Figure S1. Distributions of π_{nor} of coding substitutions on the *D. melanogaster* branch. Data of 200 bp window are shown. The distribution of π_{nor} is approximately exponential (A), with a gap between zero and non-zero bins (B).

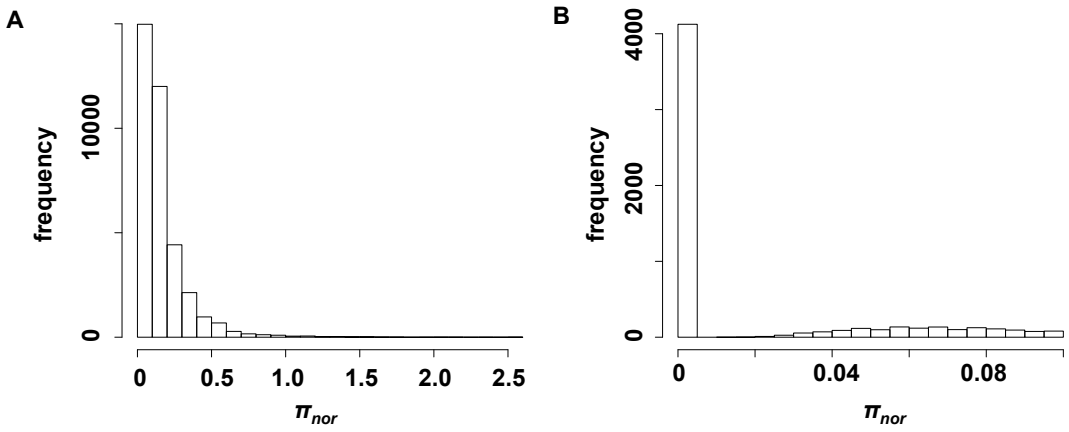


Figure S2. Statistical significance for comparisons of $\varepsilon_{\pi nor}$ of fixations in *single-substitution* genes *vs* other genes while controlling for the effect of exon size. Substitutions are categorized into four equal bins according to the size of exons they are located in (the 25th, 50th, and 75th percentiles of exon size are 418 bp, 798 bp, and 1350 bp). Substitutions in *single-substitution* gene are compared to substitutions in other genes of the same exon-size category. "ns." are comparisons for nonsynonymous substitutions while "s." are comparisons for synonymous substitutions. Upper and lower graphs are for comparisons using $\varepsilon_{\pi nor}$ from linear regression and logistic regression respectively. All *p-values* were calculated using *Mann-Whitney U test*. Increased statistical significance is represented with darker color. The categories with lower $\varepsilon_{\pi nor}$ are consistent with results based on all substitutions (presented in the main text): substitutions in *single-substitution* gene (nonsynonymous substitutions) and substitutions in other genes (synonymous substitutions).

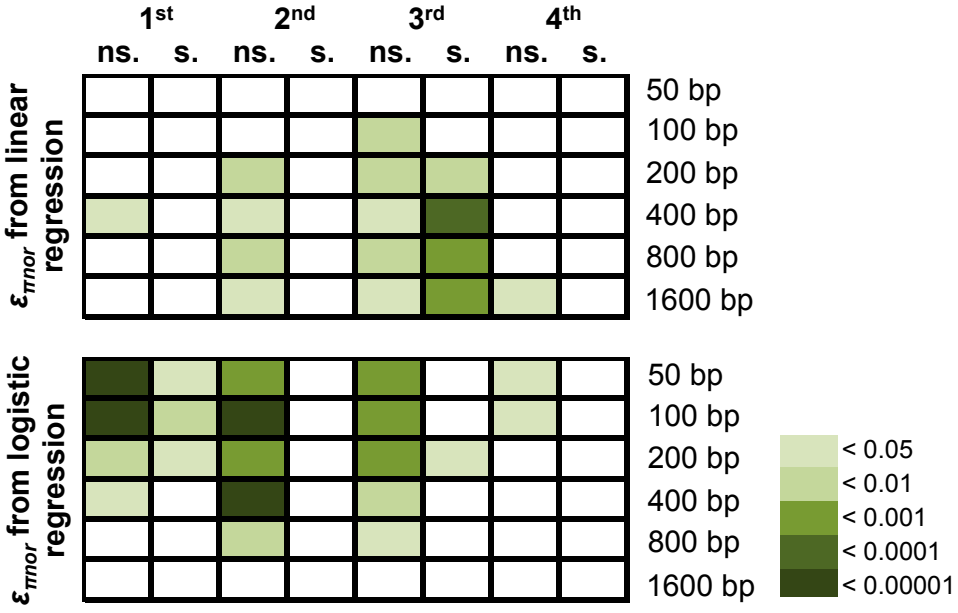


Figure S3. Boxplots for the distributions of factors that correlate with π_{nor}. Plots shown are distribution of recombination rate around the focal substitution (*recomb*), the distance from the focal substitution to the next nonsynonymous substitution (d_{ns}) and synonymous substitution (d_s), and the distance from the focal substitution to the exon-intron boundary (d_{intron}), the edge of 5' UTR (d_{5UTR}), the edge of 3' UTR (d_{3UTR}), and GC content of 4-fold degenerate sites (*GC*).

