Codon degeneracy as well as pretermination codons influence transition to transversion ratio in coding sequences

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Abstract

Transition to transversion ratio (ti/tv) is important in the study of molecular evolution, which provides the understanding of mutational bias in an organism. Since, synonymous polymorphism for *two-fold degenerate* codons depends only on transitions, *two-fold degenerate* codons and *four-fold degenerate* codons are different regarding (Ti/Tv) ratios both for synonymous as well as non-synonymous mutations. Therefore, various coding sequences in any organism having difference in degenerate codon composition is likely to affects the ti/tv ratio. In addition to the above, compositional differences among the coding sequences regarding pretermination codon is also likely to influence the ti/tv ratio because purifying selection is stronger on non-synonymous substitutions leading to termination codons. In this study we have improve the method of finding the ratio between transition to transversion, synonymous transition to synonymous transversion and non-synonymous transition to non-synonymous transversion and also developed a computer program to calculate the values. Our analyses in 2516 coding sequences in the *E. coli* genome suggested a strong correlation between codon degeneracy with synonymous transition to transversion ratio, also we have observed a moderate correlation between pretermination codons

and non-synonymous transition to transversion. The percentage change between the conventional and improved method was the most striking part of our observation.

Keywords

Substitution mutation, Nucleotide polymorphism, Transition to transversion ratio, Codon degeneracy, Pretermination codon

Introduction

In the universal genetic codon table out of 64 codons, 61 codons encode for 20 amino acids whereas 3 codons act as termination signal in the protein synthesis process. The redundancy or the degeneracy nature of the genetic codon table suggests that a single amino acid can be coded by multiple codons. There is different magnitude of degeneracy among the 61 sense codons ranging between 0-6. AUG and UGG encode for Met and Trp having no degeneracy at all. Codons such as UUU and UUC encode for Phe and belong to the category of *two-fold degenerate* (TFD) codons. AUU, AUC and AUA encode for Ile and belong to the category of three-fold degenerate codons. Codons such as GGU, GGC, GGA and GGG encode for *four-fold degenerate* (FFD) codons. Codons such as CUN and UUR encode for Leu and are known as *six-fold* (SFD) codons. Because of different degeneracy among codons the number of synonymous polymorphism and the number of non-synonymous polymorphism are not equal among the codons. Every codon can undergo 9 different single nucleotide polymorphisms (SNPs), out of which 3 substitutions are transition (ti) and 6 substitutions are transversion (tv) substitutions. In case of a TFD codon, there is only single synonymous transition (Sti), zero synonymous transversion (Stv), two non-synonymous transition (Nti) and six non-synonymous transversion (Ntv) possible. In case of a FFD codon one Sti, two Stv, two Nti and four Ntv are possible (Fig.

S1A). In a standard genetic codon table out of 549 total SNPs involving 61 sense codons, only 134 SNPs are synonymous (24.4%). Among synonymous (Sti and Stv) and non-synonymous (Nti and Ntv) polymorphisms, the theoretically estimated ($_e$) numbers of Sti, Stv, Nti and Ntv in a genetic codon table are 62,72,121 and 294 respectively (Table S1). Yet the observed synonymous polymorphisms are always higher than the expected proportion across genes (Tamura 1992; Wolfe et al. 1989; Moriyama and Powell 1997; Ngandu et al. 2008).

It is already known that synonymous polymorphisms are more preferred over non-synonymous polymorphisms because purifying selection is stronger on non-synonymous substitution (Hurst and Pál 2001). Therefore, it is expected that, number of substitutions per codon is likely to be higher in case of a FFD codon (Aziz et al. 2022). Hence, it is expected that a gene having higher proportion of FFD codons than TFD codons will obtain more substitutions than the others. Coding sequences are likely to be varying from each other regarding the composition of TFD & FFD.

Among 61 sense codons there are 18 Pre-termination codons (PTC) which can lead to stop codons by a single substitution at certain positions in the PTC (Modiano et al. 1981) (Fig. S1B). Out of 18 PTC, UUA, UCA, UAY and UGG are such 5 PTC, in which out of nine possible outcomes two possible outcomes can lead to stop codons. For example, out of 9 substitutions, 2 in UUA and 1 in UUG can lead to stop codon. Hence non-synonymous substitution count is different for UUA and UUG (Fig. S1C). Hence PTC compositions are also likely to influence the non-synonymous polymorphism because purifying selection is stronger on non-sense substitutions (van den Eynden et al. 2016; Morales et al. 2021; Li et al. 1981). Therefore, PTC composition in a gene is likely to influence the polymorphism. Therefore, the theoretically estimated Sti, Stv, Nti and Ntv vary among codons, subsequently codon composition in a gene

alternatively affects the ti to tv ratio, which is self-explanatory regarding the codon degeneracy. By acknowledging the disproportion of polymorphism patterns affected by codon degeneracy as well as PTC, now the theoretically estimated polymorphisms can be calculated in the form of Sti, Stv, Nti and Ntv for each codon (Table 1). Also, we have summarized the total numbers of theoretically estimated synonymous ti (tv) and non-synonymous ti (tv) in the genetic codon table considering (Table 2)

Base substitution mutation is the major driving force of molecular evolution. Transition (*ti*) base substitution that defines replacement of a purine (or pyrimidine) by another purine (or pyrimidine) is two times more frequent than transversion (*tv*) substitution that defines replacement of a purine (or pyrimidine) by another pyrimidine (or purine) (Seplyarskiy et al. 2012; Duchêne et al. 2015; Lewis Jr et al. 2016; Stoltzfus and Norris 2016; Lyons and Lauring 2017; Schroeder et al. 2017). It is already known in the literature that the rate of ti and rate of tv are not similar. ti is known to be approximately four times more frequent than tv (Sen et al. 2022; Aziz et al. 2022), this is known as Kimura's two parameter model (Kimura 1980). However, all the ti rates and tv rates are not identical. In this context, it is important to understand ti/tv or Sti/Stv or even Nti/Ntv rate in a gene by finding out the ratio between ti/tv. It is known that Sti and Stv sites or Nti and Ntv sites are not equally proportional among the codons.

In this study we have found that compositional difference of different degenerate codons is influencing the ti/tv ratio along with the composition of PTC in a gene. As there is a difference among the different degenerate codons regarding Sti, Stv, Nti and Ntv. Further, it has been demonstrated that TFD: FFD values indeed influence the ti/tv values. Also, we have considered the compositional difference among the coding sequences regarding the PTC while calculating the ti/tv by the improved method. We have also proved that PTC% influences the ti/tv ratio.

Based on the work by Ina 1998 we tried to establish the biological significance of codon composition and ti/tv ratio across 2516 coding sequences of *E. coli*. The new method which we developed may be apparent to study different ti/tv (Sti/Stv and Nti/Ntv) across coding sequences.

