Title: Population Level Variation of Transposable Elements in a Maize Diversity Panel

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ABSTRACT

Intact transposable elements (TEs) account for 65% of the maize genome and can impact gene function and regulation. Although TEs comprise the majority of the maize genome and affect important phenotypes, genome wide patterns of TE polymorphisms in maize have only been studied in a handful of maize genotypes, due to the challenging nature of assessing highly repetitive sequences. We implemented a method to use short read sequencing data from 509 diverse inbred lines to classify the presence/absence of 494,564 non-redundant TEs that were previously annotated in four genome assemblies including B73, Mo17, PH207, and W22. Different orders of TEs (i.e. LTRs, Helitrons, TIRs) had different frequency distributions within the population. Older LTRs were generally more frequent in the population than younger LTRs, though high frequency very young TEs were observed. Age and frequency estimates of nested elements and the outer elements in which they insert revealed that most nesting events occurred very near the timing of the outer element insertion. TEs within genes were at higher frequency than those that were outside of genes and this is particularly true for those not inserted into introns. Many TE insertional polymorphisms observed in this population were not tagged by SNP markers and therefore not captured in previous SNP based marker-trait association studies. This study provides a population scale genome-wide assessment of TE variation in maize, and provides valuable insight on variation in TEs in maize and factors that contribute to this variation.

INTRODUCTION

Transposable elements (TEs) are present in all eukaryotic genomes (BENNETZEN 2000; WICKER *et al.* 2007). In maize, 65% of the genome is made up of intact TEs (JIAO *et al.* 2017), and another 20% is comprised of fragmented TEs (SCHNABLE *et al.* 2009). There are many examples of phenotypic effects of TEs from null mutations, such as maize kernel color (SELINGER AND CHANDLER 2001), white wine grapes (CADLE-DAVIDSON AND OWENS 2008), and color variation in the common morning glory (CLEGG AND DURBIN 2000). TE insertions can also positively or negatively affect gene regulatory functions, such as insertion of a long terminal repeat LTR retrotransposon into the promoter region of the Ruby gene in oranges that leads to red fruit flesh of blood oranges (BUTELLI *et al.* 2012), and an LTR retrotransposon that is associated with red skin color in apples (ZHANG *et al.* 2019). TE insertions in maize have also been associated with genes that are upregulated in response to abiotic stress (MAKAREVITCH *et al.* 2015).

TEs are classified into two classes depending on how they replicate and from there into superfamilies and families by sequence similarity (WICKER *et al.* 2007). Class I elements, or retrotransposons, replicate via an RNA intermediate (BENNETZEN 2000; LISCH 2013). Long terminal repeat retrotransposons (LTR) are the most abundant type of retrotransposons in maize (BENNETZEN 2000) and intact elements account for over half of the maize genome by sequence length (ANDERSON *et al.* 2019; STITZER *et al.* 2019). Class II elements, or DNA transposable elements, replicate via a DNA intermediate, and the two largest orders are terminal inverted repeat (TIR) and Helitron elements. TIRs are defined by terminal inverted repeat sequences at both ends of the TE (WICKER *et al.* 2007) and intact TIRs make up around 3% of the maize genome (ANDERSON *et al.* 2019). Helitrons are defined by their 'rolling circle'

replication mechanism (LISCH 2013) and intact Helitrons make up around 4% of the maize genome (ANDERSON *et al.* 2019). TEs are found throughout the maize genome, they can be found near and even within genes, can be anywhere from a few hundred base pairs to >10 kb in length (BENNETZEN 2000), and range in age from very recent insertions to >2 million years old insertions (STITZER *et al.* 2019).

Studies done on TEs in maize have shown extensive variation in TE insertion presence/absence patterns at specific loci across maize inbred lines (SANMIGUEL *et al.* 1996; FU AND DOONER 2002; MORGANTE *et al.* 2005; DOONER *et al.* 2019). Early work on the *bronze* locus in multiple maize lines found that different lines differed in not only the gene order and content but also in TE content (FU AND DOONER 2002). More recent work on mutations in the same region found that not only were high mutation rates due to TE insertions, but that different TEs were inserting in different maize lines (DOONER *et al.* 2019). Whole genome analysis of four maize genomes with *de novo* TE annotations revealed extensive TE polymorphism between maize lines on a whole genome scale (ANDERSON *et al.* 2019). On average, about 500 Mb of TE sequence, or ~20% of the maize genome, was variable between the four inbred lines (B73, Mo17, PH207 and W22). Another 1.6 Gb of TE sequence was only shared between two or three of the lines.

Despite the widespread prevalence and polymorphism of TEs in the maize pan-genome, as well as many examples connecting TE insertions to functional phenotypic variation, there have been very few scans of specific TE insertion frequencies in divergent maize populations. The analysis of the frequency of TE insertions can provide insights into the level of variability for TEs and help understand the presence/absence of common and rare TE variants. To understand

patterns of TE polymorphism on a genome-wide scale, we utilized short read sequencing of 509 diverse maize lines to score the presence/absence of 494,564 non-redundant TEs that were annotated in four reference genome assemblies (B73, Mo17, PH207, and W22). This study provides a genome-wide analysis of TE presence/absence polymorphism across a large panel of diverse maize genotypes as we continue to try to understand how TEs contribute to phenotypic variation and adaptation within the species.

MATERIALS AND METHODS

Whole genome resequencing: A subset of 509 lines from the Wisconsin Diversity Panel (HANSEY *et al.* 2010; HIRSCH *et al.* 2014; MAZAHERI *et al.* 2019) was used for this study (Table S1). For 57 genotypes, available short-read sequence data was downloaded from the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA; Table S1). These samples ranged in theoretical coverage of 10-55x sequencing depth based an estimated genome size of 2.4 Gb. For 454 genotypes, plants were grown under greenhouse conditions (27C/24C day/night and 16 h light/8 h dark) with five plants of a single genotype per pot. Plants were grown in Metro-Mix 300 (Sun Gro Horticulture) with no additional fertilizer. Tissue was harvested for DNA extractions at the Vegetative 2 developmental stage. The newest leaf of each seedling in the pot was collected and immediately flash frozen in liquid nitrogen. Tissue was ground in liquid nitrogen using a mortar and pestle. DNA was extracted using a standard cetyltrimethylammonium bromide (CTAB) DNA extraction protocol (SAGHAI-MAROOF *et al.* 1984), and treated with 25 uL of PureLink RNase A (Invitrogen) at 39°C for 30 minutes. Genomic DNA for each genotype was submitted to Novogene (Novogene Co., Ltd., Beijing, China) for whole

genome sequencing with 150 base pair paired end reads generated on a HiSeq X Ten sequencing machine. For each genotype at least 20x theoretical sequencing depth was achieved.

Read Alignment and processing: Quality control analysis of the sequence data was conducted using fastqc version 0.11.7 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adapter sequence and low-quality base trimming was done using cutadapt version 1.18 (MARTIN 2011) and sickle version 1.33 (https://github.com/najoshi/sickle) both with default parameters. Sequence reads were aligned to the B73 v4 (JIAO *et al.* 2017), Mo17 v1 (SUN *et al.* 2018), PH207 v1 (HIRSCH *et al.* 2016), and W22 v1 (SPRINGER *et al.* 2018) genome assemblies. Alignment of reads was conducted using SpeedSeq version 0.1.2 (CHIANG *et al.* 2015), which efficiently parallelizes bwa mem (LI AND DURBIN 2009), with read groups labelled separately for each FASTQ. Alignments were subsequently filtered to require a minimum mapping quality of 20 using samtools view (LI *et al.* 2009). To assess sample integrity, single nucleotide polymorphisms (SNPs) were called relative to the B73 v4 genome assembly using Platypus v 0.8.1 (RIMMER *et al.* 2014) with default parameters. A subset of 50,000 random bi-allelic SNPs with less than 50% missing data and less than 10% heterozygosity was used to calculate Nei's pairwise genetic distances using StAMPP version 1.5.1 and a neighbor joining tree was generated with nj() in ape version 5.3 all using R version 3.6.2 (R DEVELOPMENT CORE TEAM 2020). Three samples with discordance to known pedigree relationships were removed from downstream analysis to result in the 509 genotypes included in Table S1.

