A simple mathematical method is developed to estimate the number of nucleotide substitutions per site between two DNA sequences, by extending Kimura's (1980) two-parameter method to the case where a G+C-content bias exists. This method will be useful when there are strong transition-transversion and G+C-content biases, as in the case of Drosophila mitochondrial DNA.

Introduction

In the study of molecular evolution, it is important to know the number of nucleotide substitutions per site \( (d) \) between DNA sequences. There are many different methods for estimating this number (for reviews, see Nei 1987, chap. 5; Gojobori et al. 1990), but all of them depend on some simplifying assumptions and do not always give reliable estimates. Two important factors that should be considered in the estimation of \( d \) are the inequality of the rates of transitional and transversional nucleotide substitution (transition-transversion bias) and the deviation of the G+C content (\( \theta \)) from 0.5 (G+C-content bias).

Kimura (1980) developed a method of estimating \( d \) for the case where the transition-transversion bias exists. In his method, however, the frequencies of the four nucleotides A, T, C, and G are all assumed to be equal to 0.25, throughout the evolutionary time. In practice, this assumption usually does not hold. For example, the G+C content at the third-codon positions of the coding region of Drosophila mitochondrial DNA (mtDNA) is \( \sim 0.1 \) (Clary and Wolstenholme 1985). It is therefore useful to develop a method that will take care of both the transition-transversion and G+C-content biases. In the following, I present one such method and compare the method's reliability with that of others.

Theory

I consider the model of nucleotide substitution shown in table 1, where \( \alpha \) and \( \beta \) denote the rates of transitional and transversional changes, respectively. This model extends Kimura's (1980) two-parameter model to the case where \( \theta \neq 0.5 \). This model also is a special case of Hasegawa et al.'s (1985) model, where the frequency of A plus G is assumed to be equal to that of T plus C. This model assumes that the pattern of

1. Key words: nucleotide substitutions, transition-transversion bias, G+C-content bias.
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nucleotide substitution is the same for all nucleotide sites, irrespective of the location of the nucleotide. This assumption does not seem to hold in many cases, but we can extract particular nucleotide sites at which this assumption approximately holds. For example, third-codon positions or fourfold-degenerate sites in protein-coding sequences can be regarded as such sites.

In the present case, the rate of nucleotide substitution per site is given by \( \theta\alpha + \beta \) for nucleotides A and T and by \( (1 - \theta)\alpha + \beta \) for C and G. The average rate of nucleotide substitution per site \( \lambda \) is therefore given by

\[
\lambda = (1 - \theta)(\theta\alpha + \beta) + \theta[(1 - \theta)\alpha + \beta] = 2\theta(1 - \theta)\alpha + \beta ,
\]

whereas the total \( d \) since the time of divergence between two sequences is given by

\[
d = 4\theta(1 - \theta)\alpha t + 2\beta t .
\]

Here \( t \) is the divergence time (measured in years) between the two sequences, generations, or any other time units. The estimate of \( d \) is obtained by the following equation:

\[
d = 2\theta(1 - \theta)\left[ -\log_e \left( 1 - \frac{1}{2\theta(1 - \theta)} \hat{P} - \hat{Q} \right) + \frac{1}{2} \log_e (1 - 2\hat{Q}) \right] - \frac{1}{2} \log_e (1 - 2\hat{Q})
\]

\[
= -2\theta(1 - \theta)\log_e \left( 1 - \frac{1}{2\theta(1 - \theta)} \hat{P} - \hat{Q} \right) - \frac{1 - 2\theta(1 - \theta)}{2} \log_e (1 - 2\hat{Q}) ,
\]

where \( \hat{\theta} \) is the estimate of \( \theta \) and where \( \hat{P} \) and \( \hat{Q} \) are the estimates of the proportions of the nucleotide sites showing, respectively, transitional and transversional differences between the two sequences (for the analytical solution, see the Appendix). We can easily calculate these estimates from the observed number of the different nucleotide matches between the two sequences compared. In this equation, \( \hat{\theta} \) is the estimate of \( \theta \) for the two sequences. In practice, however, the \( \theta \)'s may not be the same for the two sequences. In this case, the following equation seems to be useful:

\[
d = -\left( \hat{\theta}_1 + \hat{\theta}_2 - 2\hat{\theta}_1 \hat{\theta}_2 \right) \log_e \left( 1 - \frac{1}{\hat{\theta}_1 + \hat{\theta}_2 - 2\hat{\theta}_1 \hat{\theta}_2} \hat{P} - \hat{Q} \right) - \frac{1}{2} \log_e (1 - 2\hat{Q}) ,
\]
where $\hat{\theta}_1$ and $\hat{\theta}_2$ are the estimates of the $\theta$'s for the two sequences, respectively. This modification is due to the work of Bulmer (1991).

The sampling variance of $\bar{d}$ can be obtained by

$$V(\bar{d}) = \left(\frac{\partial d}{\partial P}\right)^2 V(P) + \left(\frac{\partial d}{\partial Q}\right)^2 V(Q) + 2\left(\frac{\partial d}{\partial P}\right)\left(\frac{\partial d}{\partial Q}\right) \text{cov}(P,Q),$$

where $V(P) = \hat{\theta}(1-\hat{\theta})/n$, $V(Q) = \hat{\theta}(1-\hat{\theta})/n$, and $\text{cov}(P,Q) = -\hat{\theta}\hat{\theta}/n$. Here $n$ denotes the number of nucleotide sites examined. It becomes

$$V(\bar{d}) = \left[a^2\hat{\theta} + b^2\hat{\theta} - (a\hat{\theta} + b\hat{\theta})^2\right]/n,$$

where

$$a = \frac{1}{1 - \frac{\hat{\theta}}{2\hat{\theta}(1-\hat{\theta})} - \hat{\theta}}$$

$$b = 2\hat{\theta}(1-\hat{\theta})a + \frac{1 - 2\hat{\theta}(1-\hat{\theta})}{1-2\hat{\theta}}.$$

**Numerical Example**

Satta et al. (1987) determined the DNA sequences of parts of the NADH dehydrogenase subunit 2 (ND2) and cytochrome oxidase subunit 1 (COI) genes of mtDNAs from *Drosophila melanogaster*, *D. simulans*, and *D. mauritiana*. The sequences for these regions of mtDNA are also available from *D. yakuba* (Clary and Wolstenholme 1985). I therefore compared the third-codon positions ($n = 254$) of these sequences, between the four species. Table 2 shows the observed number ($n_{ij}$) of the different nucleotide pairs, at the third-codon positions, between the *D. simulans*

<table>
<thead>
<tr>
<th>No. OF DIFFERENCES FOR NUCLEOTIDE PAIR</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPECIES COMPARED</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td><em>D. simulans</em> vs.</td>
</tr>
<tr>
<td><em>D. mauritiana</em></td>
</tr>
<tr>
<td><em>D. simulans</em> vs.</td>
</tr>
<tr>
<td><em>D. melanogaster</em></td>
</tr>
<tr>
<td><em>D. simulans</em> vs.</td>
</tr>
<tr>
<td><em>D. yakuba</em></td>
</tr>
</tbody>
</table>
and each of the *D. mauritiana, D. melanogaster,* and *D. yakuba* sequences. The relative frequency of nucleotide pair *i* and *j* (*x*<sub>ij</sub>) can then be obtained by dividing *n*<sub>ij</sub> by the total number of nucleotides used. For example, *x*<sub>AG</sub> between *D. simulans* and *D. yakuba* in table 2 is given by 9/254 = 0.0354. Then *P*, and transversional *Q*, and *θ* are given by

\[ P = x_{AG} + x_{TC} = 0.0866, \quad Q = x_{AT} + x_{AC} + x_{TG} + x_{GC} = 0.0591, \quad \theta = (x_{AG} + x_{AC} + x_{TC} + x_{TG})/2 + x_{GC} + x_{GG} + x_{CC} = 0.0689. \]

Therefore, equation (3) gives an estimate of \( \hat{d} = 0.225 \), whereas the variance of \( \hat{d} \) given by equations (6) and (7) is 0.00466. The standard error of \( \hat{d} \) is then 0.068. Similar computations give \( \hat{d} = 0.086 \pm 0.034 \) between *D. simulans* and *D. mauritiana* and \( \hat{d} = 0.112 \pm 0.043 \) between *D. simulans* and *D. melanogaster*.

