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2 An averaging model for analysis and interpretations of high-order genetic interactions

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SUMMARY

While combinatorial genetic data collection from biological systems in which quantitative phenotypes are controlled by functional and non-functional alleles in each of multiple genes (multi-gene systems) is becoming common, a standard analysis method for such data has not been established. A common additive model of the non-functional allele effects contrasted against the functional alleles, based on ANOVA with interaction, has three issues. First, although it is a long tradition of genetics, modeling the effect of the non-functional allele (a null mutant allele) contrasted against that of the functional allele (the wild-type allele) is not suitable for mechanistic understanding of multi-gene systems. Second, an additive model is highly problematic when the system has more than two genes and a limited phenotypic range: errors propagate toward higher order interactions. Third, interpretations of higher-order interactions defined by an additive model are not intuitive. I propose an averaging model, which is suitable for mechanistic understanding of multi-gene systems. The effect of the functional allele is contrasted against the effect of the non-functional allele for easier mechanistic interpretations. Errors in interactions across the orders consistently stay low, which makes the model highly scalable to systems with many genes. The interactions defined by the averaging model are highly intuitive regardless of the orders. Yet, it is still a general linear model, so model fitting is easy and accurate using common statistical tools.

INTRODUCTION

Accumulation of genetic knowledge in many biological systems and technological advances that made combining multiple genetic loci easier have facilitated combinatorial genetic analysis among multiple genes, each of which has the functional (“wild-type”) and non-functional (null “mutant”) allele states, involved in single quantitative traits [1-4], which I here call multi-gene systems. However, conventional genetics is not well built for analysis and interpretation of high-order genetic interactions among multiple genes involved in a single quantitative trait. This is because conventional genetics is an extension of early objectives of analyzing functionally independent and/or qualitative genes. First, comparing multiple mutant phenotypes to the wild-type phenotype does not allow simple mechanistic interpretations. The phenotype of a particular genotype should be compared to the phenotype of the most disrupted mutant state (e.g., a quadruple null mutant in a 4-gene system) for simple mechanistic interpretations. Second, how to define and interpret genetic interactions among multiple genes is not definitively integrated. The main topic of this paper concerns this second point. An additive model based on ANOVA with interaction is a simple implementation for analysis of high-order genetic interactions. However, such an additive model requires conservation of the distributive law involving addition and interaction (e.g., $(A + B):C = A:C + B:C$, where “:” indicates the interaction defined in the additive model). I demonstrate that this requirement is not generally satisfied in high-order genetic interactions in a typical biological system, in which possible phenotypic values for a trait are bounded in a limited range. Previously we proposed a network reconstitution (formerly called signaling allocation) general linear model (NR model), which assumes violation of the distributive law [2]. This non-distributivity assumption led to use of the average of interactions in each order (not including 1-gene effects) in estimation of the highest order interaction in question. Whereas the NR model resolved problems caused by non-distributivity in genetic interactions, I recently recognized an inconsistency in the NR model, which was caused by the assumption of additive relationships among the 1-gene effects (i.e., non-interactive, main effects). This inconsistency was previously overlooked because the non-distributivity assumption did not constrain the 1-gene effects. The inconsistency was resolved by extending the averaging procedure to the 1-gene effects. I call the resulting model an averaging model. I demonstrate that the behavior of the averaging model is consistent regarding the level of representation of each of the 1-gene effect and multiple-gene interaction estimates. Furthermore, the averaging model allows consistent and intuitive interpretations of genetic interactions throughout all orders involved: a genetic interaction in the averaging model is a deviation of the phenotypic value of a multi-gene genotype from the average of the phenotypic values of all the genotypes that have one gene fewer than the interaction in question (e.g., $A;B;C = ABC - (AB + AC + BC) / 3$, where “;” indicates the interaction defined in the averaging model and the italicized upper-case letters denote genotypes carrying various combinations of wild-type alleles *A*, *B*, and *C*). I propose the averaging model as a standard general linear model for study of multi-gene interactions.