Transposable element identification in the Wisconsin Diversity Panel: Transposable element (TE) annotations for the B73, Mo17, PH207, and W22 genome assemblies were obtained from https://mcstitzer.github.io/maize_TEs. Mean coverage over the genome windows adjacent to each of the TE boundaries was calculated using multicov within bedtools version 2.29.2 (QUINLAN AND HALL 2010). The windows ranged in size from 1 to 10 bp, and included the internal and external sequences that flanked the start and stop boundaries of each of the annotated TEs for four windows per TE (Figure S1). Coverage down sampling to 20%, 32%, 45%, 66% and 83% of their original coverage levels was performed using samtools view (LI *et al.* 2009). A non-redundant set of TEs with presence/absence scores based on whole-genome comparisons of B73, Mo17, PH207, and W22 was used as previously reported (ANDERSON *et al.* 2019) for analysis of the true positive, true absent, false positive, and false absent rates of the short read based presence/absence calls.

After assessing accuracy of different TE calling parameters the following criteria were chosen to balance accuracy and the number of ambiguous calls: 1) A TE was called present if the mean coverage over the 10 bp just after the start and just before the end of the TE were both greater than or equal to 8x depth, 2) A TE was called absent if the mean coverage over the 10 bp just after the start and just before the end of the TE were both less than 7x depth, and 3) all other TEs were called as ambiguous that did not meet either of these criterion. TEs for which the alignment of short reads to its own genome assembly did not result in a present call were removed from downstream analyses. The threshold for calling a TE as present in the reference genome samples was less stringent than the final selected criteria for other genotypes (greater than 0x coverage), because there was no need to balance calling a true present versus true

absent TE. Method development showed that a low coverage threshold, 1x or 2x coverage, for calling a TE as present had a very high accuracy rate, but that the accuracy of calling a TE as absent was much lower at this low coverage.

This method was applied to the short read alignments across the four genome assemblies for the full set of sequenced lines described above to classify a TE as present, absent, or ambiguous in a genotype. If a TE was classified as ambiguous in more than 25% of genotypes it was excluded from further analysis (B73 - File S1; Mo17 – File S2; PH207 – File S3; W22 – File S4). The non-redundant TE dataset from Anderson et al 2019 was used to combine TE population frequencies across homologous TEs from the different genome assemblies. The geometric mean of the population frequency for each TE was calculated between homologous TEs (File S5). TE metadata (e.g. location relative to the nearest gene or family) for the TE in the reference genome it was first identified in was used in downstream analyses using a previously described order for adding in TEs from each of the reference genome assemblies (ANDERSON *et al.* 2019).

Information about TE family/superfamily size and age of LTR retrotransposons was obtained from the previously published TE annotation gff files (ANDERSON *et al.* 2019). Gene annotations from B73 version 4 (JIAO *et al.* 2017), Mo17 (SUN *et al.* 2018), PH207 (HIRSCH *et al.* 2016), and W22 (SPRINGER *et al.* 2018) reference genomes were used to identify TE locations relative to genes. All metadata is included in File S5.

The Kolmogorov-Smirnov test was used to test whether the frequency distribution of nested and non-nested TEs was different. This test was implemented using the ks.test function in base R version 3.6.2 (R DEVELOPMENT CORE TEAM 2020).

SNP identification and analysis: Joint SNP calling was performed across all genotypes using *freebayes* v1.3.1-17 (GARRISON AND MARTH 2012) for the alignments to the B73 v4 reference genome assembly with scaffolds removed. Sites with less than 1x average coverage over the whole population or greater than 2x the population level mean coverage were excluded from SNP calling. SNP calls were quality filtered using GATK (v4.1.2) (VAN DER AUWERA *et al.* 2013) recommended filters as follows: QualByDepth (QD) less than 2, FisherStrand (FS) greater than 60, root mean square mapping quality (MQ) less than 40, MappingQualityRankSum (MQRankSum) less than -12.5, or ReadPosRankSum less than -8. If a SNP failed (e.g. MQ < 40) any one of those filters it was removed from the dataset. Finally, *vcftools* (v 0.1.13) (DANECEK *et al.* 2011) was used to filter sites for a minimum quality score of 30, a minimum allele count of 50 (called in at least 25 homozygotes or 50 heterozygotes) and to filter out genotypes called in fewer than 90% of the individuals in the population. In total, 3,146,253 SNPs remained after all of these filtering steps (File S6).

These SNP calls were used to conduct linkage disequilibrium analysis with the TE presence/absence scores described above. For this analysis, TE presence/absence data from only the alignments to B73 reference genome assembly were used. Any SNPs located within TEs were removed. Linkage disequilibrium between SNPs and TEs was calculated using plink v1.90b6.16 (PURCELL *et al.* 2007) with the --make-founders option to calculate LD among all inbred lines, --allow-extra-chr to calculate LD in extra scaffolds, --ld-window-r2 0 to report r^2 in the 0 to 1 range (default is 0.2 to 1), --ld-window 1000000 --ld-window-kb 1000 to calculate LD within 1mb windows, and --r2 dprime with-freqs to report both D' and r^2 and to display minor

allele frequencies in the output. If more than one SNP had the same highest LD in either the r^2 or D' analysis, the SNP that was physically closest to the SNP was used for downstream analyses. Principal components analysis of SNPs and TEs were conducted using plink v1.90b6.16 (PURCELL *et al.* 2007). Pairwise genetic distance matrices for SNPs and TEs were calculated as 1 minus identity by state (IBS) using TASSEL version 5.2.64 (BRADBURY *et al.* 2007).

Data Availability: All sequence data is available on the NCBI SRA (BioProject PRJNA661271, Table S1). Code for this study is available at https://github.com/HirschLabUMN/TE_variation.

RESULTS AND DISCUSSION

Using short read sequence data to identify TE presence/absence: For this study, we implemented an approach for scoring TE presence/absence variation from short read sequence alignment using the average coverage of windows surrounding the boundaries of previously annotated TEs. To assess classification accuracy, presence/absence scores defined by previous comparison of TE content generated for four maize genome assembles including B73, Mo17, PH207, and W22 were used as truth sets (ANDERSON *et al.* 2019). It should be noted that some polymorphisms in this 'truth' set might be wrong for reasons described in the publication from which they were generated (ANDERSON *et al.* 2019). As such, 100% accuracy in comparison to this set is not possible unless the same miscalls are generated in two independent methods. Still, this set represents a high quality set of TE polymorphisms for which to assess the relative accuracy of different parameters. The following parameters were factorially tested: 1) mean coverage of the windows used to classify a TE as present (2x-8x coverage), 2) size of the

windows around the start and end of the TE (1, 3, 5, and 10 bp), and 3) coverage before and after the start and end of each TE (4 sides) versus only coverage just after the start and just before the end of the TE (2 sides) (Figure S1).

Initially, a TE was only classified as present or absent. To be classified as present the mean coverage over all genome fragments used had to be greater than or equal to the coverage threshold. If mean coverage was less than the threshold over any genome fragment at the start or end of the TE in question, the TE was classified as absent. From this initial factorial analysis, coverage threshold was identified as an important factor for TE calling accuracy (Figure 1). In contrast, window size did not have a major impact on TE calling accuracy, nor did using coverage information from only just after the start and just before the end of a TE versus before and after the start and end of the TE boundaries (Figure 1). It is notable that the TE classification accuracy rate patterns differed in W22, compared to Mo17 and PH207, where the mean genome-wide coverage was substantially lower (6x average realized coverage for W22 versus 28x and 30x for Mo17 and PH207).