Table 3 shows the estimates of \( \hat{d} \) that are obtained by the Jukes and Cantor (1969) (JC), Kimura (1980) two-parameter (K2), Tajima and Nei (1984) (TN), Takahata and Kimura (1981) four-parameter (TK), and Gojobori et al. (1982a) six-parameter (GIN) methods and by equation (3). The estimates obtained by equation (3) are largest for all pairwise comparisons of species. If we consider that there are strong transition-transversion and G+C-content biases in the nucleotide *Drosophila* mtDNA (see table 4), this result suggests that all methods other than equation (3) give serious underestimates of \( d \).

**Computer Simulations**

To examine the efficiency of equation (3) relative to that of other methods, in obtaining reliable estimates of \( d \), I conducted a computer simulation. In this simulation, a random nucleotide sequence was generated as the common ancestral sequence for the two sequences compared under the condition that the expected nucleotide frequency is \( x_i = (1-\theta)/2 \) for \( i = A \) or \( T \) and \( i = \theta/2 \) for \( i = G \) or \( C \), as before. I assumed that \( \theta = 0.1 \) and \( \alpha/\beta = 10 \) in this simulation. An extremely biased condition such as this has actually been observed in *Drosophila* mtDNA (table 4; also see DeSalle et al. 1987; Satta et al. 1987). The two descendant sequences were then generated from this ancestral sequence by a Monte Carlo simulation, with either \( d = 0.5 \) or \( d = 1.0 \). The number of nucleotides examined was 300, 1,000, or 3000. The estimates of \( d \) between two descendant sequences were obtained by the JC, K2, TN, TK, and GIN methods and by equation (3). This process was repeated 100 times.

Table 5 shows the mean and standard deviation (SD) for each estimates obtained, as well as the number of cases in which a particular method was not applicable because of negative values of the argument of the logarithms involved. The JC, K2, and TN
Table 4
Estimated Percent of Relative Substitution in Third-Codon Positions of Cytochrome b and NADH Dehydrogenase Subunit 1 Genes in Drosophila mtDNA

<table>
<thead>
<tr>
<th>ORIGINAL NUCLEOTIDE</th>
<th>ESTIMATED % RELATIVE SUBSTITUTION FOR MUTANT NUCLEOTIDE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>A</td>
<td>1.7</td>
</tr>
<tr>
<td>T</td>
<td>1.7</td>
</tr>
<tr>
<td>C</td>
<td>3.5</td>
</tr>
<tr>
<td>G</td>
<td>33.1</td>
</tr>
</tbody>
</table>

NOTE.—These rates were obtained by the method of Gojobori et al. (1982b), from DNA sequence data for the D. nanata species subgroup (six species and subspecies). The number of nucleotide sites examined is 698. Data are from Tamura (1991).

methods almost always give underestimates of \( d \), although there are no inapplicable cases. The TK and GIN methods give better estimates, but there are many inapplicable cases when \( d = 1.0 \). Equation (3) gives even better estimates than do the TK and GIN methods, and there are fewer inapplicable cases. However, in all cases the GIN method gives a smaller SD than do the TK method and equation (3).

Discussion

From the present study, equation (3) is the best among the methods examined, for estimating the number of nucleotide substitutions when there are strong transition-transversion and G+C-content biases. Although these conditions may not exist widely, equation (3) is useful for analyzing Drosophila mtDNA. A high G+C-content bias has also been observed at the third-codon positions of the major histocompatibility complex class I loci (Hughes and Nei 1988). If the extent of these biases is not strong, the TK, GIN, and TN methods are useful, but equation (3) is much simpler than these methods, and the simplicity of a method is important for practical data analysis.

