

Vann *et al.*: Variation in teosinte at the *tb1* locus

Natural variation in teosinte at the domestication locus *teosinte branched1* (*tb1*)¹

Laura Vann², Thomas Kono^{2,3}, Tanja Pyhäjärvi^{2,4}, Matthew B. Hufford^{2,5,7}, and Jeffrey
Ross-Ibarra^{2,6,7}

²Department of Plant Sciences, University of California Davis

³Department of Agronomy and Plant Genetics, University of Minnesota Twin Cities

⁴Department of Biology, University of Oulu

⁵Department of Ecology, Evolution, and Organismal Biology, Iowa State University

⁶Center for Population Biology and Genome Center, University of California Davis

⁷Author for Correspondence

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1 Abstract

2 *Premise of the study:* The *teosinte branched1* (*tb1*) gene is a major QTL controlling
3 branching differences between maize and its wild progenitor, teosinte. Previous work has
4 shown that the insertion of a transposable element (*Hopscotch*) upstream of *tb1* enhances
5 the gene's expression, causing much of the reduction in tillering observed in maize.
6 Observations of the maize *tb1* allele in teosinte and estimates of an age of insertion of the
7 *Hopscotch* element that predates domestication led us to investigate its prevalence and
8 potential role in teosinte.

9 *Methods:* Prevalence of the *Hopscotch* element was assessed across an Americas-wide
10 sample of 1110 maize and teosinte individuals using a co-dominant PCR assay.
11 Population genetic summaries were calculated for a subset of individuals from four
12 teosinte populations in central Mexico. Phenotypic data were also collected from a single
13 teosinte population where *Hopscotch* was found segregating.

14 *Key results:* Genotyping results suggest the *Hopscotch* element is at higher than expected
15 frequency in teosinte. Analysis of linkage disequilibrium near *tb1* does not support recent
16 introgression of the *Hopscotch* allele from maize into teosinte. Population genetic
17 signatures are consistent with selection on this locus revealing a potential ecological role
18 for *Hopscotch* in teosinte. Finally, two greenhouse experiments with teosinte do not
19 suggest *tb1* controls tillering in natural populations.

20 *Conclusions:* Our findings suggest the role of *Hopscotch* differs between maize and
21 teosinte. Future work should assess *tb1* expression levels in teosinte with and without the
22 *Hopscotch* and more comprehensively phenotype teosinte to assess the ecological
23 significance of the *Hopscotch* insertion and, more broadly, the *tb1* locus in teosinte.

24 **Key words:** domestication; maize; teosinte; *teosinte branched1*

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

24 **Sequencing**—In addition to genotyping, we chose a subset of *parviglumis*
25 individuals for sequencing. We chose twelve individuals from each of four populations
26 from Jalisco state, Mexico (San Lorenzo, La Mesa, Ejutla A, and Ejutla B). For

1 amplification and sequencing, we selected two regions approximately 600bp in size from
2 within the 5' UTR of *tb1* (Region 1) and from 1,235bp upstream of the start of the
3 *Hopscotch* (66,169bp upstream from the start of the *tb1* ORF; Region 2). We designed
4 the following primers using PRIMER3 (Rozen and Skaletsky, 2000): for the 5' UTR,
5 5'-GGATAATGTGCACCAGGTGT-3' and 5'-GCGTGCTAGAGACACYTGTTGCT-3'; for the
6 66kb upstream region, 5'-TGTCCTCGCCGCAACTC-3' and
7 5'-TGTACGCCCCGCCCTCATCA-3' (Fig. S1). We used Taq polymerase (New England
8 Biolabs Inc., Ipswich, Massachusetts, USA) and the following thermal cycler conditions to
9 amplify fragments: 94°C for 3 min, 30 cycles of 92°C for 40 s, annealing for 1 min, 72°C
10 for 40 s, and a final 10 min extension at 72°C. Annealing temperatures for Region 1 and
11 Region 2 were 59.7°C and 58.8°C, respectively. To clean excess primer and dNTPs we
12 added two units of Exonuclease1 and 2.5 units of Antarctic Phosphatase to 8.0 μ L of
13 amplification product. This mix was placed on a thermal cycler with the following
14 program: 37°C for 30 min, 80°C for 15 min, and a final cool-down step to 4°C.