Methods

Coding sequences considered

In this study, we carried out a SNPs based computational analysis of 2516 coding sequences across 157 strains of E. *coli* (Thorpe et al. 2017). The information regarding the dataset selected for our study are made available in SI *Appendix*, Table S2. Although the public database has ~4500 coding sequences but we excluded coding sequences showing length variation and improper alignment/annotation. Codons having polymorphisms in more than one position or polymorphisms leading to ambiguous nucleotides represented as 'N' were not considered in the analyses of the polymorphisms in this study. A detailed procedure followed for the same is represented in SI *Appendix*, Table S3 using example of a hypothetical sequence. Further, the SNPs observed (SNP_o) at different positions of each codon were categorized into Sti_o, Stv_o, Nti_o and Ntv_o, where S for synonymous, N for non-synonymous, ti_o for transition observed and tv_o for transversion observed, and subsequently ti_o ($Sti_o + Nti_o$) and tv_o ($Stv_o + Ntv_o$) for each gene were calculated (*SI Appendix*, Table S4). Since we got the observed ($_o$) polymorphism values for a gene, now it was required to calculate the theoretically estimated ($_e$) polymorphism values for the gene. The reference sequence which was previously derived from the sequence alignments was used to calculate the codon count of each gene. After finding the codon count, each codon count value was multiplied by theoretically estimated polymorphism values for each codon. For example, if the codon frequency of GGG is observed to be 10 in a gene, then the total

theoretically estimated value for Sti, Stv, Nti and Ntv are 10,20,20 and 40 (as estimated Sti, Stv,Nti and Ntv for each GGG is 1,2,2 and 4 respectively). The total theoretically estimated polymorphism values for each codon in a gene, we then summed up the individual polymorphism values for 61 sense codons to get the total theoretically estimated ($_e$) individual substitution values (Sti_e, Stv_e,Nti_e and Ntv_e) for the gene (*SI Appendix*, Table S5). The Sti_o, Stv_o, Nti_o, Ntv_o, ti_o and tv_o values as well as Sti_e, Stv_e Nti_e, Ntv_e, ti_e and tv_e of a coding sequence are the used in the modified equation to find out different $\frac{ti}{tv}$ ratio (*SI Appendix*, Table S6). A detailed workflow is provided in Fig. 1. The calculations were executed through a programing language written in 'C language' to find out the ti to tv (Sti/Stv and Nti/Ntv) ratio in the conventional as well as our improved method.

Improved equations

$$\frac{ti'}{tv'} = \frac{\left({ti_o}/{ti_e}\right)}{\left({tv_o}/{tv_e}\right)} \qquad \textbf{Equation 1}$$

$$\frac{Sti'}{Stv'} = \frac{\left({Sti_o}/{Sti_e}\right)}{\left({Stv_o}/{Stv_e}\right)} \qquad \textbf{Equation 2}$$

$$\frac{Nti'}{Ntv'} = \frac{\left({Nti_o}/{Nti_e}\right)}{\left({Ntv_o}/{Ntv_e}\right)} \qquad \textbf{Equation 3}$$

Where, $\frac{ti'}{tv'}$ is improved $\frac{ti}{tv}$ ratio, $\frac{Sti'}{Stv'}$ is improved $\frac{Sti}{Stv}$ ratio, $\frac{Nti'}{Ntv'}$ is improved $\frac{Nti}{Ntv}$ ratio. ti_o is transition observed, ti_e is the theoretically estimated transition, tv_o is transversion observed, tv_p is transversion estimated, Sti_o is synonymous transition observed, Sti_e is the theoretically estimated synonymous transition, Stv_o is synonymous transversion observed, Stv_e is the theoretically estimated synonymous transversion, Nti_o is non-synonymous transition observed, Nti_e is the theoretically

estimated non-synonymous transition, Ntv_o is non-synonymous transversion observed, Ntv_e is the theoretically estimated non-synonymous transversion.

Comparative representation of $\frac{ti}{tv}$ through conventional and improved method

We have done a comparative presentation of $\frac{ti}{tv}$ through both the existing and the improved method to support the usefulness of our method. Also, we have calculated the TFD: FFD and PTC% of each gene and performed statistical analysis between different parameters. A summary of the different parameters observed throughout the dataset is represented in Table 3.

Statistical analyses

Between the conventional and improved $\frac{ti}{tv}$ values different statistical methods were implemented to show the importance of improved method. OriginPro 2022, OriginLab Corporation, Northampton, MA, USA was used to draw the Box-plot as well as to perform the Mann-Whitney test (Nachar 2008) to find out the *p*-value test. Correlation plot was drawn for R^2 value and Pearson's correlation value was also found between conventional and improved $\frac{ti}{tv}$ values (Benesty et al. 2009).

Software and program availability

A software is developed for finding the values of different $\frac{ti}{tv}$ using 'C programing' language.

Results

Compositional differences among coding sequences regarding TFD: FFD codons and PTC codons

We found out the total numbers of TFD, FFD and PTC codons across 2516 coding sequences of *E. coli*. The % of TFD, FFD and PTC was also calculated (refer Table S2). The compositional differences in the entire coding sequences regarding TFD%, FFD% and PTC% is represented in box-plot (Fig. 2). A variable range of TFD: FFD ratio in the coding sequences of *E. coli* was observed. The minimum to maximum TFD:FFD ratio was observed as 0.229 and 3.333 in *sugE* and *mntS* respectively. It suggests that FFD codons are present ~four times more frequently than TFD codons in certain coding sequences, similarly TFD codons are present ~three times more frequently than FFD codons in the selected coding sequences in certain coding sequences. Among 2516 coding sequences only 35 coding sequences possessed the TFD:FFD ratio as 1.000. Hence, this observation suggests that there is a huge compositional asymmetry regarding the TFD and FFD codons in the coding sequences. The minimum to maximum PTC% was observed in *atpE* as 15% and in *chaB* as 57.14%. The compositional variation among the coding sequences were analyzed in the context of different ti/tv ratio in the following section.

Impact of codon degeneracy and PTC on $\frac{Sti}{Stv}$ and $\frac{Nti}{Ntv}$ values

The complete set of observed and estimated value of different polymorphisms is listed in Table S7. These values were used to calculate the $\frac{ti}{tv}$, $\frac{Sti}{Stv}$ and $\frac{Nti}{Ntv}$. Further the improved equation was used to calculate the $\frac{ti'}{tv'}$, $\frac{Sti'}{Stv'}$ and $\frac{Nti'}{Ntv'}$ through our improved method. We performed a comparison between the conventional method ($\frac{ti}{tv}$, $\frac{Sti}{Stv}$ and $\frac{Nti}{Ntv}$)values with the improved method ($\frac{ti'}{tv'}$, $\frac{Sti'}{Stv'}$ and $\frac{Nti'}{Ntv'}$) values for each gene individually (Table S8) (see Methods section for details). A box-plot showing the comparison between the values of different ti/tv values through both the conventional and improved method is represented in Figure 3. We talso calculated the % change in $\frac{Sti}{Stv}$ and $\frac{Nti}{Ntv}$ values from conventional to improved method. The minimum % change in $\frac{Sti}{Stv}$ between conventional and improved method was observed in *garP* as -30.99 and maximum in *yfhL* as 30.76. Similarly, the minimum % change in $\frac{Nti}{Ntv}$ between conventional and improved method was observed in *thiS* as -194.675 and maximum in *bhsA* as -129.600. The detailed % change in $\frac{Sti}{Stv}$ and $\frac{Nti}{Ntv}$ is represented in Table S9. While it is evident that, TFD codons and FFD codons have different probabilities of obtaining synonymous polymorphisms, the composition of TFD: FFD was cross-checked against the % change in $\frac{Sti}{Stv}$ for each gene. Likewise, PTC% was also considered to be cross-checked against % change in $\frac{Nti}{Ntv}$ values.

