**Introduction**

Synonymous mutations are those mutations in the coding sequence (CDS) that do not change the amino acids (AAs). However, this does not mean that synonymous mutations are free from natural selection.1-3 It was reported that some synonymous mutations could affect messenger RNA (mRNA) splicing2 and the splicing patterns might consequently affect the biological processes.4 These splicing-related synonymous mutations were likely to be selected against.4 Another well-known impact of synonymous mutation is the influence on synonymous codon usage. The G/C-ending synonymous codons seem to appear more frequently in the genome compared to the neutral expectation.6 Theories were established to explain the preference for G/C-ending codons. One potential advantage of the G/C-ending synonymous codons is that they are decoded at higher rates during mRNA translation elongation 7-10 due to the putatively higher transfer RNA (tRNA) availability. Accordingly, G/C-ending synonymous codons usually have higher codon adaptation index (CAI)11 or tRNA adaptation index (tAI)12 values than their A/T-ending counterpart. Codon adaptation index describes the relative usage of synonymous codons in the genome and tAI incorporates the tRNA copy number of the corresponding codon. Faster translation rates might provide higher probability for the G/C-ending codons to be selectively maintained by natural selection.

In addition, there are also other potential impacts of synonymous mutations, such as the changes in thermodynamic stability of the secondary structures of mRNA (termed minimum free energy [MFE]) or methylation contexts. RNA structures could affect many aspects of RNA biology, such as the RNA-binding proteins (RBP) binding efficiency and the movement of ribosomes on RNAs. If a synonymous mutation altered the structure of RNAs, this change could be either deleterious, beneficial, or neutral and consequently subjected to natural selection.

Despite the established hypotheses explaining the putative advantage of G/C-ending codons, it would be interesting to directly verify the selection force acting on those synonymous mutations that change the codon usage patterns. If the G/C-ending codons are advantageous, then the synonymous mutations that change A/T to G/C would be “optimal” compared to the opposite ones. In theory, signals of positive selection should be observed on these “optimal” synonymous mutations.

I fully take advantage of the single-nucleotide polymorphism (SNP) data from 1135 Arabidopsis thaliana lines (the 1001 genome project). If all synonymous mutations are strictly neutral, then the different types of mutations (eg, A-to-C, G-to-T, T-to-C) would exhibit similar degrees of derived allele frequencies (DAFs). If particular types of synonymous changes (eg, T-to-C) are more advantageous than the opposite ones (C-to-T), then the optimal synonymous changes should be positively selected and exhibit relatively higher DAF.

Moreover, it is necessary to test whether the observed pattern on synonymous DAF is affected by population structures.
Population structure reflects the ancestry of different groups within the population. If an expected pattern is unbiased, it should not be affected by population structure and should be observed across all the representative subpopulations.

In this study, by analyzing the frequency spectrum of the synonymous mutations, I draw to the conclusion that synonymous mutations in natural populations are not strictly neutral: the synonymous mutations that increase GC content (from A/T to G/C) tend to have higher DAF and therefore are likely to be positively selected. This pattern is not affected by the population structure as it is observed in all representative subpopulations. My current study suggests that synonymous mutations are not strictly neutral and should not be automatically ignored in evolutionary analyses.

**Materials and Methods**

**Data collection**

I download the SNP data as well as the genome-wide Fst values (window size 10000bp) of the 1,135 natural inbred lines of *A thaliana* from the 1001 genome project (http://1001genomes.org/data/GMI-MPI/releases/v3.1). The annotation of each SNP is included in the downloaded variant calling format (vcf) files. Note that I only used the SNPs in the main chromosomes and discarded the very few SNPs in the chloroplast or mitochondrial genomes. Furthermore, the admixture groups defined by the original work were retrieved. Some subpopulations had only a few alternative alleles so that the allele frequency might be very low and fluctuating due to sampling bias. In the subpopulation analyses, I only chose 3 representative subpopulations with at least 10 alternative alleles to ensure that the allele frequencies were not affected by sampling bias.

**Inferring the DAF using an outgroup**

I aligned the CDSs sequences between *A thaliana* (TAIR 10 version, link: https://www.arabidopsis.org/download_files/Sequences/TAIR10_blastsets/TAIR10_cds/20101214_.updated) and *Arabidopsis lyrata* (from Ensembl Plant, link: ftp://ftp.ensemblgenomes.org/pub/release-46/plants/fasta/arabidopsis_lyrata/cds/) using the BLAST+ package. The best-matched pairs of CDSs in 2 species are regarded as orthologous genes. For each polymorphic site in *A thaliana*, the orthologous site was extracted from the orthologous gene of *A lyrata*.

If the nucleotide in *A lyrata* is the same as the reference allele of *A thaliana* (most cases), then DAF is the alternative allele frequency.

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If the nucleotide in *A lyrata* does not match either the reference or alternative allele in *A thaliana* (very few cases), then these SNP sites are discarded.