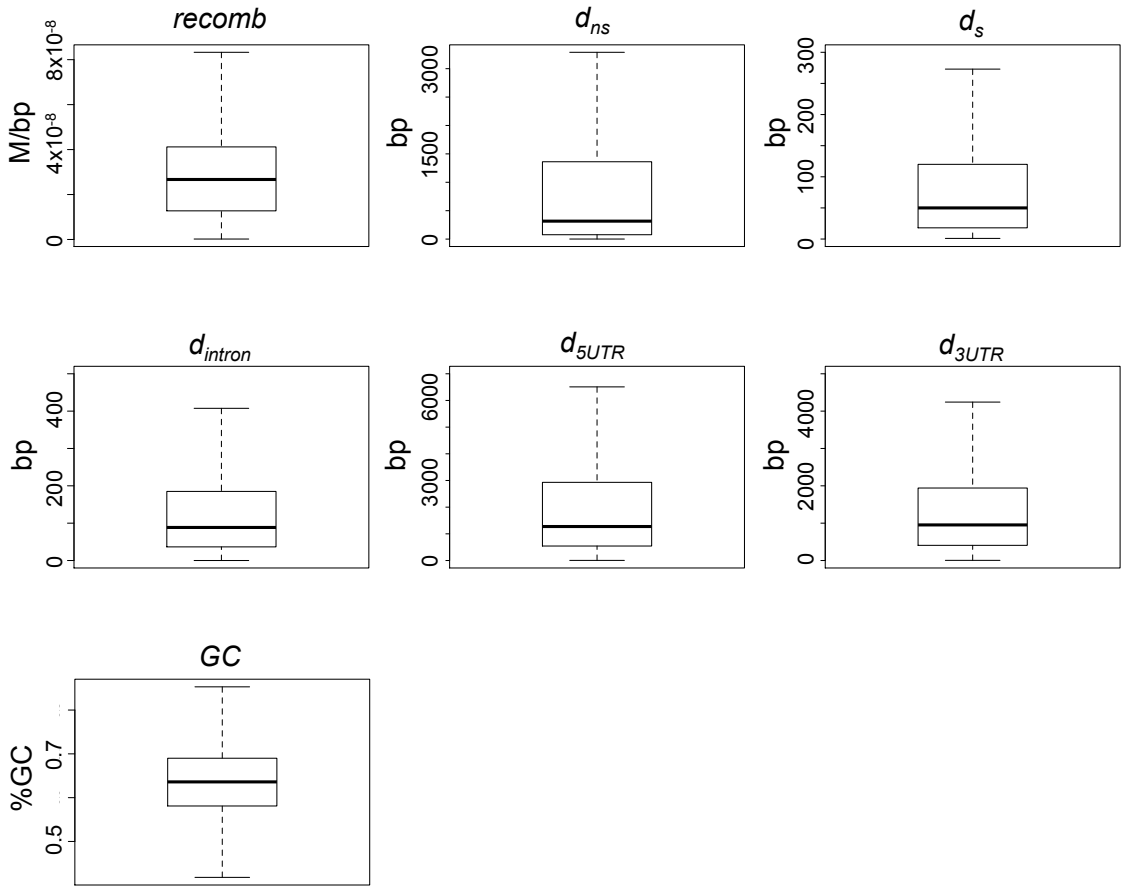


Figure S4. Barplots for the number of nonsynonymous substitutions other than the focal substitution in a window (n_{ns}).

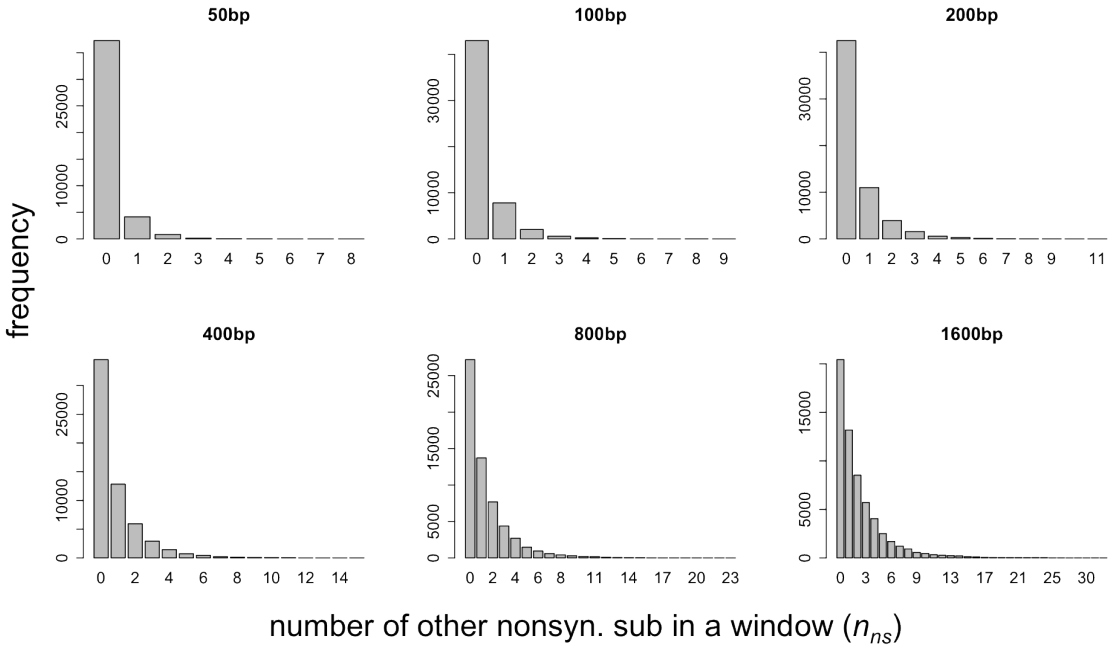


Figure S5. Barplots for the number of synonymous substitutions other than the focal substitution in a window (n_s).