To study the impact of codon degeneracy (TFD: FFD) and PTC% on the % change in $\frac{Sti}{Stv}$ and $\frac{Nti}{Ntv}$ various statistical methods were used. The Pearson correlation coefficient value between TFD: FFD and % change in $\frac{Sti}{Stv}$ (conventional to improved) was obtained to be 0.891; a very strong correlation coefficient was observed. Similarly, the Pearson correlation coefficient value between PTC% and % change in $\frac{Nti}{Ntv}$ was obtained to be -0.488; a moderate negative correlation between the

two variables. The strong correlation between TFD: FFD and % change in $\frac{Sti}{Stv}$ suggested that there is an influence of the TFD: FFD codons in overall synonymous $\frac{ti}{tv}$. Similarly, the moderate negative correlation also suggested the contribution of PTC codons in overall non-synonymous $\frac{ti}{tv}$. The results were found to be significantly different at $p<0.01$, for $\frac{ti}{tv}$ & $\frac{ti'}{tv'}$, $\frac{Sti}{Stv}$ & $\frac{Sti'}{Stv'}$ and $\frac{Nti}{Ntv}$ & $\frac{Nti'}{Ntv'}$. Further the correlation graph between % change in $\frac{Sti}{Stv}$ and TFD: FFD and % change in $\frac{Nti}{Ntv}$ and PTC% were found to be supported by our earlier observation regarding role of codon composition and $\frac{ti}{tv}$ ratio (Fig 4A & 4B). In the trendline analyses the % Change in $\frac{Sti}{Stv}$ was perceived to be directly influenced by the TFD: FFD with a R^2 value of 0.795. A weak negative association was obtained between % change in $\frac{Nti}{Ntv}$ and PTC% with a R^2 value of 0.216. Hence, it is pertinent to note that the overall % change in $\frac{Sti}{Stv}$ and $\frac{Nti}{Ntv}$ were directly influenced by the TFD and PTC codon composition across the coding sequences. This relative analyses correctly attributes to the % change in $\frac{Sti}{Stv}$ and $\frac{Nti}{Ntv}$ with respect to TFD: FFD and PTC% respectively. The conventional method does not account for the codon composition as a parameter which can ultimately affect the $\frac{ti}{tv}$ ratio, certainly the improved method has vindicated regarding the importance of codon composition while judging the $\frac{ti}{tv}$ ratio across the genome.

Discussion

In this study we have demonstrated that, coding sequences are highly variable in terms of compositions of different degenerate codons and PTC. The improved equations used in this study is helpful to estimate the ti and tv rate differences in coding sequences by considering the compositional difference of degenerate codons and PTC. Though it is known that TFD codons only

undergo synonymous ti, the ti/tv ratio among the coding sequences in relation to their compositional difference in terms of TFD and FFD has not been reported earlier. The improved equation used to estimate Sti, Stv distinctly vindicated the role of codon degeneracy on ti and tv values. This is in support to the published work by (Aziz et al. 2022).

We believe our improved method is more compatible in the calculation of different ti to tv in coding sequences. By taking care of possible polymorphism of individual codons now we have done the normalization of the ti to tv ratio. If $\frac{Sti'}{Stv'}$ is different between genes it must be compared with TFD:FFD ratio also the gene expression values can have an impact on the ratio directly. Similarly, if $\frac{Nti'}{Ntv'}$ is different between genes it must be compared with TFD:FFD ratio, PTC and expression value of the gene. Though in this work we were not able to conclude whether observed Sti is influenced by higher degeneracy or not. It is ascertained that, TFD codons only undergo Sti whereas FFD codons obtain synonymous polymorphism by Sti and Stv. Hence, to discover the observed Sti in the different degeneracy will be an interesting work. However, two genes with almost similar estimated values of polymorphism can have different ratios of ti to tv, because of different intrinsic and extrinsic factors like gene expression, selection, and acceptance of one amino acid instead of the other. Since the magnitude of synonymous polymorphism is limited to 6 for a codon, the selected non-synonymous polymorphism for a codon has an ample number of possibilities in any organism.

This work gives us the insight in the analyses of different degenerate codon composition and PTC composition across the coding sequences. Is it because of the amino acid usage or it has any other evolutionary significance, it is yet to be discovered. The influence of different degenerate

codons and PTC on gene expression is not known yet, hence it will be an interesting finding in the future.

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Authors' Contributions

Conceptualization: PKB, RA, SSS and SKR; Methodology: PKB, PS, EF, SSS and SKR; Execution, Writing, review, and editing: PKB, PS, RA, SD, NDN, EF, SSS and SKR; Supervision: SSS and SKR. All authors endorsed the manuscript.

Author Disclosure Statement

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Figure_legend_Table

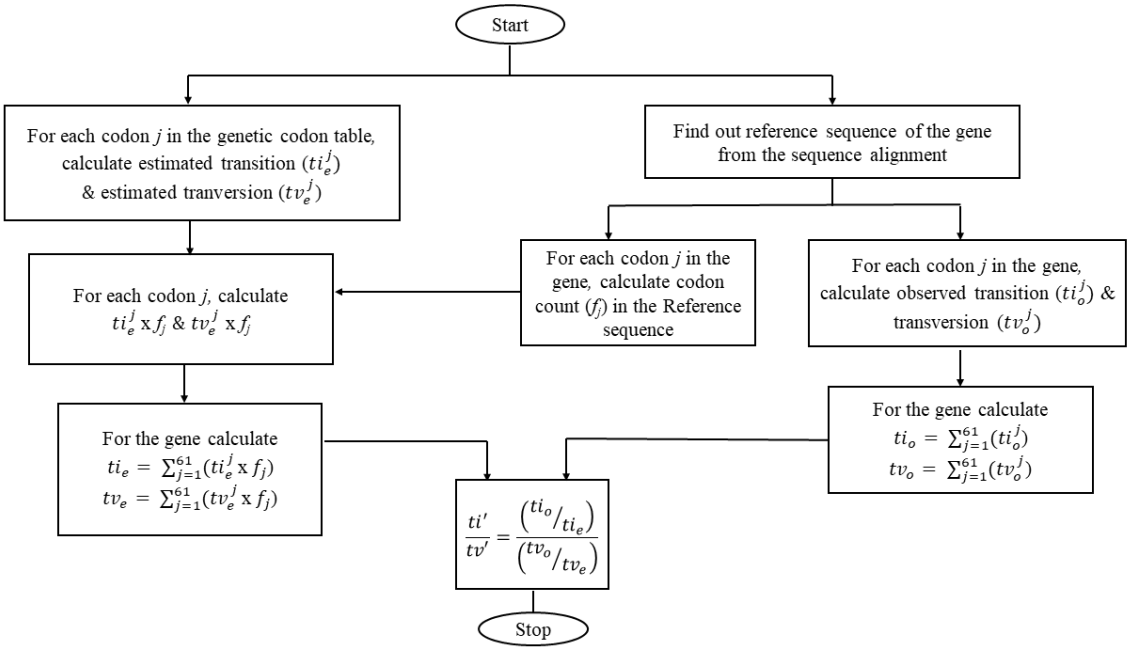


Fig. 1. The workflow of the process shows the steps to execute our improved method. By considering each codon as j, ti_e^j and tv_e^j can be calculated for each codon as presented in Table 1. The reference sequence needs to be calculated from the coding region(gene/query). From the