RESULTS AND DISCUSSION

Objective of the study

I define a multi-gene system as one in which multiple genes affect a single quantitative trait while each of the genes can have two states, functional and non-functional, arising from the wild-type and null mutant alleles, respectively. Such a system necessarily implies a gene network, in which the gene functions are not organized in a series (i.e., not in a single pathway). This is because a series of genes, each of which can only take a functional or non-functional state, can only generate an on or off output, so it is not quantitative. Instead, such a gene network must have a converging node(s) to generate a single trait. Converging nodes are sources of complex system behaviors [5, 6]. For a data set, I consider the measurement of the quantitative trait as the phenotype and measurements made with exhaustively combinatorial genotypes (i.e., for a n -gene system, the number of the exhaustively combinatorial genotypes is 2^n).

The objective of this study is to best describe the output of such a multi-gene system using the general linear model framework to facilitate mechanistic interpretations of the system behavior. Limiting the approach to a standard approach using the general linear model framework is associated with drawbacks because many biological systems contain non-linear components. However, a standard approach using the general linear model framework has practical advantages in actual applications. In a multi-gene system, usually we do not have sufficient knowledge to assume a particular, parameterized non-linear model for the system. In addition, we often lack quantitative input-output relationship information, which would help to constrain parameter values in a more complex model. Furthermore, fitting a general linear model is computationally easier and more accurate compared with fitting complex non-linear models. The general linear model could serve as a simple and versatile platform in many cases.

General notation rules

In this paper, I assume that all the genes of interest are homozygous for diploid organisms. A single gene is denoted by a single alphabetical letter in italics, with the upper-case letter for the wild-type allele and the lower-case letter for the null mutant allele. For example, *ABc* represents the genotype with the wild-type alleles for genes *A* and *B* and the mutant allele for gene *C*. When a description does not require noting the mutant alleles, I also use the genotype notation omitting the mutant alleles, such as *AB* instead of *ABc* for the purpose of simplicity, clarity, and generalization. The phenotype of a particular genotype is represented by the genotype notation. The non-italic lower-case letters, such as *a*, *b*, and *c*, represent the mutant allele effects defined in comparison to the wild-type alleles. The wild-type allele effects, represented by non-italic upper-case letters, such as *A*, *B*, and *C*, are defined in comparison with the mutant allele. The additive effect of *A* and *B* is denoted using a plus sign between them, *A + B*. The interaction between *A* and *B* effects on the phenotype in the additive model context is denoted using a colon between them, *A:B*. I will define another type of interaction between *A* and *B* effects on the phenotype in the averaging model context below, which is denoted using a semicolon between them, *A;B*. In a mechanistic network model underlying the phenotype observation, the node corresponding to gene *A* and the output of the node are denoted as *nA*.

I typically use a 3-gene system, *ABC*, as an example for the sake of simplicity. I also use systems with more genes for cases in which this makes the impacts in question clearer. I typically omit the intercept term in linear models for simplicity. The points discussed in the following text can be generalized to a system consisting of an arbitrary number of genes.

Comparing to the most disrupted state instead of the intact state gives better interpretability

A convention in genetics is to compare a mutant phenotype to the wild-type phenotype. Here I argue that instead, comparing a phenotype of any genotype to the phenotype of the most disrupted state, e.g., comparing to the triple mutant state in a 3-gene system, leads to much better mechanistic interpretations. In this section, for the sake of simplicity, I use a system defined by an ANOVA-based, 3-gene additive model although I will subsequently point out a separate issue associated with the additive model for a multi-gene system.

Fig. 1. A simple network behavior can be well described by the wild-type allele effects of a multi-gene system but not by the mutant allele effects. (a) A mechanistic model of a network containing 3 nodes that can be mutationally manipulated (a 3-gene system). The network consists of 6 nodes, among which *nA*, *nB*, and *nC* are mutationally manipulable and *nX*, *nY*, and *nZ* are not. The output of each node is given either as a value or an equation. The output of *nZ* is the quantitative phenotype of the system. (b) The phenotype values of all 8 combinatorial genotypes. (c) The values for the mutant allele effects and interactions. (d) The values for the wild-type allele effects and interactions.