According to these classification criteria, the direction of mutation is from ancestral to derived. However, *A lyrata* may experience the same type of selection on synonymous mutations. It should be mentioned that using only one species as an outgroup does not provide a certain ancestral inference. More closely related species could be used in future analyses to give a more accurate ancestral inference.

The allele counts used to calculate allele frequencies are provided in the SNP file. The vcf format contains the position and mutation-type columns as well as the “info” column. The “info” column includes information of annotation (genic or intergenic, coding or noncoding, missense or synonymous) and allele counts (the reference and alternative allele counts from the reads mapped to this position).

**Classification of synonymous mutations**

Synonymous mutations were classified into 3 categories according to whether they increase (from A/T to G/C), decrease (from G/C to A/T), or maintain (the remaining) the GC content. Among the 12 types of mutations, A-to-C, T-to-C, A-to-G, and T-to-G increase the GC content. C-to-A, C-to-T, G-to-A, and G-to-T decrease the GC content. C-to-G, G-to-C, A-to-T, and T-to-A do not affect the global GC content.

**Regression analysis**

The regression analysis was performed using “lm(Y ~ X1 + X2 + . . . + Xn)” in the R platform (http://www.R-project.org/). Y is the DAF of each synonymous mutation; X1 is the change of CAI value caused by the synonymous mutation (from −1 to 1); X2 is the change of tAI value (from −1 to 1); X3 is whether the mutation increase CG content (1 = increase, −1 = decrease, and 0 = unchanged); X4 is whether the SNP site is immediately upstream/downstream or exactly at a methylation site (1 = yes and −1 = no; if yes, that means this SNP may affect the methylation status); X6 is the MFE (per Kb) of host genes (normalized to −1 to 1); X5 is whether the SNP site is immediately upstream/downstream or exactly at a methylation site (1 = yes and −1 = no). The regression analysis will provide us an estimate of the relative contribution of X1, X2, . . . , Xn to Y, termed regression coefficient. Note that X1, X2, . . . , Xn all range from −1 to 1, so that their regression coefficients are comparable.

Codon adaptation index and tAI were defined by early studies, which were the parameters for codon bias and described the synonymous codon preference and tRNA availability of each codon. My group and other group(s) have previously done works investigating the selection patterns on CAI and tAI and the calculations were performed with the same pipeline.
folded the CDSs of *A. thaliana* using RNAfold. The MFE value of each CDS sequence was extracted from the output file. The MFE per kilobase was calculated for each CDS.

**Statistical analysis**

All statistical analyses and graphic work were conducted in the R environment (http://www.R-project.org/). When comparing 2 sets of mutations (eg, missense vs synonymous mutations), if synonymous mutations have a globally higher DAF spectrum than missense mutations, then this would indicate a stronger purifying selection on missense mutations. In other words, synonymous mutations are “less harmful” (more advantageous) than missense mutations. Likewise, when comparing different sets of synonymous mutations, the group with higher DAF is likely to be advantageous and positively selected. The statistical tests (comparing DAF values) could be the non-parametric tests like Wilcoxon rank sum tests.

**Results and Discussion**

**Variations in the 1135 natural inbred lines of Arabidopsis thaliana**

A total number of 11,609,631 SNP sites and 1,271,972 indels are included in the vcf file that I have downloaded (see Materials and Methods section). According to the annotation provided in the vcf file, 1,135,084 SNPs are missense (nonsynonymous), 795,623 SNPs are synonymous, and 27,813 SNPs are nonsense mutations (that introduce in-frame stop codons in the main CDS). Apart from the mutations in the coding region, 319,647 SNPs take place in 5′ UTR and 465,647 SNPs are located in 3′ UTR. The remaining variations are non-exonic, including intronic and intergenic variations.

**Synonymous mutations that increase GC content have higher DAF**

Synonymous mutations were classified into 3 categories (see Materials and Methods section). I first looked at the fractions of the 12 types of mutations (Figure 1A). The most prevalent mutation type is C-to-T and G-to-A. This is reasonable as transitions take place more frequently than transversions. Next, I defined the DAF using the information provided in the SNP files and an outgroup (see Materials and Methods section). I found that those synonymous mutations that increase the GC content (from A/T to G/C) have significantly higher DAF than other synonymous mutations (Figure 1B, Wilcoxon rank-sum tests). This indicates that these “optimal” mutations are likely to be positively selected. To make the relationship between mutation and selection clearer, I tested the Spearman correlation between the fractions shown in Figure 1A and the median DAF plotted in Figure 1B (each of the 12 boxes in Figure 1B has a median value plotted horizontally in the boxes). No correlation was observed between these 2 aspects (Figure 1C), suggesting that my observed pattern on DAF might not be caused by the mutation bias.