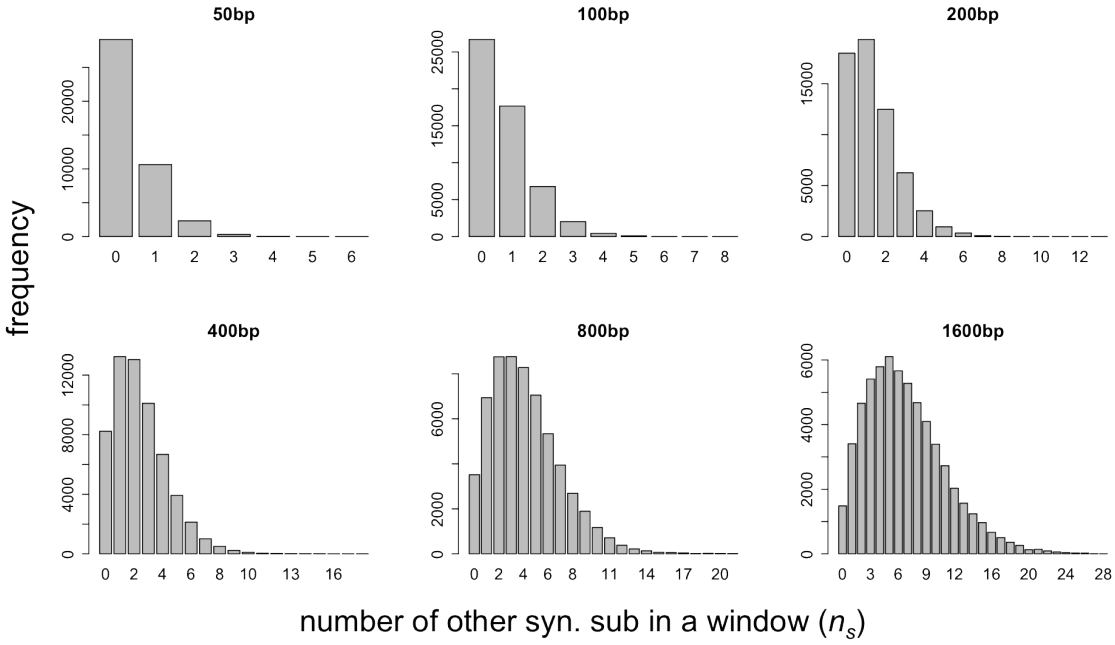


Figure S6. The distribution of non-zero π_{nor} before quantile normalization and $\varepsilon_{\pi nor}$ after quantile normalization. Data of 200bp window are shown. (A) shows the Q-Q plot of non-zero π_{nor}, whose distribution is highly non-normal. On the other hand, distributions of $\boldsymbol{\varepsilon_{\pi nor}}$ from linear regression analysis using quantile-normalized $\boldsymbol{\pi_{nor}}$ as response variable is normally distributed (B).

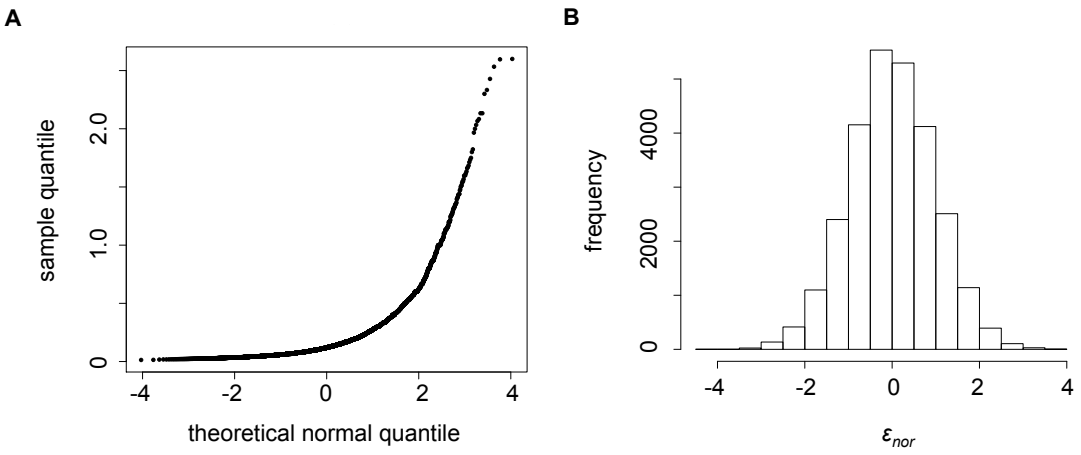


Figure S7. Statistical significance for comparisons of $\varepsilon_{\pi nor}$ of fixations associated with different biological categories using windows with no noncoding sites.

Only windows that do not span over noncoding sequences were included in the analysis [percentage of windows included in the analysis for $\boldsymbol{\varepsilon_{\pi nor}}$ of linear regression: 95.9% (50 bp), 87.9% (100 bp), 72.9% (200 bp), 50.0% (400 bp), 22.3% (800 bp), and 5.6% (1600 bp); for $\boldsymbol{\varepsilon_{\pi nor}}$ of logistic regression: 93.6 (50 bp), 81.0% (100 bp), 62.6% (200 bp), 40.2% (400 bp), 17.8% (800 bp), and 4.4% (1600 bp)]. Upper and lower graphs are for comparisons using $\boldsymbol{\varepsilon_{\pi nor}}$ from linear regression and logistic regression respectively. All *p-values* were calculated using *Mann-Whitney U test* unless otherwise specified. Increased statistical significance is represented with darker color. The categories with lower $\boldsymbol{\varepsilon_{\pi nor}}$ are consistent with the result based on all windows (Figure 3). Yet, the *p-values* are generally larger than those of using all windows (Figure 3), which is potentially due to the much smaller sample size. Comparisons shown are (A) nonsynonymous substitution *vs* synonymous substitutions, (B) nonsynonymous substitutions of *single-substitution* genes *vs* synonymous substitutions of *single-substitution* genes, (C) nonsynonymous substitutions of *single-substitution* genes *vs* nonsynonymous substitutions of other genes, (D) synonymous substitutions of *single-substitution* gene *vs* synonymous substitutions of other genes, (E) nonsynonymous substitutions that changed amino acid chemical properties in different ways (*Kruskal-Wallis test*), (F) nonsynonymous substitutions that did not change amino acid charges *vs* nonsynonymous substitutions that changed amino acid charges, (G) nonsynonymous substitutions that increased amino acid acidity *vs* nonsynonymous substitutions that increased amino acid basicity, (H) nonsynonymous substitutions that reinforced the chemical property changes of the nearest amino acid

substitutions on the same linage (both on *D. melanogaster*) *vs* those compensated for these changes, (I) nonsynonymous substitutions that reinforced the polarities changes of the nearest amino acid substitutions on the same linage (both on *D. melanogaster*) *vs* those compensated for these changes, and (J) nonsynonymous substitutions that reinforced the charge changes of the nearest amino acid substitutions on the same linage (both on *D. melanogaster*) *vs* those compensated for these changes.