Fig. 1a shows the mechanistic network underlying a system with 6 nodes, in which three nodes (*nA*, *nB*, and *nC*) can be manipulated by mutations and the other three (*nX*, *nY*, and *nZ*) cannot. Thus, for the purpose of genetic analysis, this is a 3-gene system. *nA*, *nB*, *nX*, and *nY* are input nodes, and their values are arbitrarily set at 5, -3, 4, and 2, respectively. *nZ* is the output node, and the output of *nZ* can be measured as the quantitative trait of the system. Simple additive rules at nodes *nC* and *nZ* are assumed, $nC = nA + nB + nX$ and $nZ = nC + nY$, respectively. Fig. 1b shows the *nZ* output (i.e., phenotype) of 8 exhaustively combinatorial genotypes. Fig. 1c shows the effects and interactions of the mutant alleles

that are calculated according to an ANOVA model with interaction. Fig. 1d shows the effects and interactions of the wild-type alleles that are calculated according to an ANOVA model with interaction. With Fig. 1d, it is easy to reconstitute the mechanistic network shown in Fig. 1a: there is a basal activity of 2 without any of A, B, or C; A and B are not active by themselves, while C has its own activity of 4 regardless of A and B; the connection between A and C is positive with a value of 5, and the connection between B and C is negative with a value of -3; No A:B:C interaction means that additive effects up to two-gene interactions can explain the system behavior completely. In comparison, mechanistic interpretations based on Fig. 1c are not simple.

It is intuitive that mechanistically interpreting a system with functional components (i.e., wild-type alleles) is much more straightforward than mechanistically interpreting an unknown system using its deficiencies (i.e., mutant alleles). The 3-gene example system described above clearly demonstrates this principle. I conclude that a system consisting of multiple genes should be interpreted using wild-type allele effects. I will subsequently focus on modeling a system with wild-type allele effects and their interactions.

Laws of algebra

An additive model of gene effects and interactions involves two operators: additive, “+”, and interactive, “:”. Different models can be derived if we assume different laws for these operations. Three types of laws define algebra involving two operators: commutative, associative, and distributive laws. The commutative laws are $A + B = B + A$ and $A:B = B:A$. The associative laws are $(A + B) + C = A + (B + C)$ and $(A:B):C = A:(B:C)$. The distributive law is $(A + B):C = A:C + B:C$.

I assume the commutative laws for both “+” and “:” because a single quantitative phenotype cannot experimentally distinguish $A + B$ from $B + A$ or $A:B$ from $B:A$. I also assume the associative law for “+” since without this assumption the general linear model framework cannot be used.

The associative law for “:” is also required for the general linear model framework (see below). However, as I show below, the impact of a violation of the associative law for “:” can be moderated using an averaging principle, in which the arithmetic mean of multiple different expressions for the same quantity is taken as the true value of the quantity. This moderation by the averaging principle is important in applications of the general linear model framework to multi-gene systems because we cannot generally assume that the associative law for “:” holds. For example, in the case of Fig. 1, $A:B = 0$, and thus, $(A:B):C = 0$. However, $B:C \neq 0$, so, $A:(B:C)$ may not be 0 particularly when $A:C \neq 0$. Thus, $(A:B):C \neq A:(B:C)$ could happen.

In the following sections, I will show that the distributive law is required in the additive model. I will also show that a range-limiting non-linearity of a system, such as a saturation response, would violate the distributive law. Such responses are common in biological systems. Further I will show that there is a

general linear model that allows violation of the distributive law under the assumption of the averaging principle. I call this model an averaging model.

Derivation of the averaging model

The part of the following discussion describing derivation of the NR model was modified from Text S1 in [2]. In this section, for simplicity the intercept value (i.e., the phenotype value for the most disrupted state) is subtracted from all measured values so that the intercept value is 0.