reference sequence the observed ($_o$) substitutions are calculated for the gene. The other usage of the reference sequence is to find out the codon count of the gene. Then, the respective codon count is multiplied by estimated transition and transversion for each codon. The next step is to find out the sum of ti and sum of tv in all codons to represent the estimated ($_e$) ti and tv for the gene. After getting the observed and estimated values for ti and tv, now the improved equation is applied to find out the $\frac{ti'}{tv'}$ of the gene. This scenario is only showing the $\frac{ti'}{tv'}$ ratio whereas in similar way the $\frac{Sti'}{Stv'}$ & $\frac{Nti'}{Ntv'}$ can be calculated.

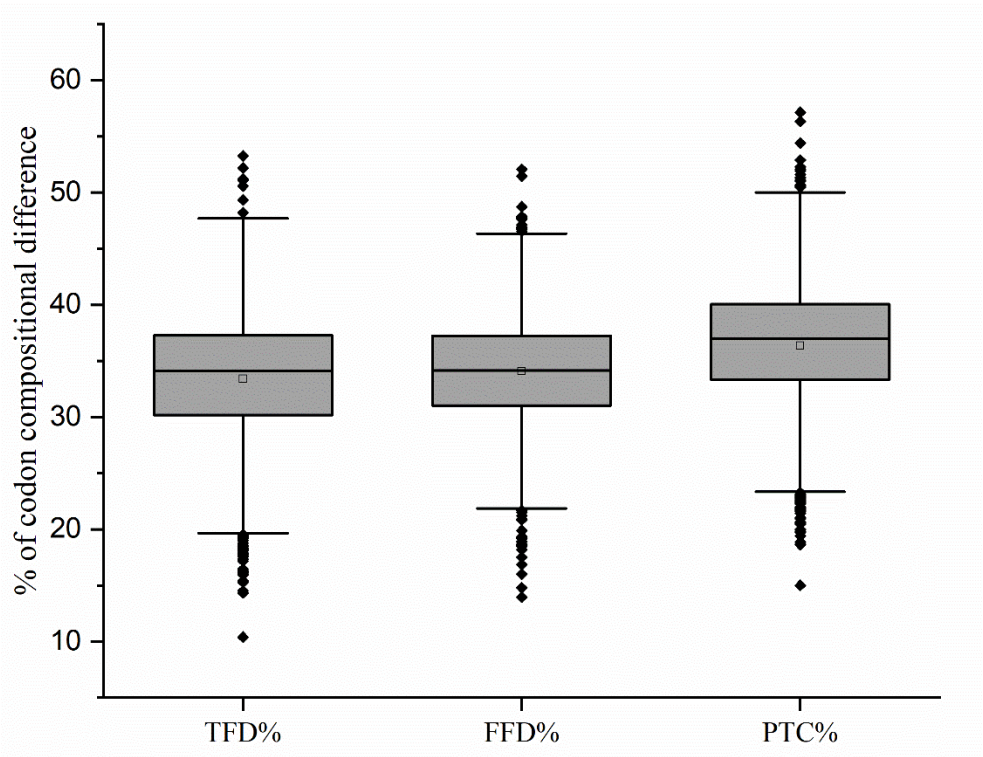


Fig. 2. A statistical representation of the compositional difference of different degenerate codons along with PTC%. The box-plot is showing the compositional difference among TFD%, FFD% and PTC% in the entire dataset.

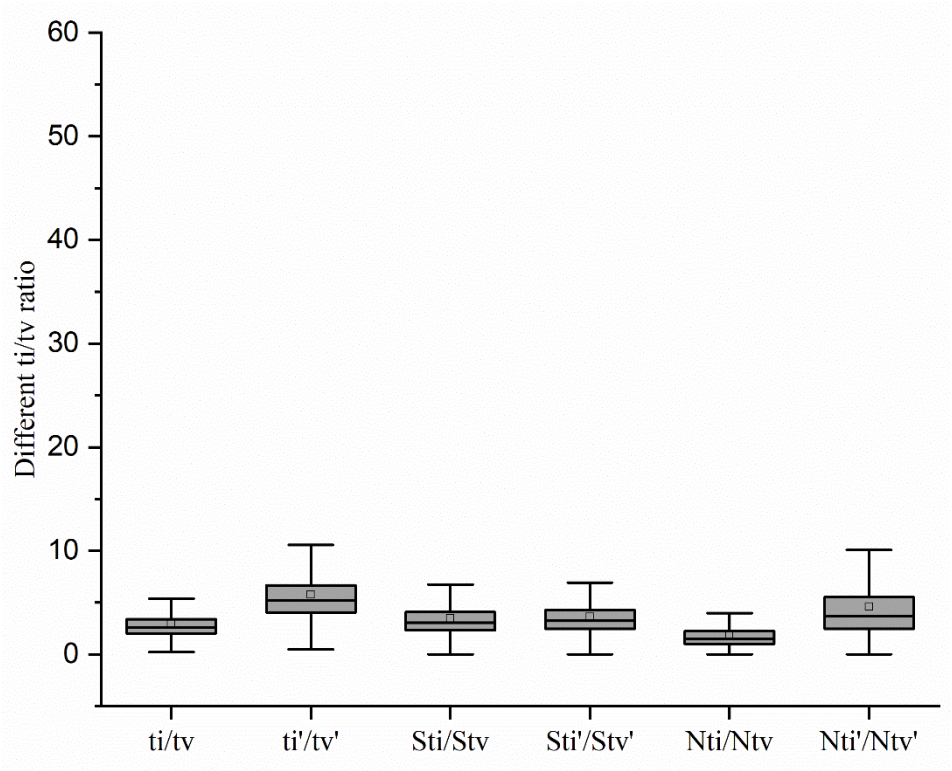
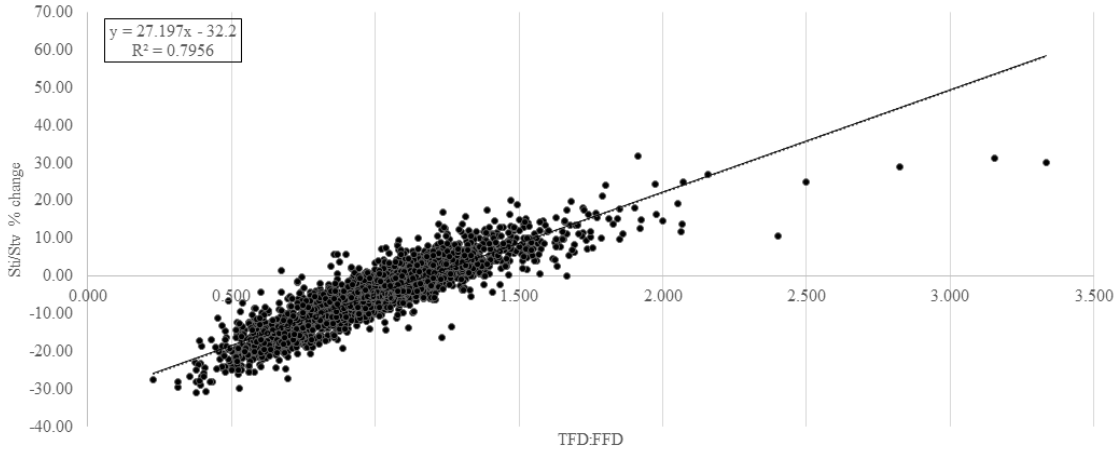