Further investigation showed that ~11% of the TEs had inconsistent coverage information, where coverage at one side of the TE was above the coverage threshold but the other side was below the threshold (Figure S2). In the original method, these TEs were called as absent, but 42% of these TEs in PH207 and 50% in Mo17 were actually present based on the prior genome-wide comparisons. This is a much higher error rate than the overall error rate of ~10%. Based on this observation, a third possible TE classification category of ambiguous was added. With this new category, the criteria for classifying a TE as present did not change (i.e. all windows included in the analysis had to be above the given coverage threshold), but the

criteria for classifying a TE as absent was changed to require that the mean coverage over all genome fragments of the TE had to be less than the coverage threshold minus one. All other TEs were classified as ambiguous (Figure S2).

Within our panel of sequenced genotypes, we had a range of observed genome wide mean coverages (Figure S3). To test the effects of coverage on TE classification accuracy rates the Mo17 and PH207 reads were down sampled to 20%, 32%, 45%, 66% and 83% of their original coverage levels. This corresponded to genome-wide mean coverages of ~6x, ~10x, ~15x, ~20x, and ~26x. Reducing mean coverage from 30x to 15x had a negligible effect on TE classification accuracy at a given coverage limit threshold (Figure S4), but reducing genome wide mean coverage from 30x to 6x had a noticeable effect on TE classification accuracy (Figure S4). As long as mean coverage was greater than 10x, classification rate accuracy was not affected by sequencing coverage. It is worth noting that the accuracy rates of Mo17 and PH207 down sampled to 6x genome wide coverage were similar to those of W22, which only had 6x mean coverage, which indicates that the down sampling accurately mimicked patterns of coverage for genotypes that have low mean coverage.

From these assessments of accuracy across different parameters, a final criterion was identified to balance inaccurate classifications and the rate of ambiguous classifications. This final criterion required the average coverage of the two 10 bp internal flanking windows to be greater than or equal to 8x to be classified as present, and both sides less than 7x average coverage to be classified as absent. All other TEs were classified as ambiguous. Highly polymorphic flanking regions in some haplotypes will result in a higher ambiguous classification rate for particular TEs that could impact downstream data analysis of TE polymorphisms. Thus,

TEs with greater than 25% ambiguous calls were filtered from further analyses. These criterion and filtering methods provided a set of TE presence/absence calls that allowed for population level analysis of TE frequencies on a genome-wide scale in maize. Still, it should be noted that rare alleles will be under-represented in this set due to the fact that only those TEs that were previously annotated in at least one of four *de novo* assembled maize genomes are included in this study.

TE frequency distribution in a panel of diverse inbred lines: The final criteria for classifying a TE as present or absent was applied to short read sequence data from 509 genotypes of the Wisconsin Diversity Panel (HANSEY *et al.* 2010; HIRSCH *et al.* 2014; MAZAHERI *et al.* 2019) mapped to the B73, Mo17, PH207, and W22 reference genome assemblies. For each annotated TE in these genome assemblies, the presence/absence frequency of the TE in this panel of diverse inbred lines was determined (Figure S5). Genetic relationships based on SNPs and TEs were assessed to further validate the quality of these TE presence/absence calls based on their consistency with other marker types and known pedigree relationships. Principle component analysis of the SNPs and TEs both revealed expected population structure based on previous pedigree information and heterotic group membership (Figure S6A), and pairwise genetic distances between individuals using SNPs and TEs were highly correlated (Figure S6B). As previously reported, there are a number of shared TEs across these four genome assemblies (ANDERSON *et al.* 2019). The population frequency of homologous TEs determined from mapping the short read data to the different genome assemblies were highly correlated (Pearson's correlation r^2 = 0.93; Figure S7), demonstrating the consistency of this pipeline, and enabling

classifications to be combined into a non-redundant set of TEs. For downstream analysis of these redundant TEs, the geometric mean was calculated across frequencies obtained from all genomes for which a homologous TE was present (Figure 2; File S5).

Different orders of TEs have different mechanisms of replication (WICKER *et al.* 2007), and families within these orders have different insertional preferences (SANMIGUEL *et al.* 1998; SULTANA *et al.* 2017; SPRINGER *et al.* 2018) and different effects on DNA methylation and chromatin accessibility (EICHTEN *et al.* 2012; CHOI AND PURUGGANAN 2018; NOSHAY *et al.* 2019). As such, we hypothesized the frequency of TEs in the population would be variable across orders of TEs and families. For non-nested elements (i.e. those not contained within another TE), a subset of the TEs were present in all or nearly all of the diverse lines included in this study for LTRs, Helitrons, and TIRs (Figure 2). However, for all three orders, there was a substantial number of TEs that were at low (<20%) to moderate (20-80%) frequency in the population. This proportion was particularly high for the LTRs where 68.1% of non-nested TEs were present in low to moderate frequency in the population. These polymorphic TEs have the potential to drive phenotypic variation as has been seen for a number of morphological/developmental (CHUCK *et al.* 2007; STUDER *et al.* 2011) and adaptive traits (YANG *et al.* 2013) in maize. The demonstrated extent of TE variability on a genome-wide scale across a large number of individuals that this study provides is critical in furthering our understanding of the contribution of variable TEs in producing phenotypic variation within species. Given that only TEs that were annotated in at least one of only four genomes were included in this study, it is expected that the proportion of polymorphic TEs at low to moderate frequency would be substantially higher than what is presented here if all TEs in all genotypes in the population were annotated. In

contrast, the majority of the high frequency TEs were likely already captured from just these four genome assemblies, and the count of these TEs would remain relatively static if all genomes were to be *de novo* assembled and annotated.

Older LTRs were generally more frequent in the population than younger LTRs : A negative relationship between age of TEs and their frequency was previously demonstrated using a limited number (n=4) of genotypes (ANDERSON *et al.* 2019). We sought to test this relationship between age and frequency on our broader set of germplasm. For LTRs the age of an element can be estimated based on the LTR similarity, where younger elements have higher LTR similarity and older elements have lower LTR similarity. For other orders of TEs there are not accurate methods to assess age. Thus, when assessing the relationship between the age of TEs and their frequency in the population the analysis was limited to LTR elements with age estimates. LTR similarity was negatively correlated with population frequency, which suggests that older LTRs were generally more frequent in the population than younger LTRs (Figure 3a). It is worth noting, however, that there were a large number (n=14,572) of LTRs that were young (LTR similarity 95-99%; n=12,701) or very young (LTR similarity >99%; n=1,871), and are at high frequency (>80%) in the population (Figure 3b), and 259 of the very young TEs were fixed in this population. Thirty-three of the fixed and very young TEs were within 5kb of a gene and may be under selection or linked to other features in the genome under selection that could have driven their rapid rise to fixation in the population. A number of these genes have been functionally characterized, such as agpll2 (HUANG *et al.* 2014), as well as trps4 (ZHOU *et al.* 2014), which may be important for tolerance to different stress conditions.

There was a large portion (76.03%) of the fixed and very young TEs that were located within previously defined low recombination regions (Table S2; (SWANSON-WAGNER *et al.* 2010; EICHTEN *et al.* 2011)). These regions were defined by comparing genetic and physical maps and defining boundaries to define the high recombination arms and the low recombination middle of each chromosomes including the centromere and pericentromere. The fixed and very young TEs were enriched (Fisher test with p-value < 0.001; Figure 3c) for being located within the low recombination regions of the genome relative to the frequency for all LTRs. However, this enrichment in the low recombination region of the genome is also observed for all fixed LTRs regardless of their age. If we look at other elements in the families from which these fixed and very young elements are in there is no significant difference between them and all LTRs or all of the very young LTRs in the genome. Thus, the enrichment of fixed and very young LTRs in the pericentromere is likely a product of their location.