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

23 Amplification products with successful incorporation of our insert were cleaned using
24 Exonuclease 1 and Antarctic Phosphatase following the procedures detailed above, and
25 sequenced with vector primers M13 Forward and M13 Reverse using Sanger sequencing at
26 the College of Agriculture and Environmental Sciences (CAES) sequencing center at UC
27 Davis. We aligned and trimmed primer sequences from resulting sequences using the

1 software Geneious (Kearse et al., 2012). Following alignment, we verified singleton SNPs
2 by sequencing an additional one to four colonies from each clone. If the singleton was not
3 present in these additional sequences it was considered an amplification or cloning error,
4 and we replaced the base with the base of the additional sequences. If the singleton
5 appeared in at least one of the additional sequences we considered it a real variant and
6 kept it for further analyses.

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

24 **Sequence analysis**—For population genetic analyses of sequenced Region 1 and
25 sequenced Region 2 we used the Libsequence package (Thornton, 2003) to calculate
26 pairwise F_{ST} between populations and to calculate standard diversity statistics (number
27 of haplotypes, haplotype diversity, Watterson’s estimator $\hat{\theta}_W$, pairwise nucleotide

1 diversity $\hat{\theta}_\pi$, and Tajima's D). To produce a visual representation of differentiation
2 between sequences and examine patterns in sequence clustering by *Hopscotch* genotype
3 we used Phylip (<http://evolution.genetics.washington.edu/phylip.html>), creating
4 neighbor-joining trees with bootstrap-supported nodes (100 repetitions). For creation of
5 trees we also included homologous sequence data from Maize HapMapV2 (Chia et al.,
6 2012) for teosinte inbred lines (TILs), some of which are known to be homozygous for the
7 *Hopscotch* insertion (TIL03, TIL17, TIL09), as well as 59 lines of domesticated maize.

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

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

27 **Phenotyping of *parviglumis***—To investigate the phenotypic effects of the

1 *Hopscotch* insertion in teosinte, we conducted an initial phenotyping trial (Phenotyping
2 1). We germinated 250 seeds of *parviglumis* collected in Jalisco state, Mexico (population
3 San Lorenzo) (Hufford, 2010) where the *Hopscotch* is segregating at highest frequency
4 (0.44) in our initial genotyping sample set. In order to maximize the likelihood of finding
5 the *Hopscotch* in our association population we selected seeds from sites where genotyped
6 individuals were homozygous or heterozygous for the insertion. We chose between 10-13
7 seeds from each of 23 sampling sites. We treated seeds with fungicide and germinated
8 them in petri dishes with filter paper. Following germination, 206 successful germinations
9 were planted into one-gallon pots with potting soil and randomly spaced one foot apart
10 on greenhouse benches. Plants were watered three times a day by hand and with an
11 automatic drip containing 10-20-10 fertilizer.

12 Starting on day 15, we measured tillering index as the ratio of the sum of tiller
13 lengths to the height of the plant (Briggs et al., 2007). Following initial measurements,
14 we phenotyped plants for tillering index every 5 days through day 40, and then on day 50
15 and day 60. On day 65 we measured culm diameter between the third and fourth nodes
16 of each plant. Culm diameter is not believed to be correlated with tillering index or
17 variation at *tb1*. Following phenotyping we extracted DNA from all plants using a
18 modified SDS extraction protocol (<http://www.ars.usda.gov>). *what is this url?* We
19 genotyped individuals for the *Hopscotch* insertion following the protocols listed above.
20 Based on these initial data, we conducted a *post hoc* power analysis using data from day
21 40 of Phenotyping 1, indicating that a minimum of 71 individuals in each genotypic class
22 would be needed to detect the observed effect of the *Hopscotch* on tillering index.

23 We performed a second phenotyping experiment (Phenotyping 2) in which we
24 germinated 372 seeds of *parviglumis*, choosing equally between sites previously
25 determined to have or not have the *Hopscotch* insertion. Seeds were germinated and
26 planted on day 7 post fruit-case removal into two gallon pots. Plants were watered twice
27 daily, alternating between fertilized and non-fertilized water. We began phenotyping

1 successful germinations (302 plants) for tillering index on day 15 post fruit-case removal,
2 and phenotyped every five days until day 50. At day 50 we measured culm diameter
3 between the third and fourth nodes. We extracted DNA and genotyped plants following
4 the same guidelines as in Phenotyping 1.