Fig. 3. The box-plot shows the different ti/tv ratio values. here we have compared the different ti/tv ratios of the entire set of genes through conventional and improved method. $\frac{ti}{tv}$ shows conventional method and $\frac{ti'}{tv'}$ shows the result of improved method. Similarly, $\frac{Sti}{Stv}$ and $\frac{Nti}{Ntv}$ represent the conventional values and $\frac{Sti'}{Stv'}$ and $\frac{Nti'}{Ntv'}$ represent the improved values. This illustration shows the median values between $\frac{ti}{tv}$ & $\frac{ti'}{tv'}$, $\frac{Sti}{Stv}$ & $\frac{Sti'}{Stv'}$ and $\frac{Nti}{Ntv}$ & $\frac{Nti'}{Ntv'}$ are different significantly (p-value < 0.01).

A



B

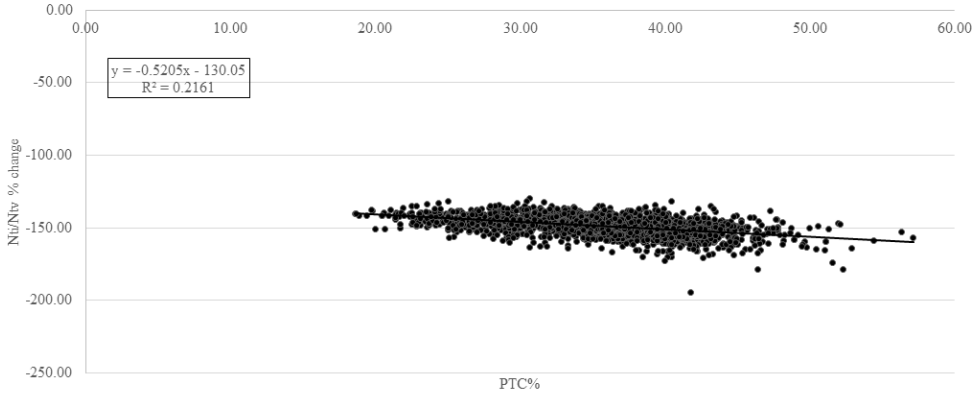


Fig. 4. The x-axis shows the codon composition (TFD: FFD and PTC%) and y-axis shows the % change in $\frac{Sti}{Stv}$ and $\frac{\mathbf{Nti}}{\mathbf{Ntv}}$. (A) % change in $\frac{Sti}{Stv}$ is showing a strong correlation with the TFD: FFD with a R^2 value of 0.763. Among few of the outliers, it can be noted that, *yhfL*, *gadE* and *mntL* obtained the highest % change in $\frac{Sti}{Stv}$ value as 31.72,31.32 and 30.00 respectively, whereas the TFD: FFD codon was noted as 1.91, 3.15 and 3.33 respectively for the genes. Similarly, it can be noted that, *garP*, *proW* and *slyB* obtained the lowest % change in $\frac{Sti}{Stv}$ value as -30.99, -30.57 and -29.68 respectively, whereas the TFD: FFD codon was noted as 0.38, 0.41 and 0.53 respectively for the

genes. (B) % change in $\frac{\mathbf{Nti}}{\mathbf{Ntv}}$ is showing a weak negative correlation with the PTC% with a a R^2 value of 0.172. While doing the similar analysis for outliers, the % change in $\frac{Nti}{Ntv}$ value and PTC% it was noted that, *thiS*, *sgrT* and *sbcC* obtained the lowest % change in $\frac{Nti}{Ntv}$ value as -194.67, -179.00 and -178.00 respectively, whereas the PTC% was noted as 41.79, 52.27 and 46.27 respectively for the genes. Similarly, bhsA, *gapA* and *yiaD* obtained the highest % change in $\frac{Nti}{Ntv}$ value as -129.67, -131.55 and -131.94 respectively, whereas the PTC% was noted as 30.68, 40.48 and 25.00 respectively for the genes.

Table 1. Theoretically estimated ($_e$) polymorphism for each codon by excluding the non-synonymous substitutions (Nti & Ntv) giving rise to stop codons through PTC.