According to the additive model, I assume $AB = A + B + A:B$... (1) as the starting point. In this case, the interaction is the deviation of the corresponding genotype from arithmetic addition of the 1-gene effects. Let's extend this to a system consisting of three genes A , B , and C . The phenotype ABC can be considered as being expressed in three different ways: adding C to the genetic background of AB ; adding A to the genetic background of BC ; or adding B to the genetic background of CA .

By adding C to AB , the genotype ABC is expressed as:

$$ABC = AB + C + AB:C = (A + B + A:B) + C + AB:C = A + B + C + A:B + AB:C \dots (2)$$

If the distributive law is not assumed, (2) cannot be simplified.

If the distributed law is assumed, (2) can be simplified to:

$$ABC = A + B + C + A:B + (A + B + A:B):C = A + B + C + A:B + B:C + C:A + (A:B):C \dots (3)$$

Similarly, if the distributive law is not assumed:

$$\text{By adding } A \text{ to } BC, ABC = A + B + C + B:C + BC:A \dots (4)$$

$$\text{By adding } B \text{ to } CA, ABC = A + B + C + C:A + CA:B \dots (5)$$

If the distributive law is assumed:

$$\text{By adding } A \text{ to } BC, ABC = A + B + C + A:B + B:C + C:A + A:(B:C) \dots (6)$$

$$\text{By adding } B \text{ to } CA, ABC = A + B + C + A:B + B:C + C:A + B:(C:A) \dots (7)$$

Although the expressions are not the same, (3), (6), and (7) must be the same in a model to explain ABC . Therefore, for this model framework to work exactly, the associative law for the interaction operator ":" is necessary,

$$(A:B):C = A:(B:C) = B:(C:A) = A:B:C \dots (8)$$

If (8) is true, (3), (6), and (7) become the same expression:

$$ABC = A + B + C + A:B + B:C + C:A + A:B:C \dots (9)$$

(9) is the additive model for three genes. This can be extended to a system consisting of more genes. In summary, the additive model is a good description of a multi-gene system if the associative law for ":" and the distributive law hold.

However, as discussed above, the associative law cannot be generally assumed for the ":" operator in a multi-gene system. This contradiction about associativity indicates a failure of the general linear model as a general description of a multi-gene system. A compromise to maintain the general linear model framework is to define ABC as the arithmetic mean of (3), (6), and (7):

$$ABC = \{[A + B + C + A:B + B:C + C:A + (A:B):C] + [A + B + C + A:B + B:C + C:A + A:(B:C)]\}$$

$$+ \{ A + B + C + A:B + B:C + C:A + B:(C:A) \} / 3$$

$$= A + B + C + A:B + B:C + C:A + \{ (A:B):C + A:(B:C) + B:(C:A) \} / 3 \dots (10)$$

I call this practical approach to avoiding the contradiction in the general linear model by averaging all possible cases an averaging principle.

Since $\{ (A:B):C + A:(B:C) + B:(C:A) \}$ cannot be expressed by the lower order terms, A , B , C , $A:B$, $B:C$, and $C:A$, it is reasonable to define $A:B:C = \{ (A:B):C + A:(B:C) + B:(C:A) \} / 3 \dots (11)$. Then,

$$ABC = A + B + C + A:B + B:C + C:A + A:B:C \dots (9)$$

Thus, with the averaging principle, the additive model can conform to the assumption of no associativity in the interaction operator “:”. This can be extended to a system consisting of more genes. In summary, the additive model should be a reasonable description of a multi-gene system if the distributive law holds.