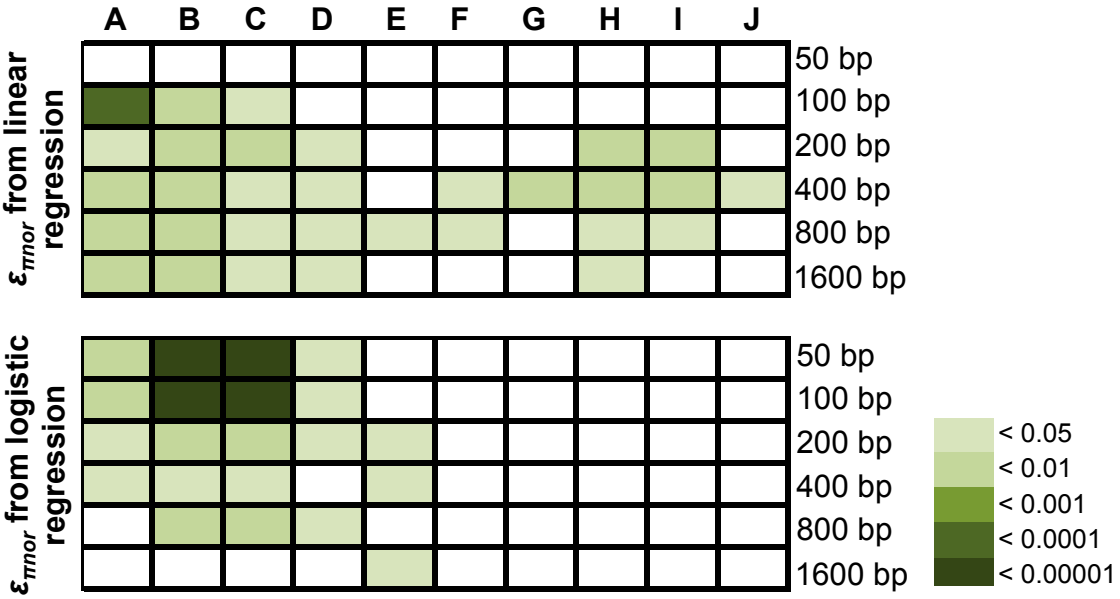
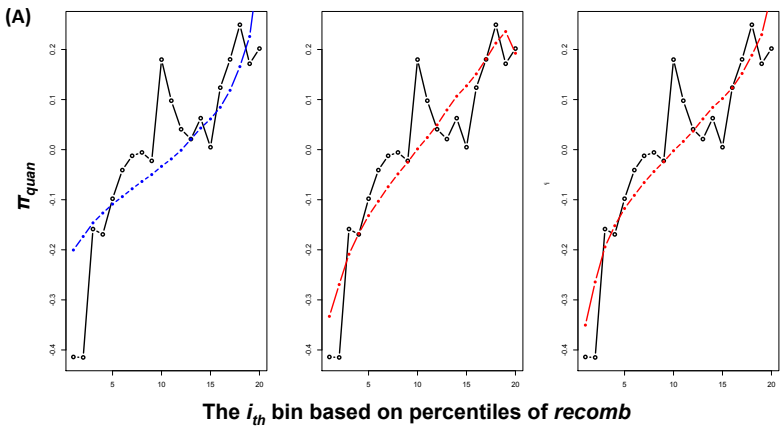
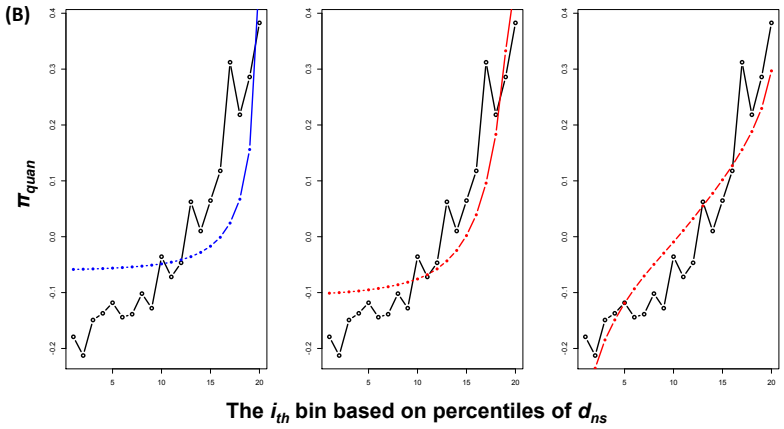


Figure S8. Single-factor linear regressions for identifying the appropriate regression model. We performed linear regression on π_{quan} (of 400bp window size) and considered one predictor variable at a time. Based on the fitted model, we calculated the predicted π_{quan}. We divided the observed windows into 20 bins according to the percentiles of the predictor variable and calculated the mean of observed π_{quan} (shown in black) and predicted π_{quan} (shown in blue or red) for each bin. For each of the following figure, the three regression models used are linear (left), quadratic (middle), logarithmic (right). We chose the model that has the largest R^2 and empirically fits the data best from the plotted figure, and presented the result based on this regression model in the main text. When R^2 do not differ greatly between two regression models, we considered both in our following analysis that evaluates the sensitivity of our conclusions to the regression model chosen (see main text, Figure S8 and Table S2). We showed the result of three predictors as examples: (A) *recomb,* the quadratic (middle) model performs best, (B) d_{ns}, the quadratic (middle, R^2 = 2.41%) and logarithmic (right, R^2 = 2.47%) model both perform well, and (C) d_{5UTR}, the quadratic (middle) model performs best.