The elements in the very young and high frequency group (not just fixed) were from 494 different families. We sought to test if there was an enrichment within these families for elements that were at high frequency and very young relative to the frequency of this class compared to all other LTRs. Indeed, for those families with at least 20 elements in the family (n=55), 13 had a higher than expected proportion of elements that were very young and at high frequency in the population (Fisher test with p-value < 0.01; Table S3). The majority of these enriched families are within the Gypsy superfamily, including RLG00001, a large Cinful-Zeon family (SANZ-ALFEREZ *et al.* 2003) with 23,948 copies in the B73 genome that lacks homologs in Sorghum (PATERSON *et al.* 2009; JIAO *et al.* 2017; STITZER *et al.* 2019). Another family of note is RLG0009. This family was previously documented to be consistently upregulated under heat

stress across genotypes with multiple members of the family showing increased expression, potentially due to the presence of conserved cis-regulatory elements within the TE that facilitate stress-responsive expression of this family (LIANG *et al.* 2020). The potential importance of this family to stress responsiveness, the conserved response across members of the family, and the consistency in response across genotypes are all consistent with, and provide potential explanations for why, this family had enriched presence in the class of very young and high frequency TEs.

On the other end of the spectrum, for the class of old TEs (LTR similarity <95%), it is expected that some will be common as they have had time to rise in frequency in the population and other will be rare as they are being lost over time, which was the case in this population. Within the class of old TEs, 15.88% were at low frequency in the population, 37.36% were at high frequency in the population, and the remaining 46.76% were at moderate frequency in the population (Figure 3b).

Most nested TEs insertions occur near the insertion time of the outer element: Nested TEs exhibited higher levels of moderate frequency (20-80%) and low frequency (<20%) TEs as compared to the non-nested TEs (Figure 2). In all three orders, the frequency distribution of nested and non-nested TEs was significantly different (KS test, p-value < 2x10-16). A nested TE could be the product of an insertion very soon after the outer element inserted or it could be the product of a spectrum of much younger insertions that happened well after the insertion of the outer element. Genome-wide we see that nested elements are younger than non-nested elements (Figure S8).

To further explore the difference in frequency of nested and non-nested elements we looked at specific nested TEs and the non-nested element into which they inserted. Within these pairs, the nested insertion should be at the same or lower frequency compared to the outer element, as the nested insertion cannot exist in a genotype without the outer element. This was true for 67.1% of the pairs, and an additional 20.2% had less than 5% higher frequency in the nested element. Overall, there was a strong correlation between the nested TE frequency and the frequency of the TE into which it was nested (Pearson's correlation r^2 = 0.578; Figure 4a), with 82.2% having frequencies within 5% of each other, and the remaining 17.8% of pairs having a range of difference in frequencies between the inner and outer elements (Figure 4b).

Within the set of outer elements that were fixed or nearly fixed in the population (frequency greater than 0.95), there was a continuous range of frequencies for inner elements that likely represent nested insertions with a range of ages. Using LTR similarity as a proxy to LTR age, we tested if there is a relationship between the age of the nested element contained within fixed elements and their frequency in the population. This analysis could only be done for LTR nested elements as the other orders do not have age estimates. As hypothesized, those nested elements that were at lower frequency in the population were younger (higher LTR similarity) than those that were at high frequency in the population (Figure 4c), and this was observed independent of the order of the outer element (Figure S9).

To further address this question of the timing of nested insertions into outer elements we focused on the subset of nested-outer element pairs in which both elements were LTRs and therefore had age estimates. As with frequency, the quality of the age estimates was assessed with the expectation that the age of the nested element based on LTR similarity should be

younger than that of the outer element. Across the LTR nesting pairs, 84.5% had a higher LTR similarity for the inner element, indicating an overall high quality to the age estimates based on LTR similarity (Figure S10). Using the combination of frequency and age we tested the extent to which an outer element and nested element insertion occurred at a similar time or weather the nested insertions occur over a range of time (Figure 4d). The majority of nested-outer element pairs have nearly identical frequencies (Figure 4a,d), but with a range of age differences. The most likely interpretation of these results is that in these cases the nested insertion occurred very near the timing of the outer element insertion and that the distribution of mutation accumulation, used to estimate age, is different for nested versus outer elements. While this finding is true for a large majority of the nested-outer pairs it should be noted that there are still a substantial number of pairs that have a range of frequency differences and age differences that likely represent longer periods of time between the insertion of the outer element and the subsequent insertion of the nested element (Figure 4d).

Relationship between location of TEs relative to genes and frequency in the population: TEs that are located in or near genes have the potential to change expression patterns of a gene (HIRSCH AND SPRINGER 2017) or the product a gene encodes (LISCH 2013). In some cases, these functional insertions are a substantial distance from the gene, such as a 57kb upstream Harbinger-like DNA transposable element that represses *ZmCCT9* in cis and promotes flowering under long days (HUANG *et al.* 2018). Others are much closer, such as a *STONER* element that inserted 42 bp upstream of the *Cg1* transcription start site and results in a chimeric fusion of the *STONER* element and the gene impacting the juvenile to adult vegetative transition (CHUCK

et al. 2007). We hypothesized that there will be different selective pressures on TEs depending on their position relative to the nearest gene in the genome and this will affect their frequency in the population. In order to test this, we first categorized TEs into categories using the following hierarchical ordering: gene completely within the TE, TE completely within the 5' UTR, completely within the 3' UTR, completely within an exon, completely within an intron, completely encompassed by a gene, 0-1 kb upstream of a gene, 1-5 kb upstream of a gene, 5-10 kb upstream of a gene, 0-1 kb downstream of a gene, 1-5 kb downstream of a gene, 5-10 kb downstream of a gene, and intergenic (greater than 10kb from the nearest gene).

There are relatively few gene proximal TEs that are actually within the gene body (orange in Figure 5a-c). Only 4.2% of LTR proximal TEs (1,261/30,407) and 4.5% of Helitron proximal TEs (275/6,171) are actually within the gene, while 11.0% (4,874/42,598) of TIR proximal TEs are within the gene body. In general those TEs that are within the gene body are at higher frequency than those that are outside of the gene body, and this is particularly true for those that not contained within an intron. For LTRs the age of the proximal TEs could also be evaluated, and those TEs that were within the gene body were also enriched for being younger based on LTR similarity than those proximal TEs that are outside of the gene body (Figure 5d; p-value < 0.001). This finding is somewhat unexpected given the potential deleterious functions that TE insertions could have on the expression of a gene and the integrity of the encoded protein, and likely indicates a positive effect of these insertions allowing them to rise in frequency relatively quickly.

TEs that are proximal to a gene, but not contained within a gene, have much lower frequencies than was observed for TEs within gene bodies (purple and green in Figure 5a-c).

The frequency further decays at increasing distance from the gene in both the 5' and 3' directions, and this is consistent across the three orders. Proximal TEs outside of the gene body also have a relatively higher proportion of older TEs relative to those that are within genes (Figure 5d). The final class of proximal TEs, those that encompass a gene, show similar frequency to TEs that are proximal, but not within a gene, and are significantly depleted for very young elements (p-value < 0.001; pink in Figure 5a-d). The genes contained within these TEs are all in the non-syntenic gene space relative to sorghum and rice. Genes in the non-syntenic gene space account for less phenotypic diversity than those in the syntenic gene space and are likely under different selective pressures in general compared to syntenic genes and TEs contained within syntenic genes (SCHNABLE *et al.* 2011; BROHAMMER *et al.* 2018).

Many TE insertional polymorphisms are not tagged by SNP markers: There is a limited number of plant species in which a species or population level cataloging of TE presence/absence variation has been conducted at a genome-wide level (QUADRANA *et al.* 2016; STUART *et al.* 2016; CARPENTIER *et al.* 2019; CHEN *et al.* 2020; DOMINGUEZ *et al.* 2020). As such, the contribution of TEs to phenotypic variation, utility for genomic prediction, and other applications linking genotypes and phenotypes has not been directly evaluated. In many instances, a linked marker has been identified as significant and with fine-mapping it is revealed that the causative variant is in fact a polymorphic TE insertion (e.g. (STUDER *et al.* 2011)).