If the distributive law cannot be assumed, (2), (4), and (5) must still be the same to express ABC . Here again we observe a failure of the linear model as a general description of a multi-gene system. I apply the averaging principle to (2), (4), and (5) to express ABC :

$$ABC = \{ \{ A + B + C + A:B + AB:C \} + \{ A + B + C + B:C + BC:A \} + \{ A + B + C + C:A + CA:B \} \} / 3$$

$$= A + B + C + (A:B + B:C + C:A) / 3 + (AB:C + BC:A + CA:B) / 3 \dots (12)$$

Since $(AB:C + BC:A + CA:B)$ cannot be expressed by the lower order terms, A , B , C , $A:B$, $B:C$, and $C:A$, it is reasonable to define $A;B;C = (AB:C + BC:A + CA:B) / 3 \dots (13)$. I use the semicolon “;” to distinguish this different definition of interaction from that of the interaction in the additive model and call “;” an averaging interaction operator and “:” an additive interaction operator.

$$ABC = A + B + C + (A:B + B:C + C:A) / 3 + A;B;C \dots (14)$$

Therefore, if the distributive law is not assumed, the average of the 2-gene additive interactions should be used to express the all wild-type allele state of ABC . In general, the rule that the terms in each order of the interactions (2-gene additive interactions, 3-gene averaging interactions, 4-gene averaging interactions, ...) must be averaged can be derived by extending this to a system with more genes.

For example, with a system consisting of 4 genes, A , B , C , and D :

$$ABCD = A + B + C + D + (A:B + A:C + A:D + B:C + B:D + C:D) / 6 + (A;B;C + A;B;D + A;C;D + B;C;D) / 4 + A;B;C;D \dots (15)$$

This is the NR model [7] (previously called the signaling allocation model [2]). Note that in the NR model, 2-gene interactions are additive interactions while 3 or higher order interactions are averaging interactions.

The assumption of the non-distributivity does not require any more changes in the model. However, the above derivation of NR model started with an arbitrary definition of the 2-gene additive interaction, $AB = A + B + A:B \dots (1)$, which is the reason the NR model is a mixture of additive and averaging interactions. The model would be more mathematically consistent if the averaging interaction definition is extended to 1-gene effect terms to make the 2-gene interactions averaging interactions as well, i.e., $AB = (A + B) / 2 + A;B \dots (16)$. I demonstrate in a subsequent section that (16) is indeed required for mathematical consistency of the model.

By applying (16):

$$ABC = (A + B + C) / 3 + (A;B + B;C + C;A) / 3 + A;B;C \dots (17)$$

$$ABCD = (A + B + C + D) / 4 + (A;B + A;C + A;D + B;C + B;D + C;D) / 6 + (A;B;C + A;B;D + A;C;D + B;C;D) / 4 + A;B;C;D \dots (18)$$

Now the rule is that the terms in each order of the interactions, including 1-gene effects (the first order), must be averaged. (17) and (18) are equivalents of:

$$ABC = (AB + BC + CA) / 3 + A;B;C \dots (19)$$

$$ABCD = (ABC + ABD + ACD + BCD) / 4 + A;B;C;D \dots (20)$$

Thus, the highest order averaging interaction is defined as the deviation of the corresponding genotype from the average of all genotypes with one gene fewer. This definition of the averaging interaction is highly interpretable. I call this extended model with all averaging interactions an averaging model. With the definitions of the averaging interactions of different orders in (16), (19), and (20), it is clear the averaging model does not require the distributive law because these definitions do not include any terms that could be affected by whether the distributive law holds or not.

Note that the mean estimates from the additive model, NR model, averaging model, and 1-way ANOVA for all genotypes are just different ways to linearly decompose the phenotype values (when the full model terms are kept). Thus, when the models are fit to actual data with replication, all these models yield the same fitted and residual values. The numbers of estimated values are the same, i.e., the models have the same residual degree of freedom. Therefore, I use only the mean estimates of the models for my arguments in the following comparisons of the models. The coefficient matrices to solve the linear equations for the means in the three models using the genotype mean values in a 3-gene system are shown in Fig. 2.