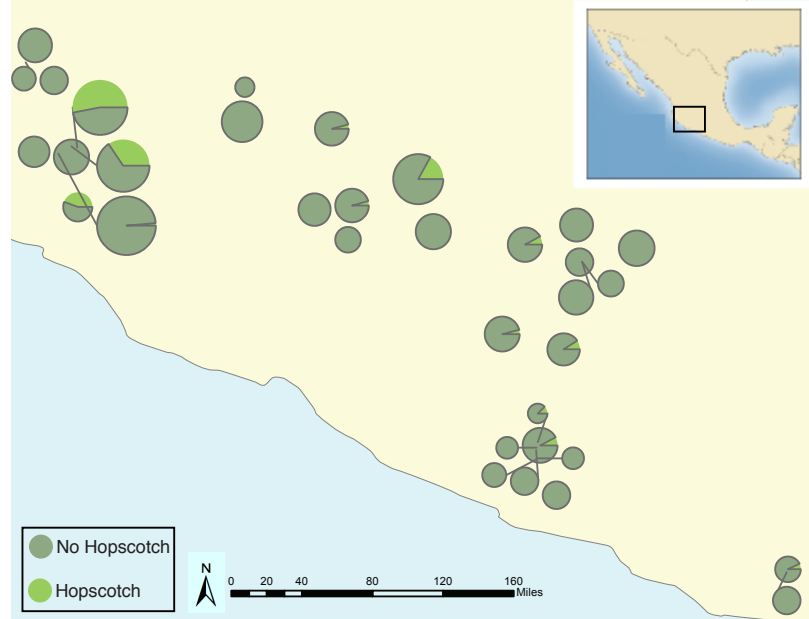
5 Tillering index data for each genotypic class did not meet the criteria for a repeated
6 measures ANOVA, so we transformed the data using a Box-Cox transformation ($\alpha = 0$
7 *what is the alpha value here?* ; Car Package for R, Fox and Weisberg 2011) to improve the
8 normality and homogeneity of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

15 RESULTS

16 **Genotyping**—Genotype of the *Hopscotch* insertion was confirmed with two PCRs
17 for 837 individuals. Among the 247 maize landrace accessions genotyped, all but eight
18 were homozygous for the presence of the insertion (Table S1 and Table S2). Within our
19 *parviglumis* and *mexicana* samples we found the *Hopscotch* insertion segregating in 37
20 and 4 populations, respectively, and at highest frequency in the states of Jalisco, Colima,
21 and Michoacán in central-western Mexico (Fig. 1).

22 Using our *Hopscotch* genotyping, we calculated differentiation between populations
23 (F_{ST}) and subspecies (F_{CT}) for populations in which we sampled 8 or more alleles. We
24 found that $F_{CT} = 0$, and levels of F_{ST} among populations within each subspecies (0.22)
25 and among all populations (0.23) *are these an average of pairwise or is this calculated among all pops?*
26 are similar to those reported genome-wide in previous studies (Pyhäjärvi et al. 2013;

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 Table 1). Although we found large variation in *Hopscotch* allele frequency among our
2 populations, BayEnv analysis did not indicate a correlation between the *Hopscotch*
3 insertion and environmental variables (all Bayes Factors < 1; Table S3).
4 **Sequencing**—To investigate patterns of sequence diversity and linkage
5 disequilibrium (LD) in the *tb1* region, we sequenced two small (<1kb) regions upstream
6 of the *tb1* ORF in four populations. After alignment and singleton checking we recovered
7 48 and 40 segregating sites for the 5' UTR region (Region 1) and the 66kb upstream
8 region (Region 2), respectively. For Region 1, Ejutla A has the highest values of
9 haplotype diversity, and θ_π , while Ejutla B and La Mesa have comparable values of these
10 summary statistics, and San Lorenzo has much lower values. Additionally, Tajima's D is
11 strongly negative in the two Ejutla populations and La Mesa, but is less negative in San
12 Lorenzo (Table 2). *need to reference Hopscotch frequencies in supplemental table somewhere* For Region
13 2, haplotype diversity and θ_π , are similar for Ejutla A and Ejutla B, while La Mesa and
14 San Lorenzo have slightly lower values for these statistics (Table 2). Tajima's D is
15 positive in all populations except San Lorenzo, *is the table wrong? MSA is the only negative value in*