Estimates of \( d \) obtained by the present method have large SDs when both biases are strong and when \( d \) is large. The high frequency of inapplicable cases observed in the simulation (table 5) reflects the large sampling errors of the estimates of \( d \). Table 6 shows the SDs calculated by equations (6) and (7), for various parameter values. In this table, we can see the large SDs in the case of \( \theta = 0.1, \alpha/\beta = 10, \) and \( d = 1.0. \) Because such large SDs are not found in the other cases, the cause of this large SD is attributable to the combined effect of the transition-transversion and G+C-content biases. Unfortunately, we cannot decrease this SD very much by increasing the number of the nucleotides examined, because the SD is inversely proportional to the square root of the number of nucleotides, as shown by equation (6). A quadrupling of the number of nucleotides decreases the SD by only half. When \( d \) increases, the SD increases rapidly. For example, the SD for \( d = 1.0 \) is about seven times greater than that for \( d = 0.5. \) This indicates that the applicability of the present method is limited to the case where \( d \) is not very large if there are strong transition-transversion and G+C-content biases.
Table 5
Means ± SDs of Estimates of d

<table>
<thead>
<tr>
<th>n</th>
<th>JC</th>
<th>K2</th>
<th>TN</th>
<th>TK</th>
<th>GIN</th>
<th>Equation (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d x 100 = 50:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>300</td>
<td>35 ± 4 (0)</td>
<td>35 ± 5 (0)</td>
<td>43 ± 7 (0)</td>
<td>47 ± 16 (5)</td>
<td>49 ± 11 (14)</td>
<td>53 ± 14 (4)</td>
</tr>
<tr>
<td>1,000</td>
<td>35 ± 3 (0)</td>
<td>35 ± 3 (0)</td>
<td>43 ± 5 (0)</td>
<td>46 ± 8 (0)</td>
<td>47 ± 6 (0)</td>
<td>51 ± 8 (0)</td>
</tr>
<tr>
<td>3,000</td>
<td>35 ± 1 (0)</td>
<td>35 ± 1 (0)</td>
<td>42 ± 2 (0)</td>
<td>44 ± 5 (0)</td>
<td>46 ± 3 (0)</td>
<td>50 ± 4 (0)</td>
</tr>
<tr>
<td>d x 100 = 100:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>300</td>
<td>54 ± 6 (0)</td>
<td>54 ± 6 (0)</td>
<td>70 ± 11 (0)</td>
<td>80 ± 37 (51)</td>
<td>84 ± 15 (60)</td>
<td>83 ± 21 (40)</td>
</tr>
<tr>
<td>1,000</td>
<td>54 ± 4 (0)</td>
<td>54 ± 4 (0)</td>
<td>70 ± 6 (0)</td>
<td>79 ± 22 (50)</td>
<td>87 ± 11 (53)</td>
<td>96 ± 26 (34)</td>
</tr>
<tr>
<td>3,000</td>
<td>54 ± 2 (0)</td>
<td>54 ± 2 (0)</td>
<td>70 ± 4 (0)</td>
<td>86 ± 23 (28)</td>
<td>87 ± 11 (27)</td>
<td>101 ± 21 (23)</td>
</tr>
</tbody>
</table>

Note.—Results are of 100 replications and have been multiplied by 100. \( \theta = 0.1; \) and \( \alpha / \beta = 10. \) The numbers in parentheses are the number of inapplicable cases.

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Appendix

Derivation of Equations (3)

Let us use the symbols given in table A1 to denote the frequencies of nucleotide pairs that are different between two homologous sequences. These frequencies at time \( t \) will be denoted by \( R_1(t), R_2(t), \) etc. We now derive equation (3) by considering the frequency change of each nucleotide pair during a short time period \( \Delta t. \) Let us first consider the change of \( P_1(t), \) i.e., the frequency of nucleotide pair AG. The frequency of \( P_1 \) at time \( t + \Delta t, \) i.e., \( P_1(t + \Delta t), \) is given by

\[
P_1(t + \Delta t) = [1 - (\alpha + 2\beta)\Delta t]P_1(t) + \{\theta[R_1(t)\alpha + Q_1(t)\beta] + \{1 - \theta\}[R_2(t)\alpha + Q_2(t)\beta + Q_3(t)\beta]\} \Delta t. \quad (A1)
\]