a Additive model								
	Intercept	A	B	C	A:B	A:C	B:C	A:B:C
triple.mut	1	0	0	0	0	0	0	0
A	1	1	0	0	0	0	0	0
B	1	0	1	0	0	0	0	0
C	1	0	0	1	0	0	0	0
AB	1	1	1	0	1	0	0	0
AC	1	1	0	1	0	1	0	0
BC	1	0	1	1	0	0	1	0
ABC	1	1	1	1	1	1	1	1

b NR model								
	Intercept	A	B	C	A:B	A:C	B:C	A:B:C
triple.mut	1	0	0	0	0	0	0	0
A	1	1	0	0	0	0	0	0
B	1	0	1	0	0	0	0	0
C	1	0	0	1	0	0	0	0
AB	1	1	1	0	1	0	0	0
AC	1	1	0	1	0	1	0	0
BC	1	0	1	1	0	0	1	0
ABC	1	1	1	1	1/3	1/3	1/3	1

c Averaging model								
	Intercept	A	B	C	A:B	A:C	B:C	A:B:C
triple.mut	1	0	0	0	0	0	0	0
A	1	1	0	0	0	0	0	0
B	1	0	1	0	0	0	0	0
C	1	0	0	1	0	0	0	0
AB	1	1/2	1/2	0	1	0	0	0
AC	1	1/2	0	1/2	0	1	0	0
BC	1	0	1/2	1/2	0	0	1	0
ABC	1	1/3	1/3	1/3	1/3	1/3	1/3	1

Fig. 2. Matrices for the linear equations to obtain model coefficients from the genotype values in a 3-gene system for (a) additive, (b) NR, and (c) averaging models. The rows are genotypes and the columns are model variables. “:” and “;” indicate the additive and averaging interactions, respectively.

Violation of the distributive law is prevalent in multi-gene systems

The averaging model does not assume the distributive law. Do we really need to consider non-distributivity in a biological system? Let's consider a simple 3-gene system, in which n_A and n_B are input nodes and n_C is the output node (Fig. 3a). Mechanistically, signals from n_A and n_B are first summed, and then modulated by a non-linear function f_1 before the signal is output from n_C . Thus,

$$(A + B):C = n_C = f_1(n_A + n_B) \dots (21)$$

$$A:C + B:C = f_1(n_A) + f_1(n_B) \dots (22)$$

Therefore, if the distributive law holds,

$$f_1(n_A + n_B) = f_1(n_A) + f_1(n_B) \dots (23)$$

Let's make f_1 a Michaelis-Menten function for a saturating response (Fig. 3b):

$$f_1(x) = \frac{10}{1 + \frac{x}{7}} \dots (24)$$

When $n_A = 5$, $n_B = 2$,

$$f_1(n_A + n_B) = f_1(5 + 2) = 5$$

$$f_1(n_A) + f_1(n_B) = f_1(5) + f_1(2) = 4.16... + 2.22... = 6.38...$$

$$\text{Thus, } f_1(n_A + n_B) \neq f_1(n_A) + f_1(n_B) \dots (25)$$

and the distributive law is violated.

Generally, non-linearity in a system leads to violation of the distributive law.

Fig. 3. Non-linearity in a system violates the distributive law. (a) a 3-gene system, in which signals from n_A and n_B feed into n_C . The output of n_C is defined as $f_1(n_A + n_B)$. (b) When $f_1(x) = \frac{10}{1 + \frac{x}{7}}$, the input-output relationships at n_C are shown. If the input is n_A , the output is expressed as $A:C$ in the additive model. This plot clearly shows that $(A + B):C \neq A:C + B:C$ (Y-axis values in orange and green, respectively), a violation of the distributive law. (the y-axis values in orange and green)