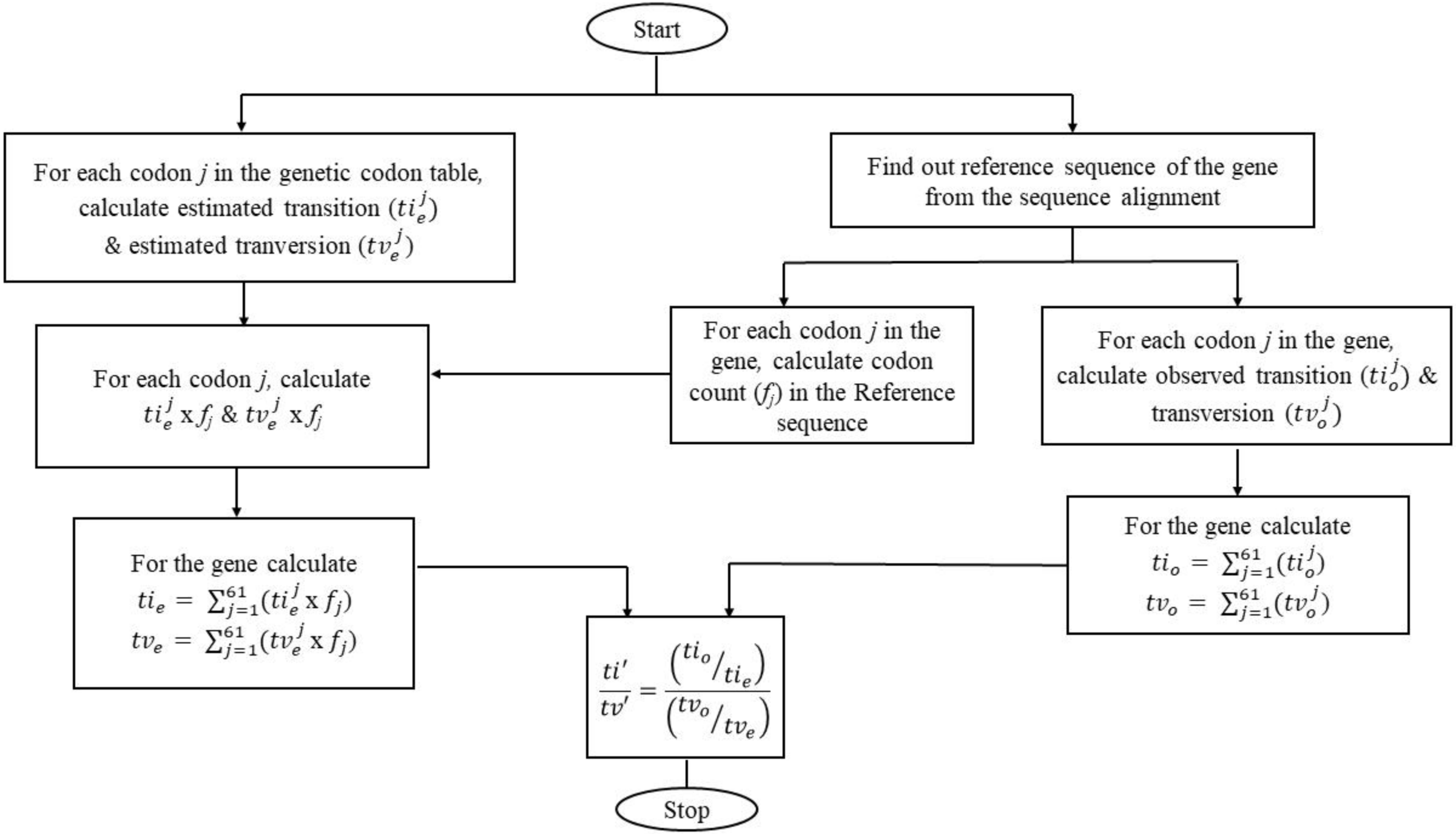
Codon	Sti_e	Stv_e	Nti_e	Ntv_e	Codon	Sti_e	Stv_e	Nti_e	Ntv_e	Codon	Sti_e	Stv_e	Nti_e	Ntv_e	Codon	Sti_e	Stv_e	Nti_e	Ntv_e
UUU	1	0	2	6	UCU	1	2	2	4	UAU	1	0	2	4	UGU	1	0	2	5
UUC	1	0	2	6	UCC	1	2	2	4	UAC	1	0	2	4	UGC	1	0	2	5
UUA	2	0	1	4	UCA	1	2	2	2	UAA	X	X	X	X	UGA	X	X	X	X
UUG	2	0	1	5	UCG	1	2	2	3	UAG	X	X	X	X	UGG	0	0	1	6
CUU	1	2	2	4	CCU	1	2	2	4	CAU	1	0	2	6	CGU	1	2	2	4
CUC	1	2	2	4	CCC	1	2	2	4	CAC	1	0	2	6	CGC	1	2	2	4
CUA	2	2	1	4	CCA	1	2	2	4	CAA	1	0	1	6	CGA	1	3	1	3
CUG	2	2	1	4	CCG	1	2	2	4	CAG	1	0	1	6	CGG	1	3	2	3
AUU	1	1	2	5	ACU	1	2	2	4	AAU	1	0	2	6	AGU	1	0	2	6
AUC	1	1	2	5	ACC	1	2	2	4	AAC	1	0	2	6	AGC	1	0	2	6
AUA	0	2	3	4	ACA	1	2	2	4	AAA	1	0	2	5	AGA	1	1	2	4
AUG	0	0	3	6	ACG	1	2	2	4	AAG	1	0	2	5	AGG	1	1	2	5
GUU	1	2	2	4	GCU	1	2	2	4	GAU	1	0	2	6	GGU	1	2	2	4
GUC	1	2	2	4	GCC	1	2	2	4	GAC	1	0	2	6	GGC	1	2	2	4
GUA	1	2	2	4	GCA	1	2	2	4	GAA	1	0	2	5	GGA	1	2	2	3
GUG	1	2	2	4	GCG	1	2	2	4	GAG	1	0	2	5	GGG	1	2	2	4

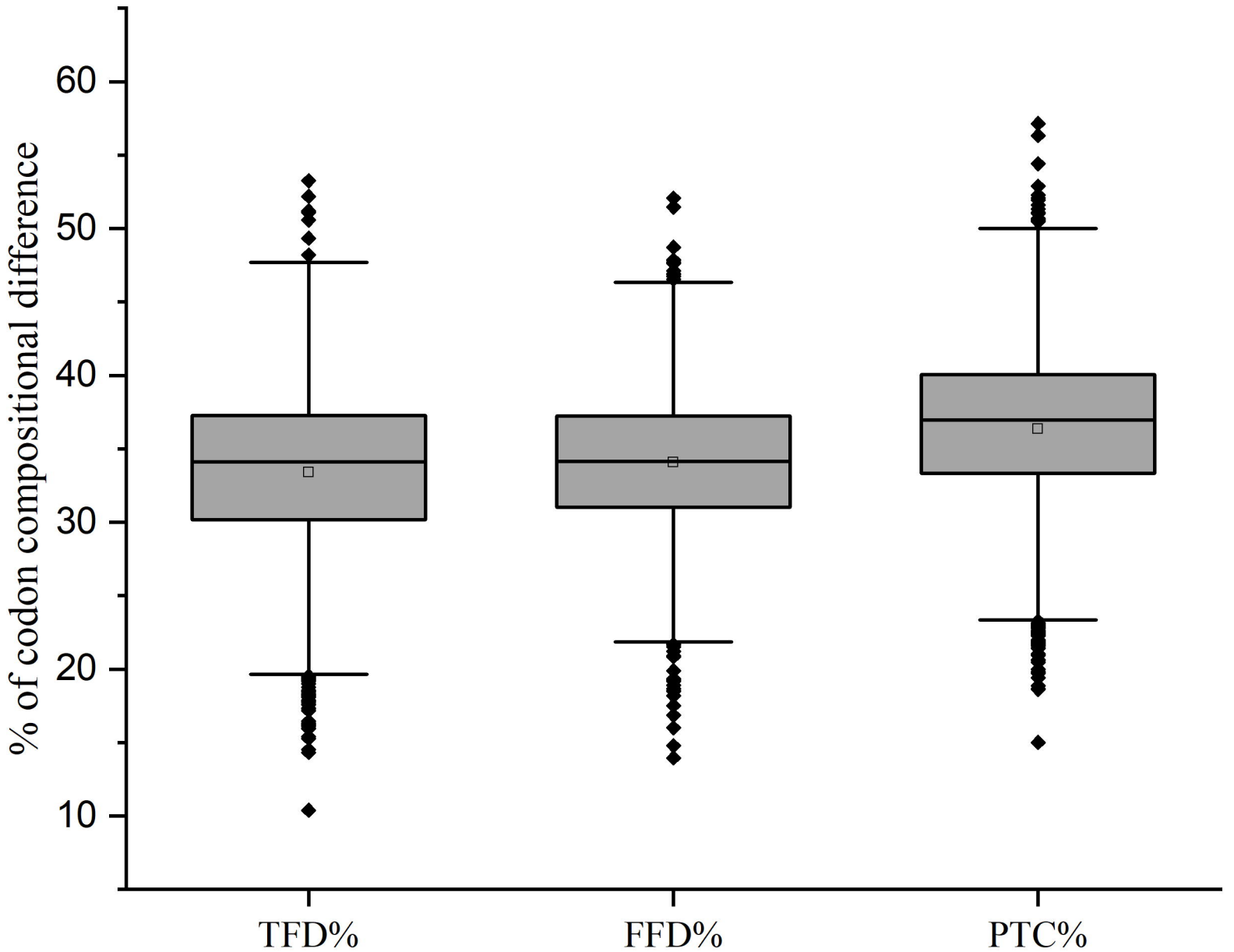
Table 2. Total estimated synonymous ti (tv) and non-synonymous ti (tv) substitutions in the genetic codon table. Numbers in (*) show the total numbers of substitutions and ratio while excluding the substitutions that lead to stop codons in PTC.