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

1 *the table* indicating an excess of low frequency variants in this population (Table 2).
2 Pairwise values of F_{ST} within population pairs Ejutla A/Ejutla B and San Lorenzo/La
3 Mesa are 0 for both sequenced regions as well as for the *Hopscotch* *table 1 shows 0.016 for*
4 *hopscotch, not 0. which is right?* , while they are high for other population pairs (Table 1).
5 Neighbor joining trees of our sequence data and data from the teosinte inbred lines (TILs;
6 data from Maize HapMapV2, Chia et al. 2012) do not reveal any clear clustering pattern
7 with respect to population or *Hopscotch* genotype (Figure S3); individuals within our
8 sample that have the *Hopscotch* insertion do not group with the teosinte inbred lines or
9 domesticated maize that have the *Hopscotch* insertion.

10 **Evidence of introgression**—The highest frequency of the *Hopscotch* insertion in
11 teosinte was found in *parviglumis* sympatric with cultivated maize. Our initial hypothesis
12 was that the high frequency of the *Hopscotch* element in these populations could be
13 attributed to introgression from maize into teosinte. To investigate this possibility we
14 examined overall patterns of linkage disequilibrium across chromosome one and
15 specifically in the *tb1* region. If the *Hopscotch* is found in these populations due to recent
16 introgression we would expect to find large blocks of linked markers near this element.
17 We find no evidence of elevated linkage disequilibrium between the *Hopscotch* and SNPs

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

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 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
7 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

8 **Phenotyping**—To assess the contribution of *tb1* to phenotypic variation in tillering

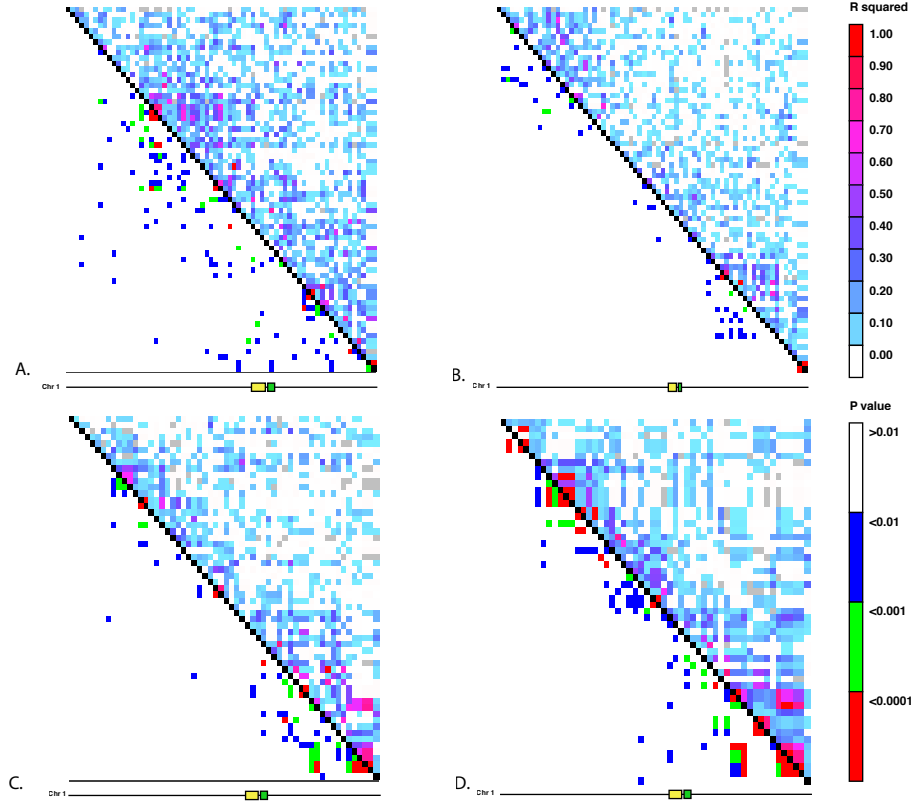


Figure 2: Linkage disequilibrium for SNPs in Mb 261-268 on chromosome 1. The yellow rectangle indicates the location of the *Hopscotch* insertion and the green represents the *tb1* ORF. A) Ejutla A; B) Ejutla B; C) La Mesa; D). San Lorenzo