(B)
TT_{quan}
The i_{th} bin based on percentiles of d_{ns}

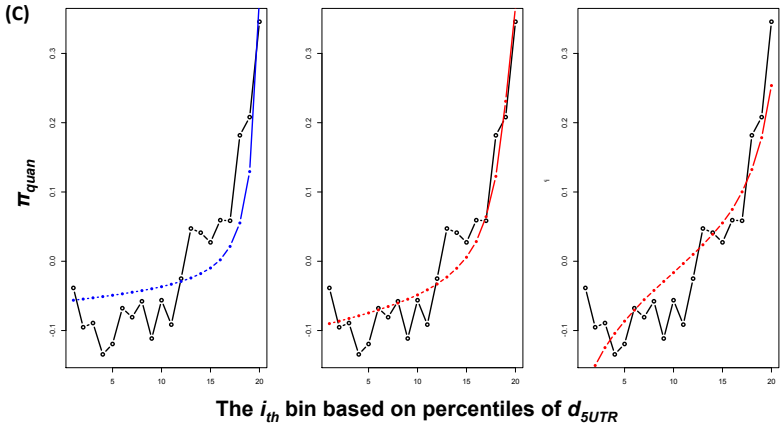
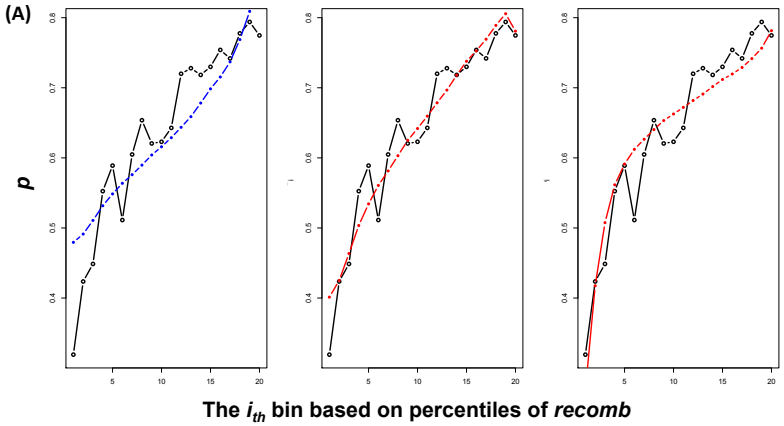
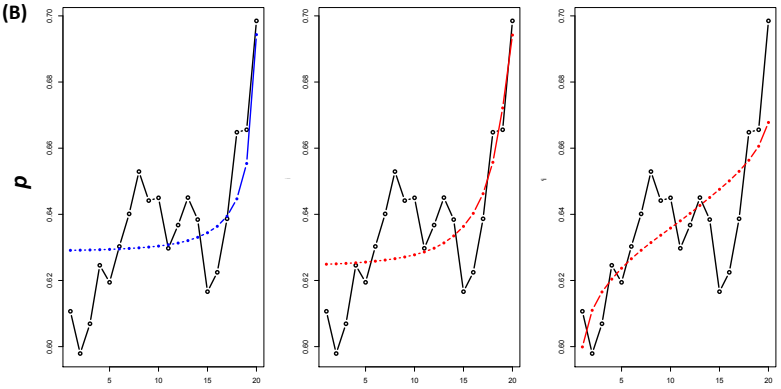

(C)
TT_{quan}
The i_{th} bin based on percentiles of d_{5UTR}

Figure S9. Single-factor logistic regressions for identifying the appropriate regression model. We performed regression on a binary variable that represents having nonzero $\boldsymbol{\pi_{nor}}$ (one) or zero $\boldsymbol{\pi_{nor}}$ (zero; of 400bp window) and considered one predictor variable at a time. Based on the fitted model, we calculated the predicted p (the probability of having nonzero π_{nor}). We divided the windows into 20 bins according to the percentiles of the predictor variable and calculated the mean of observed p (shown in black) and predicted p (shown in blue or red) for each bin. For each of the following figure, the three regression models used are linear (left), quadratic (middle), logarithmic (right). We chose the model that has the smallest AIC and empirically fits the data best from the plotted figure, and presented the result based on this regression model in the main text. When AICs do not differ greatly between two regression models, we considered both in our following analysis that evaluates the sensitivity of our conclusions to the regression model chosen (see main text, Figure S9 and Table S2). We showed the result of three predictors as examples: (A) *recomb,* the quadratic (middle) model performs best, (B) d_{ns}, the quadratic (middle, AIC = 52448) and logarithmic (right, AIC = 52461) model both perform well, and (C) d_{5UTR}, the linear (left, AIC = 52493) and quadratic (middle, AIC = 52494) both perform well.

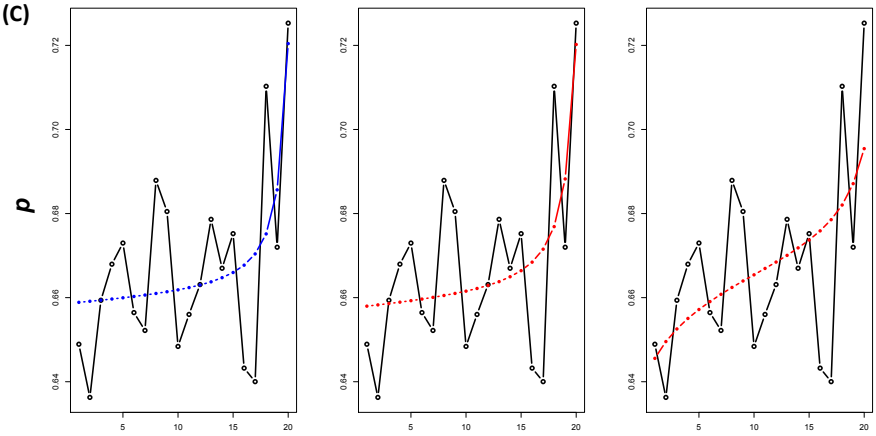


(B)



The i_{th} bin based on percentiles of d_{ns}

(C)



The i_{th} bin based on percentiles of d_{5UTR}

Figure S10. Statistical significance for comparisons of $\varepsilon_{\pi nor}$ from other linear regression models. Upper and lower graphs are $\varepsilon_{\pi nor}$ from two different linear regression models. Model 1 (regression model with all linear terms): $\pi_{quan} \sim recomb + n_{ns} + n_s + d_{ns} + d_s + d_{intron} + d_{5UTR} + d_{3UTR} + GC$,

Model 2 [original regression model with log(d_{ns}) term (see Table S2), followed by backward model selection]: $\pi_{quan} \sim recomb + recomb^2 + n_{ns} + n_s + log(d_{ns}) + d_s + d_s^2 + d_{intron} + d_{intron}^2 + d_{5UTR} + d_{5UTR}^2 + d_{3UTR} + GC$.

All *p-values* were calculated using *Mann-Whitney U test* unless otherwise specified. Increased statistical significance is represented with darker color. The categories with lower $\varepsilon_{\pi nor}$ are consistent with the result based on the regression model presented in the main text (Figure 3, upper graph). Comparisons shown are (A) nonsynonymous substitution *vs* synonymous substitutions, (B) nonsynonymous substitutions of *single-substitution* genes *vs* synonymous substitutions of *single-substitution* genes, (C) nonsynonymous substitutions of *single-substitution* genes *vs* nonsynonymous substitutions of other genes, (D) synonymous substitutions of *single-substitution* gene *vs* synonymous substitutions of other genes, (E) nonsynonymous substitutions that changed amino acid chemical properties in different ways (*Kruskal-Wallis test*), (F) nonsynonymous substitutions that did not change amino acid charges *vs* nonsynonymous substitutions that changed amino acid charges, (G) nonsynonymous substitutions that increased amino acid acidity *vs* nonsynonymous substitutions that increased amino acid basicity, (H) nonsynonymous substitutions that reinforced the chemical property changes of the nearest amino acid substitutions on the same linage (both on *D. melanogaster*) *vs* those compensated for these changes, (I) nonsynonymous substitutions that reinforced the

polarities changes of the nearest amino acid substitutions on the same linage (both on *D. melanogaster*) *vs* those compensated for these changes, and (J) nonsynonymous substitutions that reinforced the charge changes of the nearest amino acid substitutions on the same linage (both on *D. melanogaster*) *vs* those compensated for these changes.