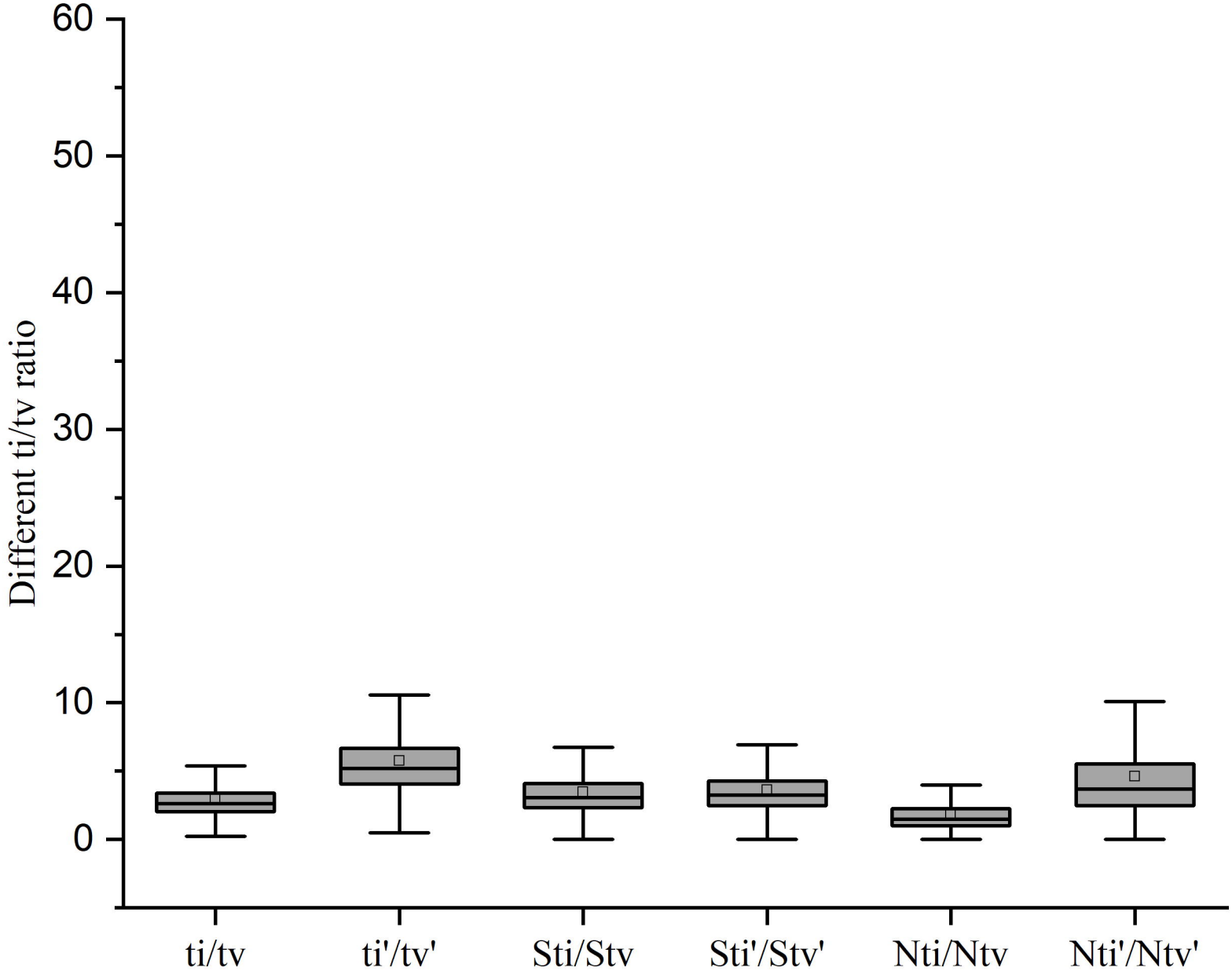
ti & tv	Synonymous	Non-synonymous (*)	Total (*)
ti	62	121 (116)	183 (178)
tv	72	294 (276)	366 (348)
ti/tv	0.861	0.412 (0.420)	0.500 (0.511)