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- 1 in a natural population, we grew plants from seed sampled from the San Lorenzo
- 2 population of *parviglumis*, which had a high mean frequency (0.44) of the *Hopscotch*
- 3 insertion based on our initial genotyping. We measured tillering index (TI), the ratio of
- 4 the sum of tiller lengths to plant height, for 216 plants (Phenotyping 1) from within the
- 5 San Lorenzo population, and genotyped plants for the *Hopscotch* insertion. We found the
- 6 *Hopscotch* segregating at a frequency of 0.65 with no significant deviations from expected

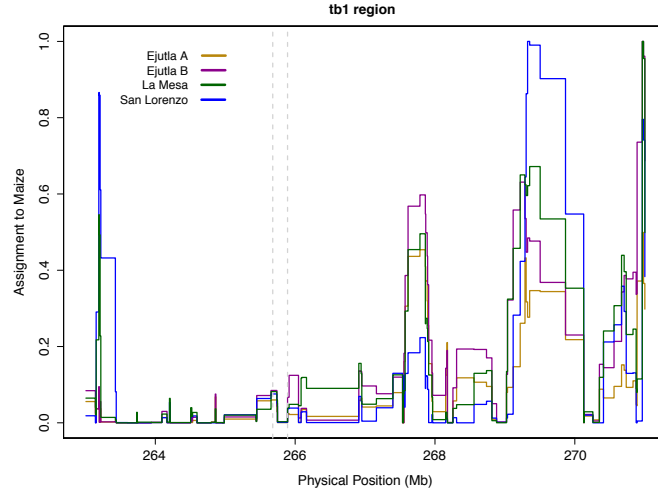


Figure 3: STRUCTURE assignment to maize across a section of chromosome 1. The dotted lines mark the beginning of the sequenced region 50kb upstream (Sequenced region 2) and the end of the *tb1* ORF.

- 1 frequencies under Hardy-Weinberg equilibrium. After performing a repeated measures
- 2 ANOVA between our transformed tillering index data and *Hopscotch* genotype we find a
- 3 weak positive correlation between presence of the *Hopscotch* and tillering index on day 40
- 4 ($p=0.0848$), a result indicating the *Hopscotch* may actually increase tillering in
- 5 *parviglumis* in contrast to its phenotypic effect in maize. We find no correlation between
- 6 tillering index and genotype on any other day (4). Additionally we find no significant
- 7 correlation between tiller number and *Hopscotch* genotype, or culm diameter and
- 8 *Hopscotch* genotype in Phenotyping 1.
- 9 We performed a second grow-out of *parviglumis* from San Lorenzo (Phenotyping 2)
- 10 to assess whether lighting conditions or sample size may have affected our ability to
- 11 detect an effect of *tb1*. For the second grow-out we measured tillering index every five
- 12 days through day 50 for 302 plants. We found the *Hopscotch* allele segregating at a