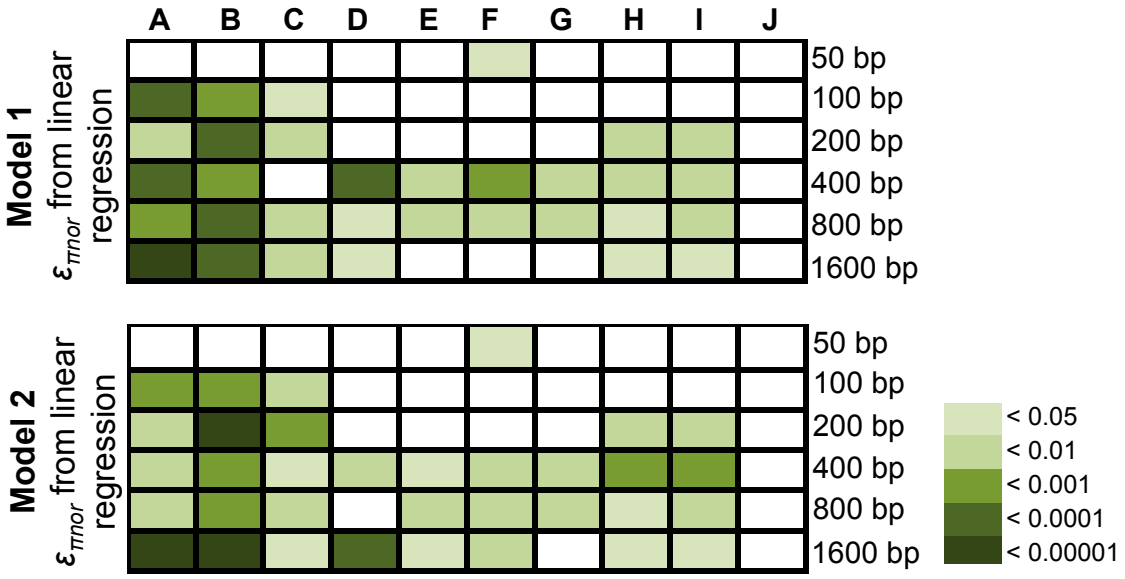


Figure S11. Statistical significance for comparisons of $\varepsilon_{\pi nor}$ from other logistic regression models. Upper and lower graphs are $\varepsilon_{\pi nor}$ from two different logistic regression models. Model 1 (regression model with all linear terms): $logit\ p \sim recomb + n_{ns} + n_s + d_{ns} + d_s + d_{intron} + d_{5UTR} + GC$,

Model 2 [original regression model with several *log* and quadratic terms that are different from the model presented in the main text (see Table S2), followed by backward model selection]: $logit\ p \sim recomb + recomb^2 + n_{ns} + n_s + log(d_{ns}) + log(d_s) + d_s^2 + log(d_{intron}) + d_{5UTR} + d_{5UTR}^2 + GC$.

All *p-values* were calculated using *Mann-Whitney U test* unless otherwise specified. Increased statistical significance is represented with darker color. The categories with lower $\varepsilon_{\pi nor}$ are consistent with the result based on the regression model presented in the main text (Figure 3, lower graph) except for those denoted with red color (see figure legend). Comparisons shown are (A) nonsynonymous substitution *vs* synonymous substitutions, (B) nonsynonymous substitutions of *single-substitution* genes *vs* synonymous substitutions of *single-substitution* genes, (C) nonsynonymous substitutions of *single-substitution* genes *vs* nonsynonymous substitutions of other genes, (D) synonymous substitutions of *single-substitution* gene *vs* synonymous substitutions of other genes, (E) nonsynonymous substitutions that changed amino acid chemical properties in different ways (*Kruskal-Wallis test*), (F) nonsynonymous substitutions that did not change amino acid charges *vs* nonsynonymous substitutions that changed amino acid charges, (G) nonsynonymous substitutions that increased amino acid acidity *vs* nonsynonymous substitutions that increased amino acid basicity, (H) nonsynonymous substitutions that reinforced the chemical property changes of the nearest amino acid

substitutions on the same linage (both on *D. melanogaster*) *vs* those compensated for these changes, (I) nonsynonymous substitutions that reinforced the polarities changes of the nearest amino acid substitutions on the same linage (both on *D. melanogaster*) *vs* those compensated for these changes, and (J) nonsynonymous substitutions that reinforced the charge changes of the nearest amino acid substitutions on the same linage (both on *D. melanogaster*) *vs* those compensated for these changes.

