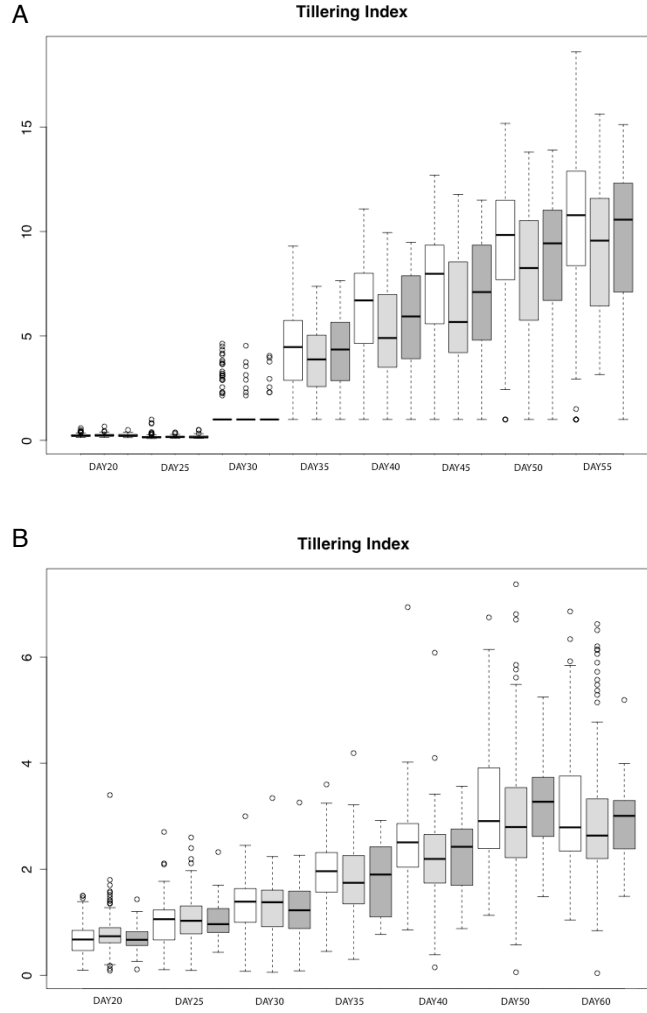


Figure 4: Box-plots showing tillering index in greenhouse grow-outs for Phenotyping 1 (A) and Phenotyping 2 (B). White indicates individuals homozygous for the *Hopscotch*, light grey represents heterozygotes, and dark grey represents homozygotes for the *teosinte* (No *Hopscotch*) allele. Within boxes,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

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

## 8 DISCUSSION

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

17 In maize, alleles at important domestication loci (*RAMOSA1*, Sigmon and Vollbrecht  
18 2010; *barren stalk1*, Gallavotti et al. 2004; and *grassy tillers1*, Whipple et al. 2011) have  
19 been shown to have been selected from standing variation, suggesting that diversity  
20 already present in teosinte may have played an important role in the domestication of  
21 maize. The *teosinte branched1* gene has long been a central focus of research concerning  
22 maize domestication, and, while previous studies have suggested that differences in plant  
23 architecture between domesticated maize and teosinte are a result of selection on  
24 standing variation, little is known about variation at this locus in teosinte (Clark et al.,  
25 2006; Studer et al., 2011). Studer et al. (2011) genotyped 90 accessions of teosinte (inbred  
26 and outbred), providing the first evidence that the *Hopscotch* insertion is segregating in

1 teosinte (Studer et al., 2011).

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

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

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

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

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

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

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

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

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

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

1 and other loci involved in branching architecture may also play roles in the  
2 regulation of tillering in teosinte. Finally, although we see no clear evidence of recent  
3 strong selection, the high frequency of the *Hopscotch* insertion in a number of  
4 populations continues to suggest to us that it plays an ecological role in teosinte. In the  
5 future, additional experiments will be needed to examine expression levels of *tb1* and  
6 additional loci involved in branching architecture (e.g. *gt1*, *tru1*, and *ter1*) in conjunction  
7 with a more exhaustive phenotyping and genotyping assay. *why not Phyb and phya? Are they*  
8 *necessary to include? I'd had them in before in a paragraph but had been voted out I'd ditch gt1 tru1 ter1 and*  
9 *maybe just cite some people including phyb etc.*  
10 *please check format of supp figs and tables; some are running off the page. you can use "longtable" to fix that*  
11 *(ask Paul for example). check fig/table references, bibliography, etc. what does "rotation" mean in supp. table 3?*  
12 *it isn't mentioned in methods. please check that all the tables and figs (including supplement) are referenced in the*  
13 *text.*