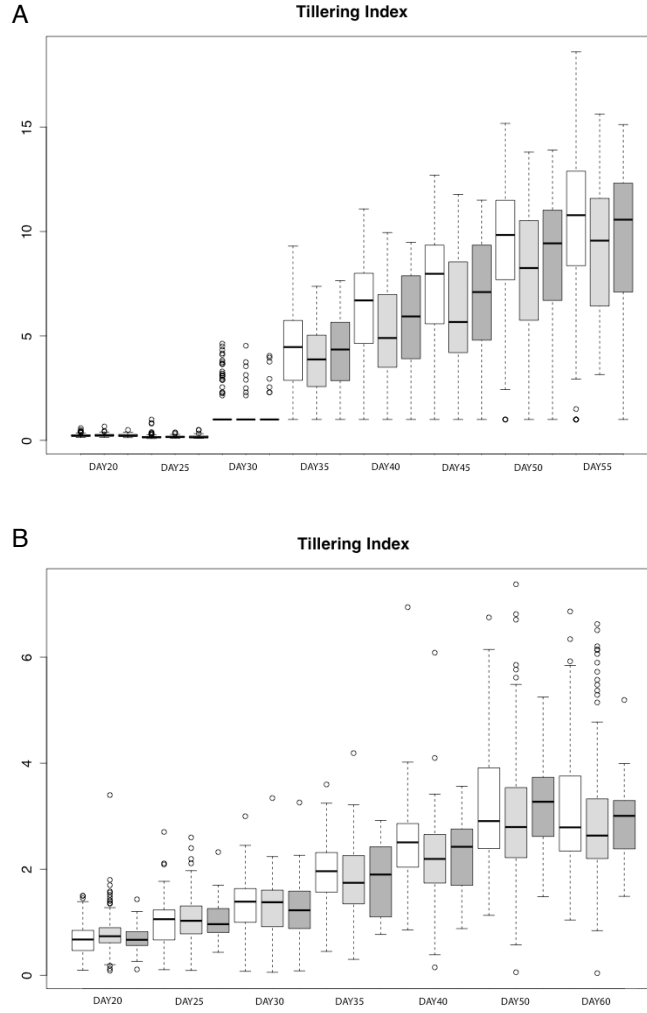


Figure 4: Box-plots showing tillering index in greenhouse grow-outs for Phenotyping 1 (A) and Phenotyping 2 (B). White indicates individuals homozygous for the *Hopscotch*, light grey represents heterozygotes, and dark grey represents homozygotes for the *teosinte* (No *Hopscotch*) allele. Within boxes, 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 frequency of 0.69, *is it in HWE in this pop?* with a 0.6 frequency of *Hopscotch* homozygotes,
2 and a 0.2 frequency of both heterozygotes and homozygotes for the teosinte allele. We
3 found similar patterns, with a weak positive correlation between tillering index and
4 *Hopscotch* genotype at day 40 ($p < 0.0611$), with no significant correlation on any day.
5 Similarly, relationships between *Hopscotch* genotype and tiller number and *Hopscotch*
6 genotype and culm diameter were not significant.

7 DISCUSSION

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

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

26 Given that the *Hopscotch* insertion has been estimated to predate the domestication

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

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

26 Although recent introgression seems unlikely, we cannot rule out ancient introgression
27 as an explanation for the presence of the *Hopscotch* in these populations. If the

1 *Hopscotch* allele was introgressed in the distant past, recombination may have broken up
2 LD, a process that would be consistent with our data. We find this scenario less
3 plausible, however, as there is no reason why gene flow should have been high in the past
4 but absent in present-day sympatric populations. In fact, early generation maize-teosinte
5 hybrids are common in these populations today (MB Hufford, pers. observation), and
6 genetic data support ongoing gene flow between domesticated maize and both *mexicana*
7 and *parviglumis* in a number of sympatric populations (Hufford et al., 2013; Ellstrand
8 et al., 2007; van Heerwaarden et al., 2011).

9 Remaining explanations for differential frequencies of the *Hopscotch* among teosinte
10 populations include both genetic drift and natural selection. Drift may have played a role
11 in the San Lorenzo *parviglumis* population. Previous studies using both SSRs and
12 genome-wide SNP data have found evidence for a population bottleneck in the San
13 Lorenzo population (Hufford, 2010; Pyhäjärvi et al., 2013), and the lower levels of
14 sequence diversity in this population in the 5' UTR (Region 1) coupled with more
15 positive values of Tajima's D are consistent with these earlier findings suggesting a
16 bottleneck. *deviations from HWE may be consistent too if we see excess of homozygotes. do we?* Such
17 population bottlenecks can exaggerate the effects of genetic drift through which the
18 *Hopscotch* allele may have risen to high frequency entirely by chance. This bottleneck,
19 however, does not explain the high frequency of the *Hopscotch* in multiple populations in
20 the Jalisco cluster. Moreover, available information on diversity and population structure
21 among Jaliscan populations (Hufford, 2010; Pyhäjärvi et al., 2013) is not suggestive of
22 recent colonization or other demographic events that would predict a high frequency of
23 the allele across populations. Finally, values of the Tajima's D statistic in the 5' UTR of
24 *tb1* are suggestive of natural selection acting upon the gene in natural population of
25 *parviglumis*. Whereas the genome-wide average of Tajima's D in genic regions of
26 *parviglumis* is 0.45 (Hufford et al., 2012b), the statistic is quite negative in the 5' UTR of
27 *tb1* (Table 2). This result is consistent with repeated selective sweeps near *tb1* and a