A saturating response limits the output range. Without non-linearity, the range of the system output is not limited, and this is the condition the additive model requires. Thus, the additive model generally cannot be used in a system consisting of multiple genes (more than 2 genes, strictly speaking: see below) when the phenotype value range is limited compared to the ranges of the gene effects and interactions. To demonstrate this point, I use a 7-gene system as this problem becomes more severe when more genes are in the system. With 7 genes, the number of exhaustively combinatorial genotypes is $2^7 = 128$. I randomly generated phenotype values by sampling from a uniform distribution ranging from 1 to 10, and each model was solved using these randomly generated data values. This procedure was repeated 10,000 times and the model estimate distributions, except for the model intercept (i.e., the septuple mutant value), were visualized as a box plot (Fig. 4). Fig. 4a shows that in the additive

model, the higher the order of interactions is, the higher the representations of the interactions are. The length of the box (the difference between the 75th and 25th percentiles) of the 7-gene interaction is about 7.5 times larger than those of the 1-gene effects. Therefore, if the additive model is used, the absolute values of higher order additive interactions are grossly overestimated in general. This problem is much smaller using the NR model (Fig. 4b). Note the scale difference in the y-axes between Fig. 4a and Figs. 4b and 4c: the distributions of the 1-gene effects are essentially the same across the models. However, the NR model still has an overrepresentation issue with the 2-gene additive interactions, suggesting that the NR model is still affected when the phenotype value range is limited. The distributions of estimates were very consistent across all the effects and averaging interactions in the averaging model (Fig. 4c). These results strongly suggest that the averaging model well handles non-distributivity arising from range-limiting non-linearity in the system response even when the number of the genes in the system is high.

Fig. 4. Distributions of the gene effects and interaction values when the phenotype values were randomly sampled from a uniform distribution with (a) additive, (b) NR, and (c) averaging models. Each order of interactions is color-coded separately, and the color coding is shown in the bottom of (a). Note that the scales in the y-axes are very different in (a) compared to (b) and (c).

Evidence of non-distributivity in an actual multi-gene data set

Do we see this problem associated with non-distributivity in actual biological systems? We initially recognized the problem in 4-gene systems [2] when we started to omit high order additive interaction terms from the full additive model. We expected that such reduced models should be good approximations of the model containing higher orders of additive interactions. However, in the full additive model, when the 4-gene additive interaction term was omitted, the estimates for the 3-gene additive interactions were reduced substantially (Fig 5a, black and red segments for the 3-gene additive interactions). Smaller, yet still substantial increases of the 2-gene additive interactions were also evident (black and red segments for the 2-gene additive interactions). Large changes of estimate values in the opposite directions for the 3-gene and 2-gene additive interactions strongly suggests artifactual overrepresentation of the 4-gene additive interaction. In the full NR model, the estimate changes in the 3-gene averaging and 2-gene additive interactions when the 4-gene averaging interaction term was omitted were much smaller (Fig. 5b). In the averaging model, the estimate changes in the 3-gene and 2-gene averaging interactions when the 4-gene averaging interaction term was omitted were almost unnoticeable (Fig. 5c).

Fig. 5. The coefficient estimates for the contribution to immunity using the data from Tsuda et al. in (a) additive, (b) NR, and (c) averaging models. The 95% confidence interval is shown as a horizontal bar, with the mean as a point. Different levels of model reduction (omitting higher order interactions from the model) are color-coded according to the color code in (a). Different shades of gray background are used to show different orders of interactions. “:” and “,” indicate additive and averaging interactions, respectively.

Another evident trend in the additive model is that the 95% confidence intervals (the lengths of the horizontal lines) were wider for the higher order additive interactions (Fig. 5a; compare the segments of the same color, which have the same order of model reduction). With the NR model, the confidence

interval was widest with the 2-gene additive interactions although overall confidence interval width differences were much smaller than in the additive model (Fig. 5b). This confidence interval width trend suggest that the overrepresentation of higher order averaging interactions was strongly reduced in the NR model compared to the additive model. In the averaging model, the widths of the confidence intervals were quite consistent across the orders of averaging interactions. The trend of the confidence interval width in the three models directly corroborates the observations made using random simulation data in Fig. 4.