Here, the first term in the right-hand side of the equation represents the probability of nucleotide pair AG remaining unchanged during time \( \Delta t, \) if we neglect the rare event that both of A and G change simultaneously. In the second term, \( R_1(t)\theta\alpha\Delta t \) represents the probability that nucleotide pair AA changes to AG during time \( \Delta t, \) again if we neglect the event of simultaneous changes of A and G. The remaining terms represent the contributions of other nucleotide pairs to the frequency of AG at time \( t + \Delta t. \) Similarly, the frequencies of other nucleotide pairs at time \( t + \Delta t \) can be written as follows:
Table 6
Expected SDs of $d$ for Various Parameter Values ($n = 1,000$)

<table>
<thead>
<tr>
<th>PARAMETERS</th>
<th>SD FOR TRUE $d$ OF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td>$\theta$</td>
<td>0.5</td>
</tr>
<tr>
<td>$\alpha/\beta$</td>
<td>1</td>
</tr>
<tr>
<td>$\theta$</td>
<td>0.5</td>
</tr>
<tr>
<td>$\alpha/\beta$</td>
<td>10</td>
</tr>
</tbody>
</table>

\[
P_2(t+\Delta t) = [1-(\alpha+2\beta)\Delta t]P_2(t)
+ \{\theta[R_2(t)\alpha + Q_1(t)\beta + Q_3(t)\beta]\]
+ (1-\theta)[R_3(t)\alpha + Q_2(t)\beta + Q_4(t)\beta]\Delta t ,
\]

\[
Q_1(t+\Delta t) = [1-2(\theta\alpha+\beta)\Delta t]Q_1(t)
+ (1-\theta)[Q_2(t)+Q_3(t)]\alpha
+ [R_1(t)+R_2(t)+P_1(t)+P_2(t)]\beta \Delta t ,
\]

\[
Q_2(t+\Delta t) = [1-(\alpha+2\beta)\Delta t]Q_2(t)
+ \{\theta[R_1(t)\alpha + Q_2(t)\beta + P_1(t)\beta]\]
+ (1-\theta)[Q_4(t)\alpha + R_3(t)\beta + P_2(t)\beta]\Delta t ,
\]

\[
Q_3(t+\Delta t) = [1-(\alpha+2\beta)\Delta t]Q_3(t)
+ \{\theta[R_3(t)\alpha + R_2(t)\beta + P_2(t)\beta]\]
+ (1-\theta)[Q_4(t)\alpha + R_4(t)\beta + P_1(t)\beta]\Delta t ,
\]

\[
Q_4(t+\Delta t) = \{1-2[(1-\theta)\alpha + \beta]\Delta t\}Q_4(t)
+ \theta\{[Q_3(t)+Q_3(t)]\alpha+[R_3(t)+R_4(t)+P_1(t)+P_2(t)]\beta\} \Delta t .
\]

Furthermore, we have

\[
\theta = R_3(t)+R_4(t)+P_1(t)+P_2(t)+Q_2(t)+Q_3(t)+2Q_4(t) ,
\]

\[
(1-\theta) = R_1(t)+R_2(t)+P_1(t)+P_2(t)+2Q_1(t)+Q_2(t)+Q_3(t) .
\]

Let $P(t)$ and $Q(t)$, respectively, be the $P$ and $Q$ between the two sequences that diverged $t$ time units ago. Then the $P$ and $Q$ at time $t+\Delta t$, i.e., $P(t+\Delta t)$ and $Q(t+\Delta t)$, respectively, can be obtained from equations (A1), . . . , (A8) as follows:
Table A1
Different Types of Nucleotide Matches between Two Homologous Sequences, and Their Frequencies