We sought to test the extent to which the extensive number of polymorphic TE insertions that were classified in this study were in linkage disequilibrium (LD) with genome-wide SNP markers to begin to understand the extent to which phenotypic variation that is

caused by TEs has or has not been accounted for in previous studies that used only SNP markers. For this analysis, all SNPs within 1Mb plus or minus a TE were evaluated and the SNP that was in the highest LD was recorded. The majority of TEs were in moderate (r^2 0.5-0.9) to high (r^2 >0.9) LD with a nearby SNP marker, but there was a subset of 36,221 TEs that were in low LD (r^2 < 0.5) with all SNPs within a 1Mb window of the TE (Figure 6a). LTRs had the highest portion of TEs in high LD with a SNP (~73%), while Helitrons had ~61% in high LD, and TIRs had only ~54% in high LD (Figure 6b). Of the subset of TEs that had a SNP in high LD (>0.9), the majority of the SNPs were within 200Kb of the TE (Figure 6c). This distance increased when TEs in all levels of LD with SNPs were included (Figure S11). TEs that were at very high or low frequency within the population were generally in low LD with SNPs, and those that were at moderate frequency in the population were in moderate to high LD with SNPs (Figure 6d-f). This trend was consistently observed across orders of TEs.

There are many metrics to assess LD and they represent different aspects of LD (FLINT-GARCIA *et al.* 2003; SLATKIN 2008). For example, D' measures only recombinational history, and r^2 measures both recombinational and mutational history. We assessed LD between SNPs and TEs using D' as the metric and observed substantially more TEs in near or perfect LD with a SNP (Figure S12a,b). Only 180 TEs had a D' value of less than 0.9 to a SNP within 1 Mb of the TE, and a relationship with population frequency was no longer observed. The distance between the SNP and TE decreased only slightly relative to r^2 for SNP in highest LD with a TE (54,606 for r^2 vs. 54,172 for D'; Figure 6c and Figure S12c). This result makes sense as TE insertions represent different mutational events from the SNPs to which they are being evaluated and this is not reflected in the D' metric.

Overall, while TE presence/absence patterns generally reflect maize breeding history (Figure S6), there are TE insertional polymorphisms that are not tagged by SNPs across different metrics (Figure 6 and Figure S12). These TEs that are not tagged by SNPs may be of important phenotypic consequence in maize, as has been shown in tomato (DOMINGUEZ *et al.* 2020) and rice (AKAKPO *et al.* 2020). The high number of TEs in low to moderate LD with SNPs based on r^2 is particularly important to this point, as r^2 directly measures how different markers correlate with each other, and therefore how well a particular SNP would correlate with a potential causative TE. Including TE insertional polymorphism will likely be important in understanding the full complexity of phenotypic trait variation and location adaptation, and developing improved maize varieties in the future.

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FIGURE LEGENDS

Figure 1. TE calling accuracy rates. a) TE calling accuracy rates using 4 windows flanking the inside and outside of the TE boundaries to call a TE as present or absent. B) TE call accuracy rates using the two internal windows (immediately after the start and immediately before the end) of the TE. All TEs were called as either present or absent in this analysis (i.e. no ambiguous call category was implemented).

Figure 2. TE frequency distribution of a non-redundant set of TEs annotated in the B73, Mo17, PH207, and W22 genome assemblies. Short read sequence data from 509 genotypes were aligned to each genome assembly. In order to call a TE as present, the average coverage of the two 10 bp internal flanking windows was required to be greater than or equal to 8x, and both sides less than 7x average coverage to be classified as absent. All other TEs were classified as ambiguous. For homologous TEs that were present in more than one assembly, the geometric mean across assemblies was used in the non-redundant set. Only TEs with less than 25% ambiguous calls are included in these plots. Percentages indicate the percent of low frequency (<20%), moderate (20-80%), and high frequency (>80%) TEs in each order.

Figure 3. Relationship between TE age and frequency in a population of diverse inbred lines. a) Heatmap of age versus frequency where white boxes indicate no TEs present at a particular frequency-by-LTR similarity. Yellow line is a LOESS curve fit through the data. b) Relationship of age in categories of old (LTR similarity <95%), young (LTR similarity between 95-99%), and very young (LTR similarity >99%) and frequency in categories of low frequency (<20%), moderate

frequency (20-80%), and high frequency (>80%). c) Proportion of different groups of LTRs in the low and high recombination portions of the genome. For a-c, age is approximated by LTR similarity as previously reported (ANDERSON *et al.* 2019). Only LTR TEs with less than 25% ambiguous calls and age estimates are included in this plot (n=149,908).

Figure 4. Relationship between population frequency of nested elements and the elements in which they are nested. a) Proportion of genotypes a TE is present in between nested TEs and the TE in which the element is nested. Only low ambiguous TEs were included in this plot. Only TEs from the B73 genome are plotted. b) Distribution of the proportion of genotypes the outer TE is present in minus proportion of genotypes in which the nested TE is present. c) Age distributions for nested elements that are nested in TEs that are fixed or nearly fixed (frequency >0.95) in the population. This plot only contains nested LTRs as age estimates are not available for other orders. d) Relationship between age and frequency for outer elements minus nested elements. Points in the gold quadrant meet biological expectations that the outer element is older and at higher frequency than the nested element. This plot only contains LTRs for the outer and nested elements. In both c and d, LTR similarity is used as a proxy to age with higher LTR similarity corresponds to a younger TE and lower LTR similarity corresponds to an older TE. Only TEs with less than 25% ambiguous calls are included in these plots.

Figure 5. Relationship between TE frequency and relative position to the nearest gene. Helitrons (a), TIRs (b), and LTRs (c) were categorized using the following hierarchy: gene completely within the TE, TE completely within the 5' UTR, completely within the 3' UTR,

completely within an exon, completely within an intron, completely encompassed by a gene, 0-1 kb upstream of a gene, 1-5 kb upstream of a gene, 5-10 kb upstream of a gene, 0-1 kb downstream of a gene, 1-5 kb downstream of a gene, 5-10 kb downstream of a gene, intergenic (not shown in figure). d) Proportion of old (LTR similarity <95%), young (LTR similarity between 95-99%), and very young (LTR similarity >99%) LTRs in each gene proximity category. Only TEs with less than 25% ambiguous calls are included in these plots.

Figure 6. Linkage disequilibrium between TEs and SNPs in a panel of diverse inbred lines. a) Linkage disequilibrium (LD) between TEs and the SNP with the highest LD within 1Mb of the middle of the TE. b) Proportion of TEs in high ($r^2 > 0.9$), moderate (r^2 0.5-0.9), and low ($r^2 < 0.5$) LD with SNPs within 1 Mb of the middle of the TE. Category is based on the SNP with the highest LD in the window. c) Distance between TEs and the SNP with the highest LD to it for TEs that had a SNP in high ($r^2 > 0.9$) LD. Distance is calculated as the middle of the TE to the SNP. Only SNPs within 1 Mb of a TE were evaluated. d-f) Density plots of population frequencies for TEs in high, moderate, and low LD with SNPs based on the highest LD within 1Mb of the middle of the TE for LTRs (d), Helitrons (e), and TIRs (f). Only TEs with less than 25% ambiguous calls are included in these plots.