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

7 Significant effects of the *Hopscotch* insertion on lateral branch length, number of
8 cupules, and tillering index in domesticated maize have been well documented (Studer
9 et al., 2011). Weber et al. (2007) have described significant phenotypic associations
10 between markers in and around *tb1* and lateral branch length and female ear length in a
11 sample from 74 natural populations of *parviglumis* (Weber et al., 2007); however, these
12 data did not include markers from the *Hopscotch* region 66kb upstream of *tb1*. Our study
13 is the first to explicitly examine the phenotypic effects of the *Hopscotch* insertion across a
14 wide collection of individuals sampled from natural populations of teosinte. We have
15 found no significant effect of the *Hopscotch* insertion on tillering index or tiller number, a
16 result that is discordant with its clear phenotypic effects in maize. One interpretation of
17 this result would be that the *Hopscotch* controls tillering in maize (Studer et al., 2011),
18 but tillering in teosinte is affected by variation at other loci. Consistent with this
19 interpretation, *tb1* is thought to be part of a complex pathway controlling branching,
20 tillering and other phenotypic traits (Kebrom and Brutnell, 2007; Clark et al., 2006). A
21 recent study by Studer and Doebley (2012) examined variation across traits in a
22 three-taxa allelic series at the *tb1* locus. Studer and Doebley (2012) introgressed nine
23 unique teosinte *tb1* segments (one from *Zea diploperennis*, and four each from *mexicana*
24 and *parviglumis*) into an inbred maize background and investigated phenotypic effects.
25 Phenotypes were shown to cluster by taxon, indicating *tb1* may underlie morphological
26 diversification of *Zea*. Additional analysis in Studer and Doebley (2012) suggested
27 tillering index was controlled both by *tb1* and loci elsewhere in the genome. Clues to the

1 identity of these loci may be found in QTL studies that have identified loci controlling
2 branching architecture (*e.g.*, Doebley and Stec 1991, 1993). Many of these loci (*grassy*
3 *tillers*, *gt1*; *tassel-replaces-upper-ears1*, *tru1*; *terminal ear1*, *ter1*) have been shown to
4 interact with *tb1* (Whipple et al., 2011; Li, 2012), and both *tru1* and *ter1* affect the same
5 phenotypic traits as *tb1* (Doebley et al., 1995). *tassel-replaces-upper-ears1* (*tru1*), for
6 example, has been shown to act either epistatically or downstream of *tb1*, affecting both
7 branching architecture (decreased apical dominance) and tassel phenotypes (shortened
8 tassel and shank length and reduced tassel number; Li 2012). Variation in these
9 additional loci may have affected tillering in our collections and contributed to the lack of
10 correlation we see between *Hopscotch* genotype and tillering.

11 In conclusion, our findings demonstrate that the *Hopscotch* allele is more widespread
12 in populations of *parviglumis* and *mexicana* than previously thought. Analysis of linkage
13 using SNPs from across chromosome 1 does not suggest that the *Hopscotch* allele is
14 present in these populations due to recent introgression; however, it seems unlikely that
15 the insertion would have drifted to high frequency in multiple populations. We do,
16 however, find preliminary evidence of selection on the *tb1* locus in *parviglumis*; this
17 coupled with our observation of high frequency of the *Hopscotch* insertion in a number of
18 populations suggests that the locus plays an ecological role in teosinte. In contrast to
19 domesticated maize, the *Hopscotch* insertion in *parviglumis* does not appear to reduce
20 tillering. Other loci involved in branching architecture may regulate tillering in teosinte.
21 Future studies should examine expression levels of *tb1* in teosinte with and without the
22 *Hopscotch* insertion and further characterize the effects of additional loci involved in
23 branching architecture (*e.g.* *gt1*, *tru1*, and *ter1*). These data, in conjunction with more
24 exhaustive phenotyping, should help reveal the ecological significance of the domesticated
25 *tb1* allele in natural populations of teosinte. *why not Phyb and phya? Are they necessary to*
26 *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*
27 *some people including phyb etc.*