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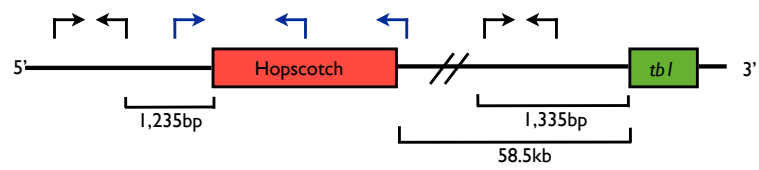
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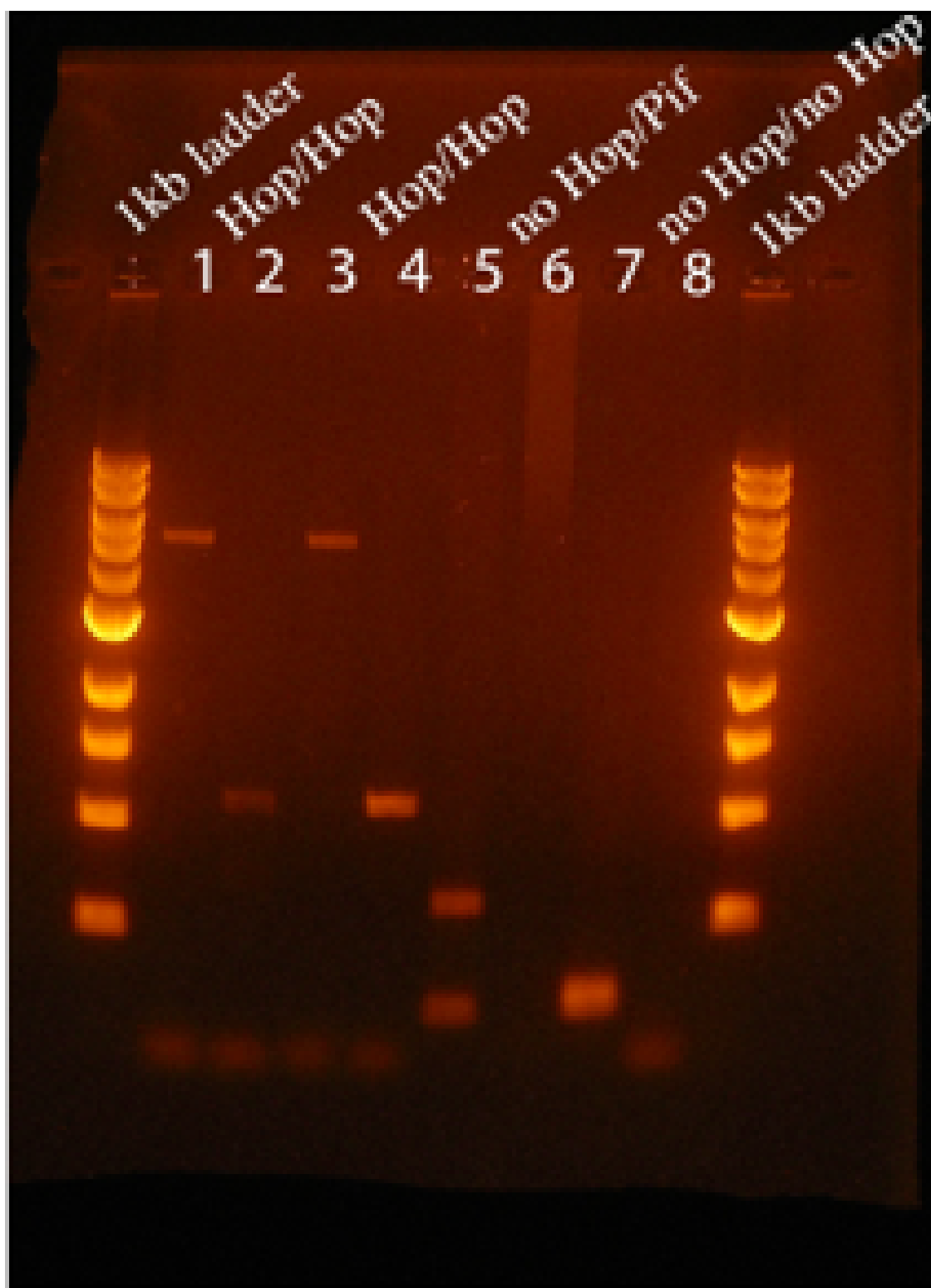
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- 22 Supplementary Materials









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	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	<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	bio4	0.244
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