<table>
<thead>
<tr>
<th>Type of Nucleotide Match</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Same:</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>$R_1$</td>
</tr>
<tr>
<td>TT</td>
<td>$R_2$</td>
</tr>
<tr>
<td>CC</td>
<td>$R_3$</td>
</tr>
<tr>
<td>GG</td>
<td>$R_4$</td>
</tr>
<tr>
<td>Total</td>
<td>$R$</td>
</tr>
<tr>
<td>Different:</td>
<td></td>
</tr>
<tr>
<td>Transition:</td>
<td></td>
</tr>
<tr>
<td>AG</td>
<td>$P_1$</td>
</tr>
<tr>
<td>GA</td>
<td>$P_1$</td>
</tr>
<tr>
<td>TC</td>
<td>$P_2$</td>
</tr>
<tr>
<td>CT</td>
<td>$P_2$</td>
</tr>
<tr>
<td>Total</td>
<td>$P$</td>
</tr>
<tr>
<td>Transversion:</td>
<td></td>
</tr>
<tr>
<td>AT</td>
<td>$Q_1$</td>
</tr>
<tr>
<td>TA</td>
<td>$Q_1$</td>
</tr>
<tr>
<td>AC</td>
<td>$Q_2$</td>
</tr>
<tr>
<td>CA</td>
<td>$Q_2$</td>
</tr>
<tr>
<td>TG</td>
<td>$Q_3$</td>
</tr>
<tr>
<td>GT</td>
<td>$Q_3$</td>
</tr>
<tr>
<td>CG</td>
<td>$Q_4$</td>
</tr>
<tr>
<td>GC</td>
<td>$Q_4$</td>
</tr>
<tr>
<td>Total</td>
<td>$Q$</td>
</tr>
</tbody>
</table>

\[
P(t+\Delta t) = 2P_1(t) + 2P_2(t) \\
= [1 - 2(\alpha + \beta)\Delta t]P(t) + 4\theta(1-\theta)\alpha\Delta t \\
- 2\{\theta[2Q_1(t) + Q_2(t) + Q_3(t)] + (1-\theta)[Q_4(t) + 2Q_6(t) + 2Q_7(t)](\alpha - \beta)\Delta t \\
= [1 - 2(\alpha + \beta)\Delta t]P(t) + 4\theta(1-\theta)[\alpha - (\alpha - \beta)Q(t)]\Delta t ,
\]

\[
Q(t+\Delta t) = 2Q_1(t) + 2Q_2(t) + 2Q_3(t) + 2Q_4(t) = (1 - 4\beta\Delta t)Q(t) + 2\beta\Delta t ,
\]

where the last equality of equation (A9) can be obtained from the following two equations:

\[
2Q_1(t) + Q_2(t) + Q_3(t) - (1-\theta)Q(t) \\
= (1 - \alpha - 3\beta)[2Q_1(0) + Q_2(0) + Q_3(0) - (1-\theta)Q(0)] = 0 ,
\]

\[
Q_2(t) + Q_3(t) + 2Q_4(t) - \theta Q(t) = (1 - \alpha - 3\beta)[Q_2(0) + Q_3(0) + 2Q_4(0) - \theta Q(0)] = 0 .
\]

These two equations can be approximated to the following set of differential equations, if $\Delta t$ is very small:

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\[
\frac{dP(t)}{dt} = -2(\alpha + \beta)P(t) + 4\theta(1 - \theta)[\alpha - (\alpha - \beta)Q(t)], \quad (A13)
\]

\[
\frac{dQ(t)}{dt} = 2\beta - 4\beta Q(t). \quad (A14)
\]

Solution of these equations with the initial condition \(P(0) = Q(0) = 0\) gives

\[
P(t) = \theta(1 - \theta)[1 + e^{-4\beta t} - 2e^{-2(\alpha + \beta) t}], \quad (A15)
\]

\[
Q(t) = \frac{1}{2} - \frac{1}{2}e^{-4\beta t}. \quad (A16)
\]

\(P(t)\) depends on the G+C content, \(\theta\), whereas \(Q(t)\) does not. \(P(t)\) is highest when \(\theta = 0.5\) and decreases as \(\theta\) deviates from 0.5. At time \(t = \infty\), \(P(t) = \theta(1 - \theta)\) and \(Q(t) = \frac{1}{2}\).

Equations (A15) and (A16) can be written as

\[
2(\alpha + \beta)t = -\log \left[1 - \frac{1}{2\theta(1 - \theta)} P(t) - Q(t)\right], \quad (A17)
\]

\[
4\beta t = -\log[1 - 2Q(t)]. \quad (A18)
\]

From these two equations and equation (2), we obtain equation (3).

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