**Relative contribution of different features to the frequency spectrum**

To better decipher the relative contribution of different features to the observed allele frequency spectrum of synonymous mutations, I perform multiple regression analysis: $Y = X_1 + \ldots + X_n$ (see Materials and Methods section). First, I listed multiple variables that might potentially affect the DAF spectrum, for example, whether the mutation increases CG content ($1$ = increase, $-1$ = decrease, and $0$ = unchanged), the
GC content of host genes, CAI, tAI, methylation status, and folding energy. It is clearly shown that “whether the mutation increase CG content” is correlated with the DAF of synonymous mutations (Figure 2A) and the changes of CAI/tAI caused by the mutation also contributed positively to the DAF spectrum (Figure 2B).

In the multiple regression analysis, the regression coefficients showed us that CAI, tAI, and the change in CG content have a remarkably larger contribution to the frequency spectrum compared to other features (Figure 2C). This indicates a role of GC content in determining the frequency spectrum. It could also be inferred from this result that the selection patterns on CG content itself might be related to the CAI and tAI parameters. Note that the contribution of MFE to DAF is negative, suggesting that the synonymous mutations on genes with lower MFE (stronger structure) tend to have higher DAF. One speculation is that the “more structured” genes have already experienced many structural changes during evolution so that the newly emerged structural changes on them tend to be less harmful. At this stage, this observation is correlative, and the detailed reason remains unexplored. One certain thing is that the increase in CG content positively contributes to the DAF spectrum.

No correlation is observed between Fst and the prevalence of optimal synonymous mutations

It is necessary to discuss and test whether the observed pattern on synonymous DAF is affected (or caused) by population structures. Fst values describe the difference in allele frequencies among populations from different locations. Higher Fst values might indicate higher divergence across different natural populations (Figure 3A). My concern is (1) if the prevalence of optimal mutations is correlated (no matter positive or negative correlation) with Fst values, then when I look at the DAF I should also consider the Fst values. The putative solution is to divide the genome into several groups with high/medium/low Fst values and then test the pattern (which has been done in.
Figure 1) within each $F_{st}$ group. (2) If the prevalence of optimal mutations is not correlated with $F_{st}$, then that means the pattern observed in Figure 1 is not affected by the population structure.

With the same window size (10,000 bp) of $F_{st}$ values provided by the 1001 genome project (see Materials and Methods section), I calculated the fraction of “optimal” synonymous mutations (among all synonymous mutations) weighted by DAF in each 10,000 bp window (Figure 3B; here “optimal” refers to A/T to G/C synonymous mutations). I intended to use this parameter to measure the prevalence of optimal synonymous mutations within each window. Higher fractions might indicate a stronger positive selection on optimal synonymous mutations (Figure 3B). However, the genome-wide $F_{st}$ shown in Figure 3A and the prevalence of optimal mutations in Figure 3B are not correlated even under such a huge sample size (Figure 3). This result might exclude the possibility that the higher DAF observed on optimal synonymous mutations (Figure 1B) is affected (or caused) by population structures.

The biased DAF spectrum is observed in all representative subpopulations

To further prove the robustness of the observed DAF spectrum of different synonymous mutations, I set out to test this pattern in different subpopulations. The admixture groups defined by the original work were retrieved (Figure 4A). I chose 3 representative subpopulations with adequate accessions with alternative alleles (Figure 4A and B). To correct the direction of mutations by using the outgroup $A. lyrata$, I further discarded the few sites with derived reference alleles so that the alternative alleles of those remaining sites are derived alleles. In the 3 representative subpopulations, the DAF distributions of different synonymous mutation types are illustrated (Figure 4B).

Those synonymous mutations that increase the GC content (orange) have remarkably higher DAF than others. Notably, this pattern appears to be stronger in the “Italy/Balkan/Caucasus” subpopulation (Figure 4B), which is possibly due to the mixed origins of these accessions. Although the reason for different selection strength across subpopulations remains speculative and uncertain, what is clear is the global pattern that the synonymous mutations that increase the GC content have higher DAF.

As mentioned in the Introduction section, the advantage of these optimal synonymous mutations (from A/T to G/C) is likely conferred by the faster translation rate during tRNA decoding. The proposed advantage might act as the selection force shaping the DAF spectrum of synonymous mutations. Nevertheless, the pure evolutionary analyses in this study did
not include any functional tests. The observed differences in DAF of those mutations serve as evidence to speculate the advantage of the optimal synonymous mutations. At this stage, the link between the biased DAF and function remains to be explored.

Conclusions
By analyzing the frequency spectrum of the synonymous mutations, I draw to the conclusion that synonymous mutations in natural populations are not strictly neutral: the synonymous mutations that increase GC content (from A/T to G/C) tend to have higher DAF and therefore are likely to be positively selected.

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Author Contributions
LW designed and supervised this research. LW analyzed the data and wrote this article.

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Data Accessibility
All data used in this study have been described in the Materials and Methods section, which are free to access.

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