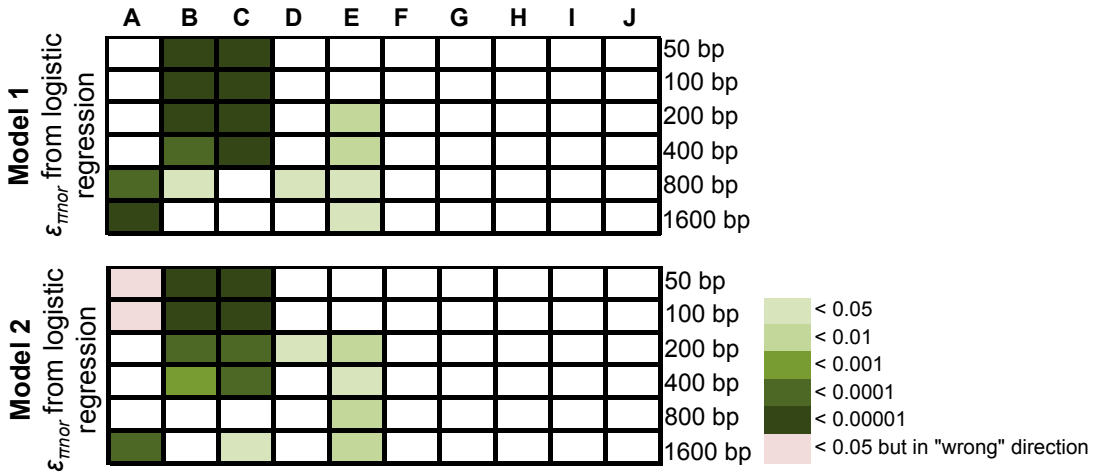


Table S1. ***Spearman rank*** ρ **between** π_{nor} **and factors.**

	50bp		100bp		200bp		400bp		800bp		1600bp	
factors	***coeff.***	***p-value***	***coeff.***	***p-value***	***coeff.***	***p-value***	***coeff.***	***p-value***	***coeff.***	***p-value***	***coeff.***	***p-value***
recomb	1.33E-01	< 2.2e-16	1.79E-01	< 2.2e-16	2.28E-01	< 2.2e-16	2.78E-01	< 2.2e-16	3.24E-01	< 2.2e-16	3.54E-01	< 2.2e-16
n_{ns}	-2.07E-02	2.03E-05	-3.67E-02	< 2.2e-16	-5.25E-02	< 2.2e-16	-7.96E-02	< 2.2e-16	-1.06E-01	< 2.2e-16	-1.35E-01	< 2.2e-16
n_s	-3.46E-02	1.01E-12	-3.92E-02	< 2.2e-16	-4.70E-02	< 2.2e-16	-5.46E-02	< 2.2e-16	-7.69E-02	< 2.2e-16	-1.01E-01	< 2.2e-16
d_{ns}	4.50E-02	< 2.2e-16	6.03E-02	< 2.2e-16	7.75E-02	< 2.2e-16	9.50E-02	< 2.2e-16	1.06E-01	< 2.2e-16	1.19E-01	< 2.2e-16
d_s	3.30E-02	1.04E-11	3.41E-02	2.40E-15	3.74E-02	< 2.2e-16	3.43E-02	< 2.2e-16	4.26E-02	< 2.2e-16	5.75E-02	< 2.2e-16
d_{intron}	1.87E-02	1.16E-04	2.85E-02	3.99E-11	3.81E-02	< 2.2e-16	2.18E-02	1.14E-07	-1.61E-03	6.94E-01	-1.49E-02	2.48E-04
d_{5UTR}	3.21E-02	3.86E-11	3.49E-02	5.55E-16	4.67E-02	< 2.2e-16	5.88E-02	< 2.2e-16	7.12E-02	< 2.2e-16	7.18E-02	< 2.2e-16
d_{3UTR}	1.28E-02	8.57E-03	1.31E-02	2.47E-03	1.50E-02	3.11E-04	5.99E-02	7.72E-03	4.62E-03	2.58E-01	4.69E-03	2.48E-01
GC	0.03249767	< 2.2e-16	0.04979838	< 2.2e-16	0.07447929	< 2.2e-16	0.1011433	< 2.2e-16	0.1252335	< 2.2e-16	0.1414383	< 2.2e-16

Significant ($p < 0.05$) positive correlations are highlighted in blue while significant negative correlations are highlighted in red.

Table S2. Regression analysis for comparisons of $\varepsilon_{\pi nor}$ of fixations in *single-substitution* genes and other genes while controlling the effect of exon size.

$\varepsilon_{\pi nor}$ of Linear Regressoin				
regression coefficient	nonsynonymous		synonymous	
	single-sub.	exon size	*single sub.*	exon size
25	not sig.	not sig.	not sig.	not sig.
50	-1.69E-01 *	not sig.	not sig.	not sig.
100	-1.93E-01 ***	not sig.	not sig.	-3.08E-05 **
200	-1.31E-01 **	-5.69E-05 ***	4.42E-02 **	-4.44E-05 ***
400	-1.51E-01 ***	-6.64E-05 ***	2.77E-02 *	-4.87E-05 ***
800	-1.37E-01 ***	-6.35E-05 ***	3.53E-02 **	-4.28E-05 ***

$\varepsilon_{\pi nor}$ of Logistic Regressoin				
regression coefficient	nonsynonymous		synonymous	
	single-sub.	exon size	*single sub.*	exon size
25	not sig.	not sig.	not sig.	not sig.
50	-2.30E-01 **	-7.81E-05 **	8.16E-02 **	not sig.
100	-1.23E-01 ***	-6.90E-05 **	8.82E-02 ***	not sig.
200	-2.17E-01 **	-9.72E-06 *	5.28E-02 *	6.40E-05 ***
400	not sig.	5.89E-05 *	not sig.	1.55E-04 ***
800	not sig.	1.44E-01 ***	not sig.	1.81E-04 ***

not sig.: the *p-value* associated with the regression coefficient is not significant
* *p-value* < 0.05, ** *p-value* < 0.01, *** *p-value* < 0.001
The regression model: $\varepsilon_{\pi nor}$ ~ *single-sub.* + exon size. "*single-sub.*" is a binary variable, describing whether the substitution is in *single-substitution* gene (1) or other genes (0). "exon size" is the size of exon (in bp) the substitution is located in. The upper and lower tables are for regression analyses using $\varepsilon_{\pi nor}$ of linear regression and $\varepsilon_{\pi nor}$ of logistic regression respectively. For both regression methods, nonsynonymous substitutions in *single-substitution* genes have lower $\varepsilon_{\pi nor}$ (negative regression coefficient for "*single-sub.*") while synonymous substitutions in other genes have lower $\varepsilon_{\pi nor}$ (positive regression coefficient for "*single-sub.*"), which supports the results without controlling for the effect of exon size.

Table S3. Chosen regression model for each predictor variable based on single-factor regression analysis.

predictor	Linear regression	Logistic regression
recomb	**quadratic**	**quadratic**
n_{ns}	**linear**	**linear**
n_s	**linear**	**linear**
d_{ns}	**quadratic/**logarithmic	**quadratic/**logarithmic
d_s	**quadratic**	**quadratic/**logarithmic
d_{intron}	**quadratic**	**logarithmic**
d_{5UTR}	**quadratic**	**linear/**quadratic
d_{3UTR}	**linear**	NA
GC	**linear**	**linear**

Regression models that have the largest R^2/smallest AIC and are included in the model presented in the main text are in bold type.