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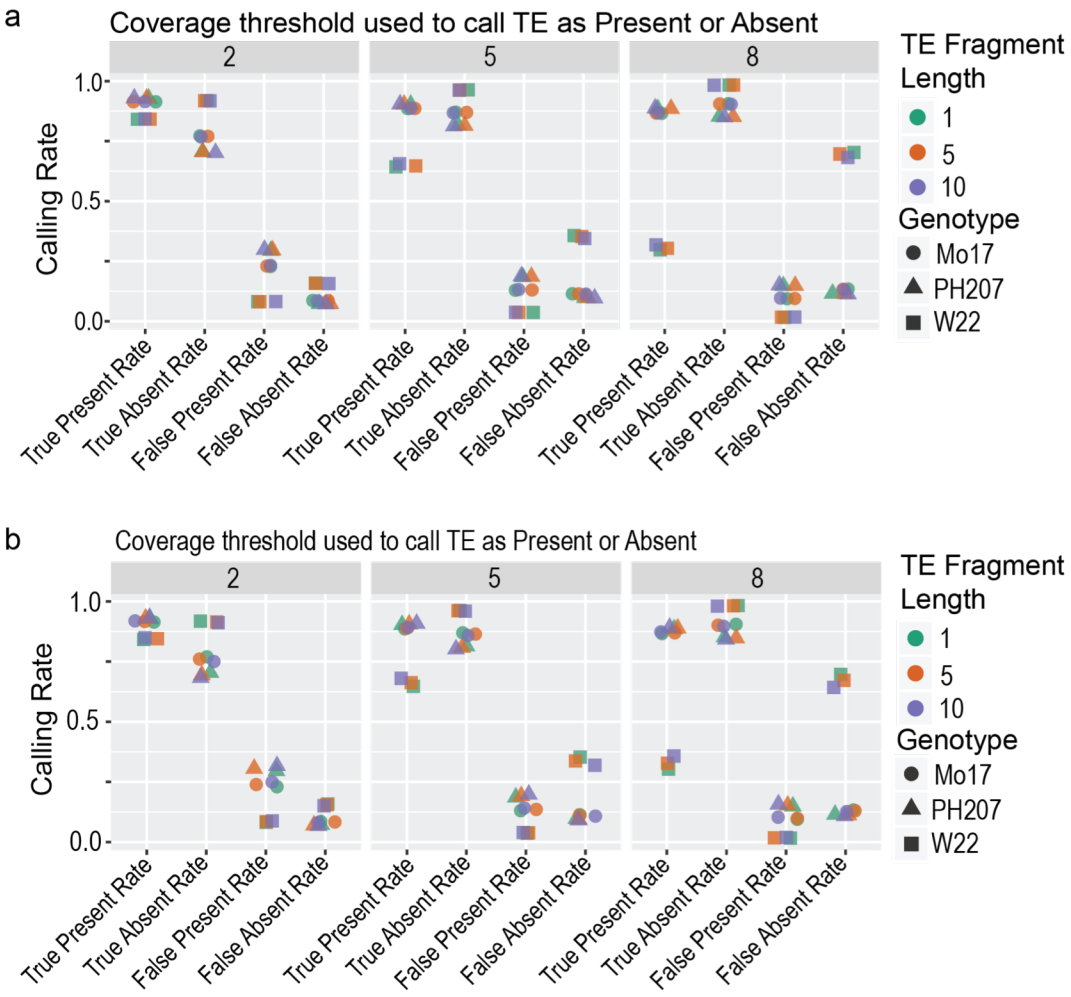

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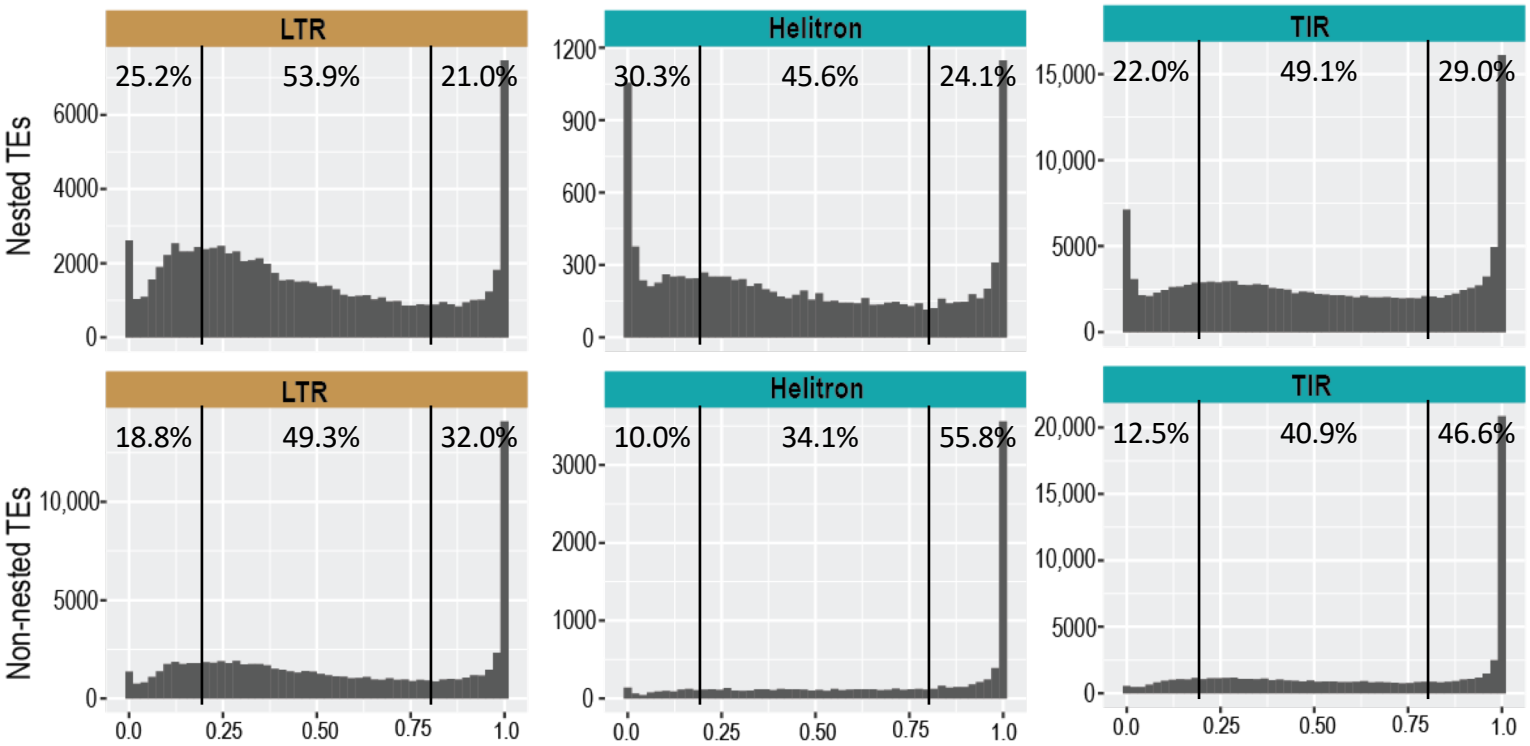