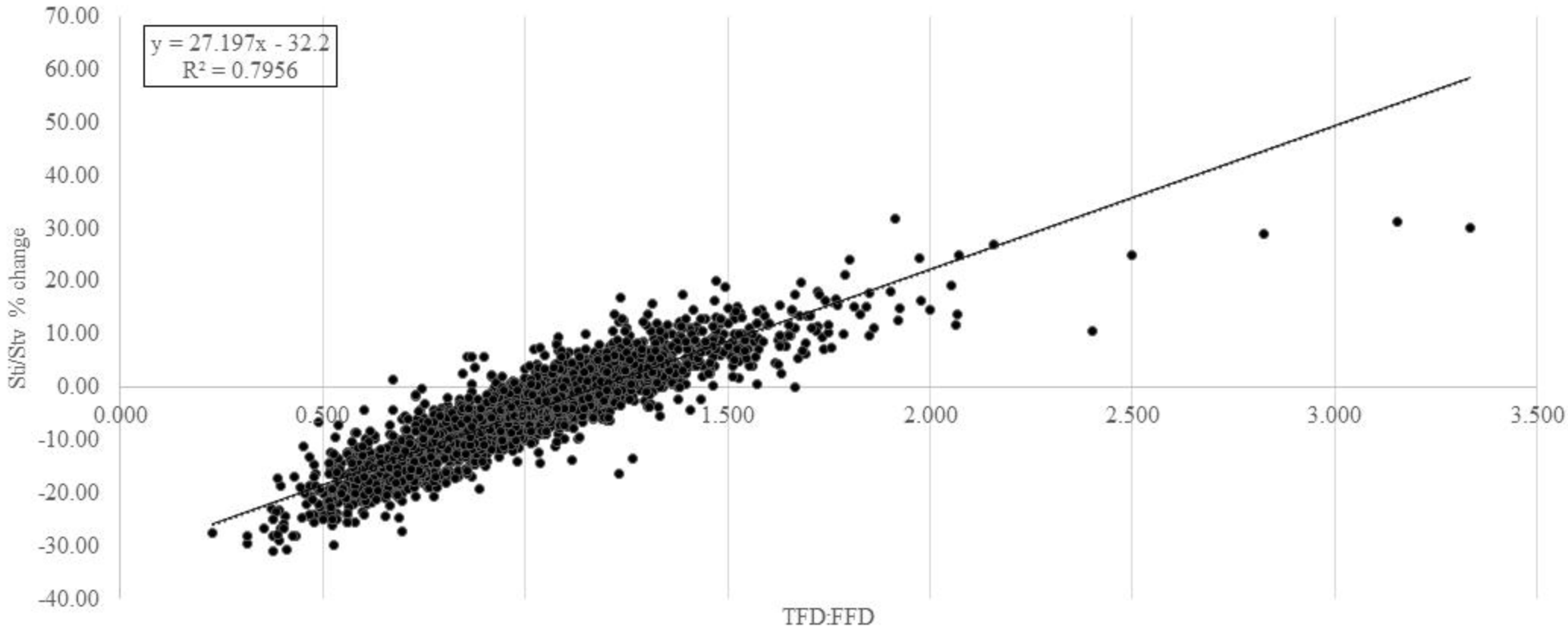
Table 3. Summary of the dataset selected for the study. It represents the minimum to maximum range of different parameters like GC%, TFD/FFD, PTC% and the mean and standard deviation (S.D) of the TFD%, FFD% and PTC%.

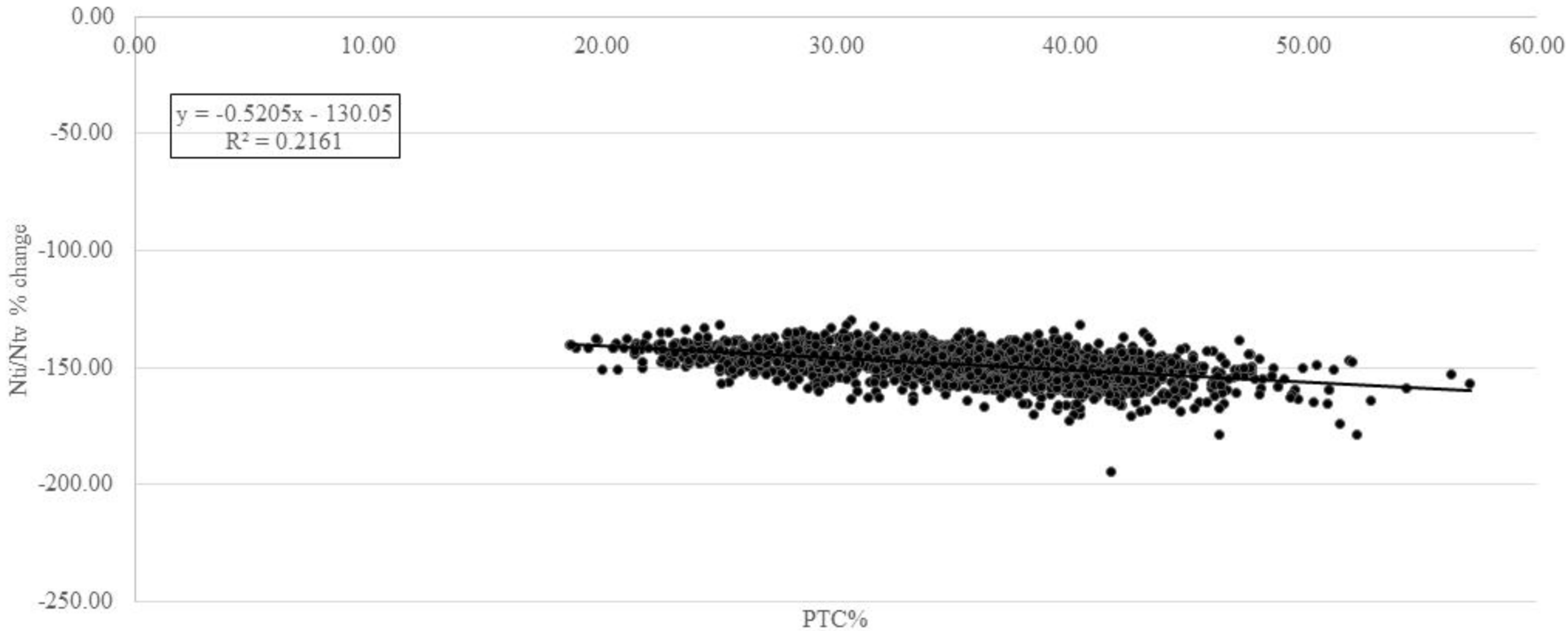
Genes considered	Size bp (Min-Max)	GC% (Min-Max)	TFD/FFD (Min-Max)	PTC% (Min-Max)	TFD%	FFD%	PTC%	
					33.384	34.084	36.350	Mean
2516	93-4554	32.95-63.80	0.22-3.33	15.00-57.14	5.726	4.761	5.625	S. D


Start
For each codon j in the genetic codon table, calculate estimated transition (ti_e^j) & estimated tranversion (tv_e^j)
Find out reference sequence of the gene from the sequence alignment
For each codon j in the gene, calculate codon count (f_j) in the Reference sequence
For each codon j in the gene, calculate observed transition (ti_o^j) & transversion (tv_o^j)
For each codon j, calculate $ti_e^j \times f_j$ & $tv_e^j \times f_j$
For the gene calculate $ti_o = \sum_{j=1}^{61}(ti_o^j)$ $tv_o = \sum_{j=1}^{61}(tv_o^j)$
For the gene calculate $ti_e = \sum_{j=1}^{61}(ti_e^j \times f_j)$ $tv_e = \sum_{j=1}^{61}(tv_e^j \times f_j)$
$\frac{ti'}{tv'} = \frac{(ti_o/ti_e)}{(tv_o/tv_e)}$
Stop


% of codon compositional difference
60
50
40
30
20
10
TFD%
FFD%
PTC%


Different ti/tv ratio
60
50
40
30
20
10
0
ti/tv
ti'/tv'
Sti/Stv
Sti'/Stv'
Nti/Ntv
Nti'/Ntv'


y = 27.197x - 32.2
R² = 0.7956
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
-10.00
-20.00
-30.00
-40.00
Sti/Stv % change
0.000
0.500
1.500
2.000
2.500
3.000
3.500
TFD:FFD


0.00
0.00
10.00
20.00
30.00
40.00
50.00
60.00
y = -0.5205x - 130.05
R² = 0.2161
-50.00
-100.00
-150.00
-200.00
-250.00
Nti/Ntv % change
PTC%