Table S4. Regression coefficients and associated *p-values* for linear regression.

predictor	50bp		100bp		200bp		400bp		800bp		1600bp	
	coeff.	*p-value*	*coeff.*	*p-value*	*coeff.*	*p-value*	*coeff.*	*p-value*	*coeff.*	*p-value*	*coeff.*	*p-value*
intercept	9.68E-03	0.762685	-8.27E-01	1.21E-10	-1.14E+00	< 2e-16	-1.49E+00	< 2e-16	-1.70E+00	< 2e-16	-1.58E+00	< 2e-16
recomb	4.25E-03	0.544976	1.50E-02	0.001226	1.04E-01	< 2e-16	1.85E-01	< 2e-16	2.76E-01	< 2e-16	3.43E-01	< 2e-16
$recomb^2$	NA	NA	NA	NA	-8.57E-03	1.42E-11	-1.48E-02	< 2e-16	-2.30E-02	< 2e-16	-2.89E-02	< 2e-16
n_{ns}	NA	NA	-4.70E-02	0.000346	-4.40E-02	7.11E-10	-4.24E-02	< 2e-16	-3.53E-02	< 2e-16	-3.25E-02	< 2e-16
n_s	NA	NA	NA	NA	-1.11E-02	0.0378	-2.72E-02	< 2e-16	-2.84E-02	< 2e-16	-2.37E-02	< 2e-16
d_{ns}	2.53E-05	0.001892	2.85E-05	8.31E-07	3.70E-05	2.32E-16	4.77E-05	< 2e-16	4.23E-05	< 2e-16	3.80E-05	< 2e-16
d_{ns}^2	-9.96E-10	0.011572	-9.65E-10	0.000657	-1.31E-09	5.15E-09	-1.74E-09	< 2e-16	-1.47E-09	< 2e-16	-1.23E-09	7.17E-14
d_s	NA	NA	8.53E-05	0.015122	1.26E-04	1.45E-05	NA	NA	NA	NA	NA	NA
d_s^2	NA	NA	NA	NA	NA	NA	4.24E-08	0.000285	3.23E-08	0.00259	NA	NA
d_{intron}	-3.81E-04	4.79E-07	-4.52E-04	< 2e-16	-7.52E-04	< 2e-16	-6.78E-04	< 2e-16	-5.19E-04	< 2e-16	-2.49E-04	< 2e-16
d_{intron}^2	1.49E-07	0.000571	1.50E-07	3.09E-07	2.63E-07	< 2e-16	1.98E-07	< 2e-16	1.36E-07	3.28E-15	2.71E-08	0.0773
d_{5UTR}	3.75E-06	0.060901	1.15E-05	0.001325	2.26E-05	< 2e-16	2.64E-05	< 2e-16	2.86E-05	< 2e-16	2.38E-05	< 2e-16
d_{5UTR}^2	NA	NA	1.15E-05	0.013366	-4.33E-10	1.53E-07	-5.25E-10	4.77E-14	-5.11E-10	< 2e-16	-3.39E-10	4.55E-09
d_{3UTR}	1.35E-05	0.010915	1.09E-05	0.00285	4.94E-06	0.0676	8.74E-06	0.000142	1.01E-05	9.22E-07	1.12E-05	2.54E-09
GC	NA	NA	1.51E+00	2.18E-10	1.92E+00	< 2e-16	2.40E+00	< 2e-16	2.57E+00	< 2e-16	2.20E+00	< 2e-16
R^2	0.009644		0.02334		0.05813		0.09847		0.1407		0.178	

Predictors that are not included in the regression model based on backward model selection are denoted as “NA”.

Table S5. Regression coefficients and associated *p-values* for logistic regression.

predictor	50bp		100bp		200bp		400bp		800bp		1600bp	
	coeff.	*p-value*	*coeff.*	*p-value*	*coeff.*	*p-value*	*coeff.*	*p-value*	*coeff.*	*p-value*	*coeff.*	*p-value*
intercept	-2.87E+00	< 2e-16	-2.87E+00	< 2e-16	-2.56E+00	< 2e-16	-2.06E+00	< 2e-16	-1.24E+00	< 2e-16	-9.90E-01	< 2e-16
recomb	4.65E-01	< 2e-16	4.91E-01	< 2e-16	5.61E-01	< 2e-16	6.57E-01	< 2e-16	7.97E-01	< 2e-16	9.54E-01	< 2e-16
$recomb^2$	-4.20E-02	< 2e-16	-4.23E-02	< 2e-16	-4.78E-02	< 2e-16	-5.50E-02	< 2e-16	-6.54E-02	< 2e-16	-7.64E-02	< 2e-16
n_{ns}	-8.37E-02	0.00954	-5.12E-02	0.00124	-4.17E-02	2.80E-05	-3.29E-02	3.15E-06	NA	NA	2.02E-02	6.77E-05
n_s	-1.26E-01	1.44E-06	-9.08E-02	4.33E-16	-6.36E-02	1.45E-11	-1.16E-02	0.04943	3.24E-02	4.79E-14	7.06E-02	< 2e-16
d_{ns}	3.83E-05	5.75E-05	4.47E-05	7.45E-10	5.12E-05	4.83E-14	3.80E-05	2.49E-07	3.79E-05	2.99E-06	6.74E-06	0.11016
d_{ns}^2	-9.56E-10	0.04233	-1.44E-09	7.96E-05	-1.72E-09	4.07E-07	-1.28E-09	0.000493	-1.34E-09	0.00131	NA	NA
d_s	4.76E-04	0.09507	NA	NA	1.33E-03	0.01412	3.40E-03	4.78E-11	4.24E-03	1.39E-12	4.82E-03	9.01E-11
d_s^2	NA	NA	NA	NA	-6.95E-06	0.00437	-1.27E-05	3.29E-07	-1.24E-05	1.93E-05	-1.17E-05	0.00138
$log(d_{intron})$	5.45E-02	1.58E+00	1.04E-01	< 2e-16	1.65E-01	< 2e-16	1.86E-01	< 2e-16	1.92E-01	< 2e-16	1.67E-01	< 2e-16
d_{5UTR}	9.90E-06	2.65E-05	9.30E-06	3.53E-07	8.45E-06	9.50E-07	1.05E-05	3.94E-08	1.24E-05	5.57E-08	1.22E-05	1.74E-05
GC	6.30E-01	0.10991	1.31E+00	3.05E-06	1.29E+00	2.38E-07	1.04E+00	5.65E-05	NA	NA	NA	NA

Predictors that are not included in the regression model based on backward model selection are denoted as “NA”.