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

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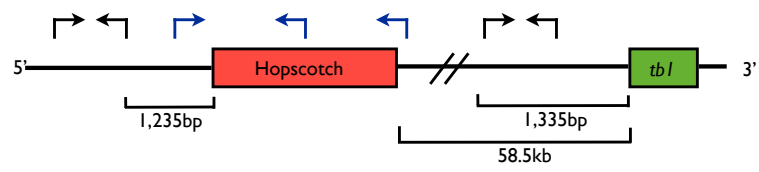
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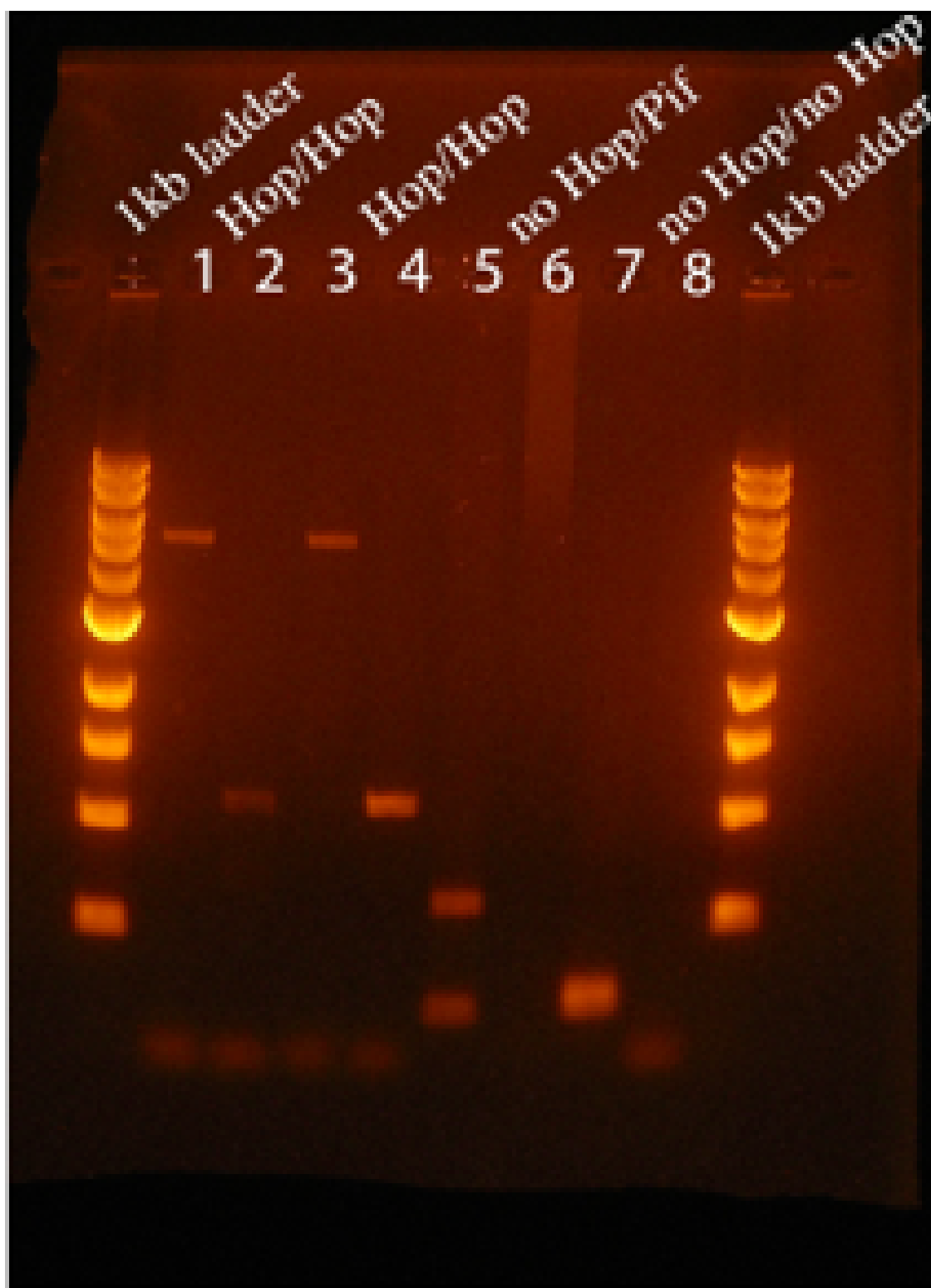
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22 Supplementary Materials







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	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