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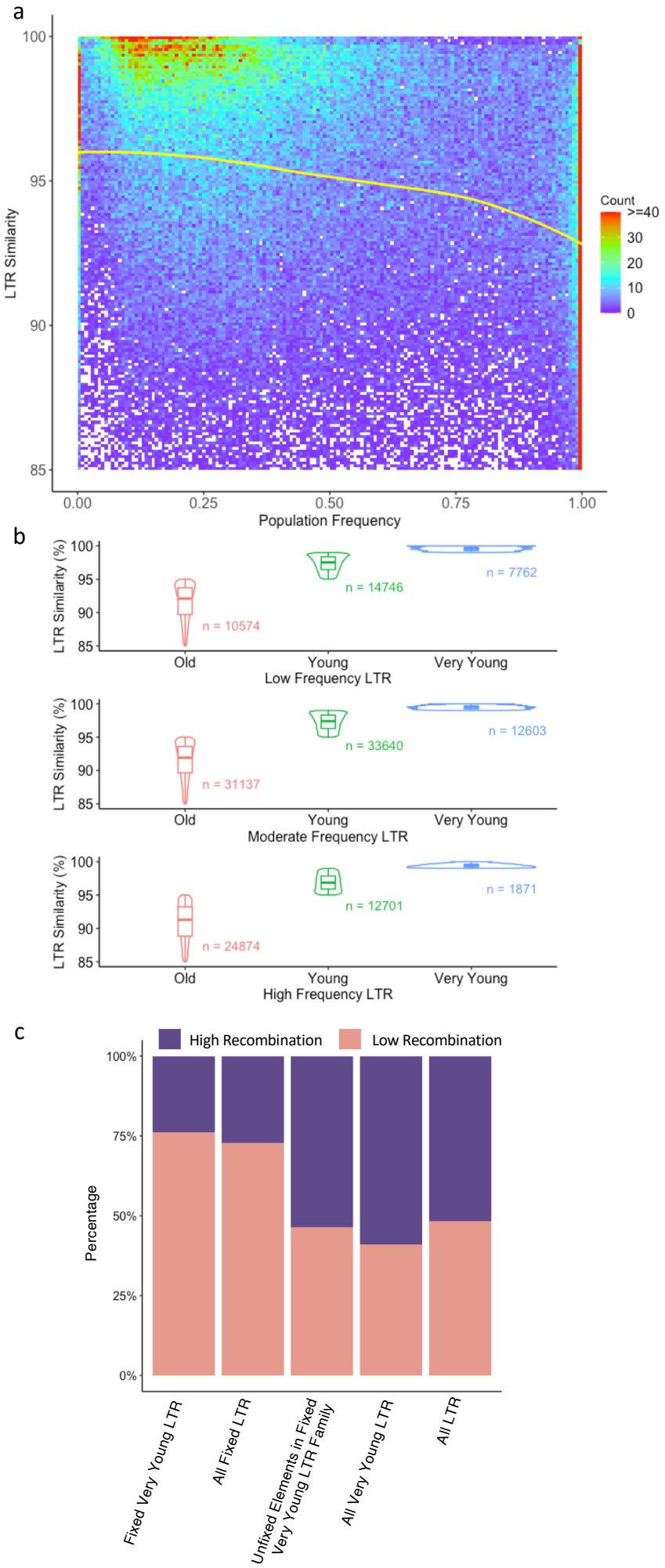

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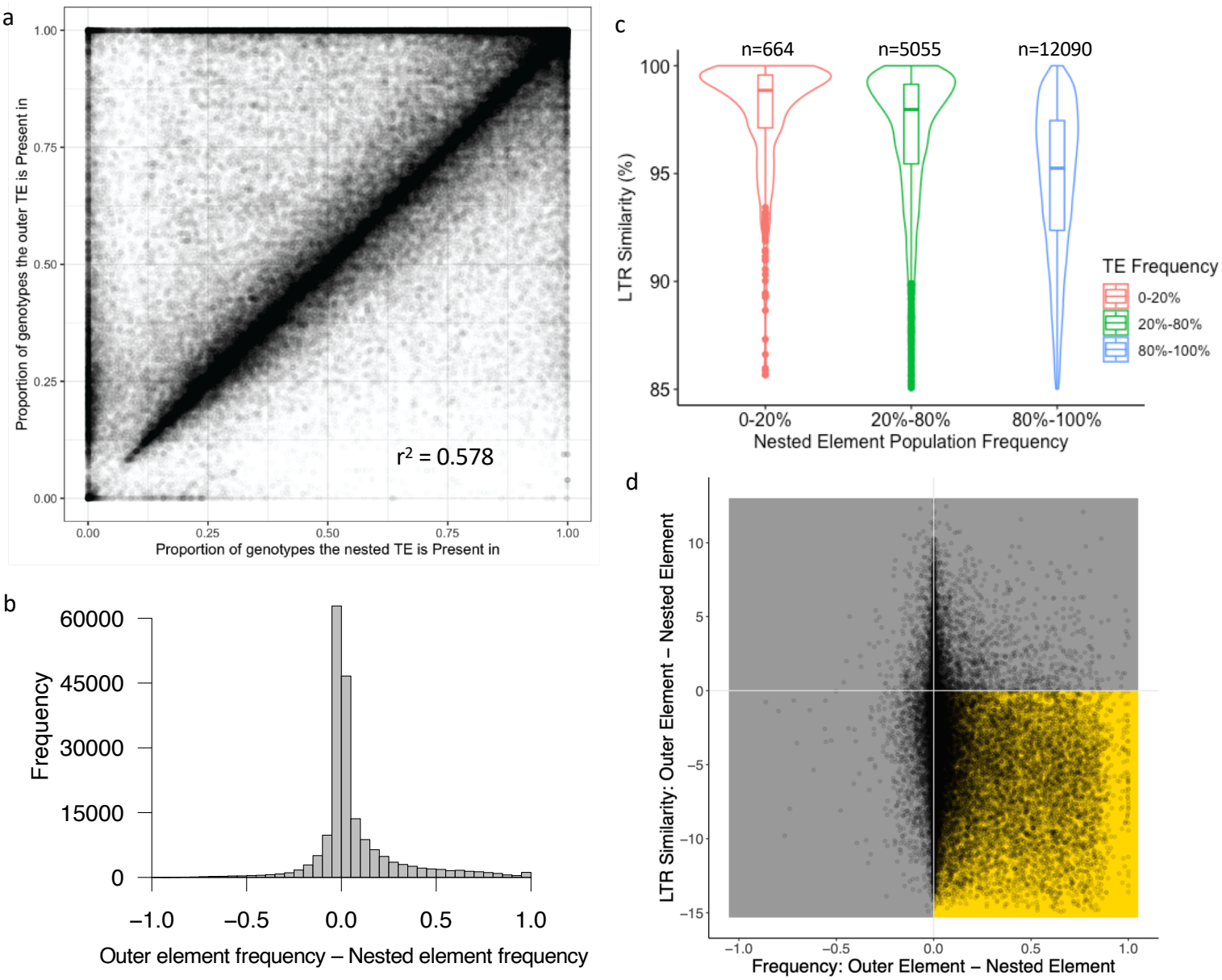

Figure 4. Relationship between population frequency of nested elements and the elements in which they are nested. a) Proportion of genotypes a TE is present in between nested TEs and the TE in which the element is nested. Only low ambiguous TEs were included in this plot. Only TEs from the B73 genome are plotted. b) Distribution of the proportion of genotypes the outer TE is present in minus proportion of genotypes in which the nested TE is present. c) Age distributions for nested elements that are nested in TEs that are fixed or nearly fixed (frequency >0.95) in the population. This plot only contains nested LTRs as age estimates are not available for other orders. d) Relationship between age and frequency for outer elements minus nested elements. Points in the gold quadrant meet biological expectations that the outer element is older and at higher frequency than the nested element. This plot only contains LTRs for the outer and nested elements. In both c and d, LTR similarity is used as a proxy to age with higher LTR similarity corresponds to a younger TE and lower LTR similarity corresponds to an older TE. Only TEs with less than 25% ambiguous calls are included in these plots.

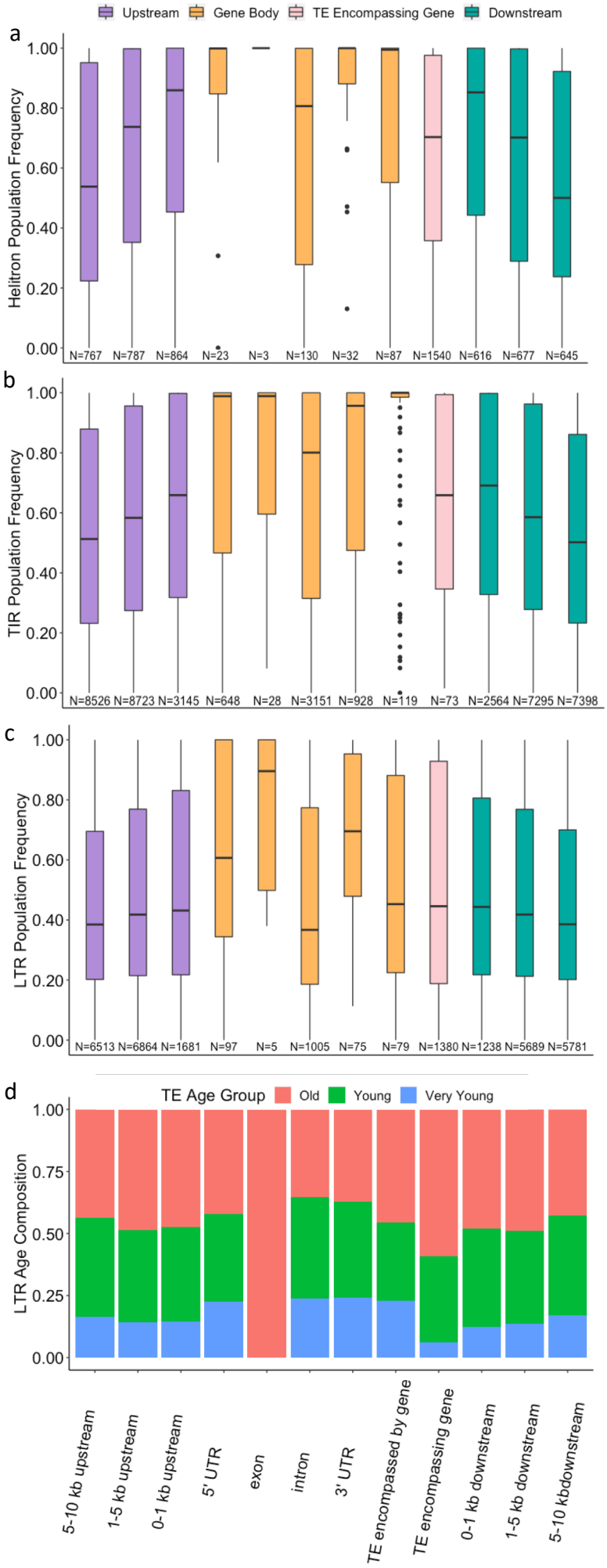

Figure 5. Relationship between TE frequency and relative position to the nearest gene. Helitrons (a), TIRs (b), and LTRs (c) were categorized using the following hierarchy: gene completely within the TE, TE completely within the 5' UTR, completely within the 3' UTR, completely within an exon, completely within an intron, completely encompassed by a gene, 0-1 kb upstream of a gene, 1-5 kb upstream of a gene, 5-10 kb upstream of a gene, 0-1 kb downstream of a gene, 1-5 kb downstream of a gene, 5-10 kb downstream of a gene, intergenic (not shown in figure). d) Proportion of old (LTR similarity <95%), young (LTR similarity between 95-99%), and very young (LTR similarity >99%) LTRs in each gene proximity category. Only TEs with less than 25% ambiguous calls are included in these plots.

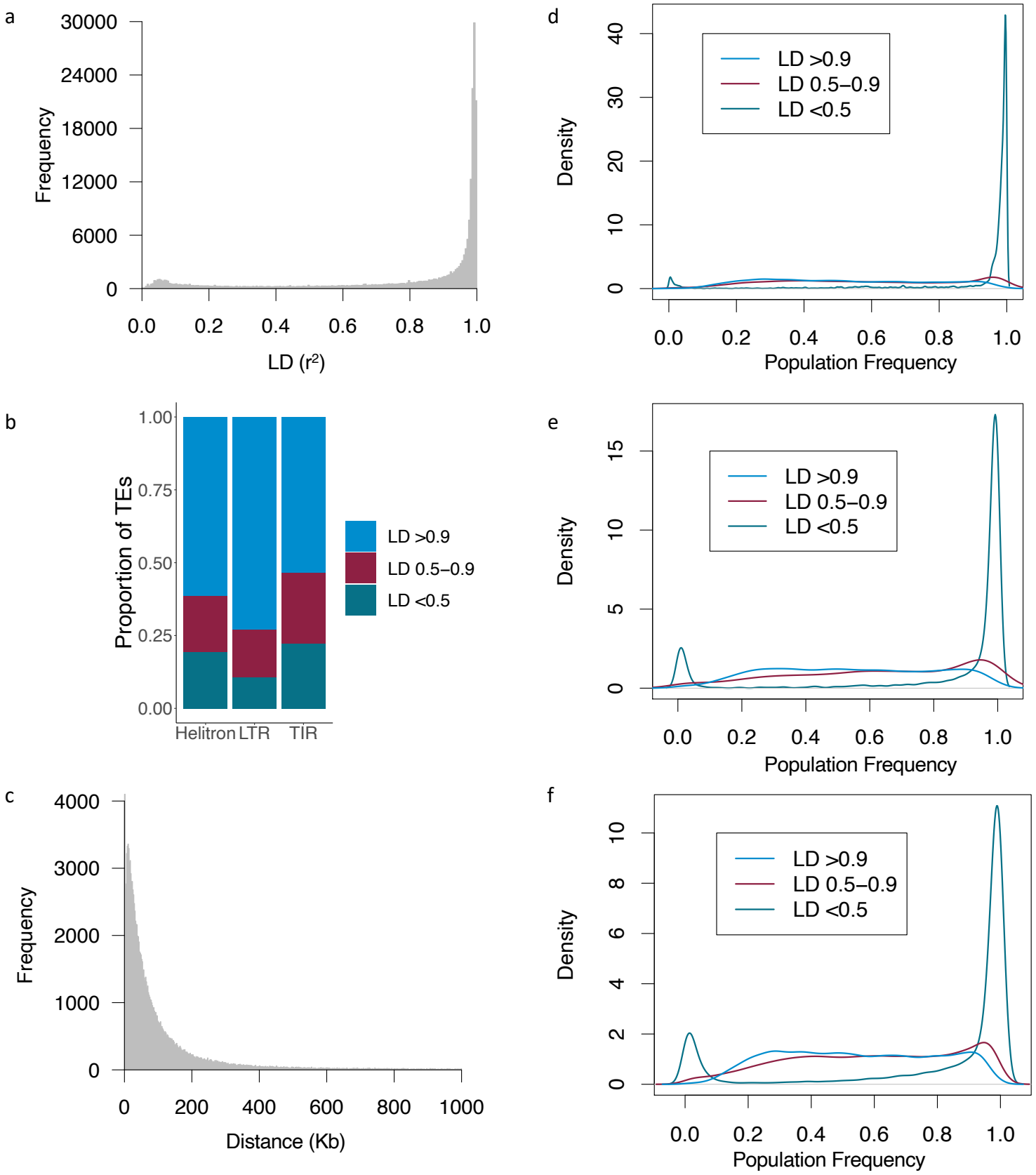

Figure 6. Linkage disequilibrium between TEs and SNPs in a panel of diverse inbred lines. a) Linkage disequilibrium (LD) between TEs and the SNP with the highest LD within 1Mb of the middle of the TE. b) Proportion of TEs in high ($r^2 > 0.9$), moderate (r^2 0.5-0.9), and low ($r^2 < 0.5$) LD with SNPs within 1 Mb of the middle of the TE. Category is based on the SNP with the highest LD in the window. c) Distance between TEs and the SNP with the highest LD to it for TEs that had a SNP in high ($r^2 > 0.9$) LD. Distance is calculated as the middle of the TE to the SNP. Only SNPs within 1 Mb of a TE were evaluated. d-f) Density plots of population frequencies for TEs in high, moderate, and low LD with SNPs based on the highest LD within 1Mb of the middle of the TE for LTRs (d), Helitrons (e), and TIRs (f). Only TEs with less than 25% ambiguous calls are included in these plots.