An additional evident trend in the confidence intervals in the additive model is that when the same coefficients (1-gene effects and additive interactions) were compared, the more reduced the model was, the narrower the confidence intervals were (Fig. 5a). With the NR model, the trend of narrower confidence intervals in the more reduced models was evident only in the 1-gene effects (Fig. 5b). This trend suggests that some problem remained with the 1-gene effect estimation in the NR model. In the averaging model, the widths of the confidence intervals were very consistent across the orders of model reduction (Fig. 5c). In summary, the problem associated with non-distributivity in this biological data set is evident in the additive model while the averaging model appears free of this problem.

Why does the averaging model describe a multi-gene system better than the additive model?

Let's consider simple additive and averaging models with no interaction using a 7-gene system. With the additive model, $ABCDEFG = A + B + C + D + E + F + G$. It is highly conceivable that the sum of all 1-gene effects could go well outside the system output range. In such a case, it is necessary for the additive model to have non-zero additive interaction(s) to keep the $ABCDEFG$ phenotype within the system output range. On the other hand, with the averaging model, $ABCDEFG = (A + B + C + D + E + F + G) / 7$, the $ABCDEFG$ phenotype range is bounded by the maximum and minimum of the 1-gene effects without non-zero averaging interactions. Thus, with the additive model, a range-limiting non-linearity generally forces non-zero additive interaction(s) in a multi-gene system while this does not occur in the averaging model.

Next, let's look at how interactions affect estimates of other interactions. A range-limiting non-linearity can be handled easily with the additive model in a 2-gene system. $AB = A + B + A:B \dots (1)$. Any non-linear effect can be attributed to the 2-gene additive interaction $A:B$, and therefore, non-linearity is not an issue. However, in a 3-gene system, $ABC = A + B + C + A:B + A:C + B:C + A:B:C \dots (9)$, the 2-gene additive interactions in (9) likely have values different from the 2-gene additive interactions in the 2-gene systems (e.g., $A:B$ in (1) and (9) should have different values) due to the non-linearity. Consequently, estimation of an additive interaction accumulates this type of non-linearity-associated errors from the lower order additive interaction estimates: i.e., non-linearity-associated errors propagate in estimation of higher-order additive interactions. This problem of propagating errors was clearly demonstrated by overrepresentation of higher-order additive interaction estimates (Fig. 4a) and by wider confidence intervals for higher-order additive interaction estimates (Fig. 5a).

On the other hand, estimation of an averaging interaction requires only observed values and does not require any of the lower-order averaging interaction estimates (e.g., equations (16), (19), and (20) for the 2-, 3-, and 4-gene averaging interactions). Thus, non-linearity-originated error is confined to each averaging interaction and does not propagate (Figs. 4c and 5c). This is the reason the averaging model performs better than the additive model when the range-limited system involves more than two genes.

Interpretation of the averaging model outcome

It should be emphasized that the definitions of the interactions are different in additive and averaging models. How do different interaction definitions affect interpretations of the 1-gene effects and interactions? With the additive model, the 2-gene additive interaction is understood as the difference from the addition of the 1-gene effects, $A:B = AB - (A + B) \dots (1)'$. When $A, B, A:B > 0$, A and B have a synergistic effect. When $A, B > 0, A:B < 0$, A and B have a compensating effect (Fig. 6a). However, such interpretations of additive interaction, synergistic or compensating, become unclear when A and B have opposite signs (Fig. 6b). In addition, with more genes in a system, the interpretation of higher-order additive interactions become nonintuitive. For example, the 3-gene additive interaction is $A:B:C = ABC - (A + B + C + A:B + A:C + B:C) \dots (9)'$ (Fig. 6c).

In contrast, the interpretation of averaging interactions in the averaging model is consistent and highly interpretable, however many genes are in the system and whatever the orders of averaging interactions are, i.e., the averaging model is highly scalable to the number of genes in the system. An averaging interaction is the deviation of the corresponding genotype from the average of all involved genotypes that have one gene fewer (equations (19) and (20)). For example, in a 2-gene system, $A;B = AB - (A + B)/2 \dots (13)'$ (Fig. 6d). Note that not just the values but also the signs of the interaction could be different between the additive and averaging interactions (compare AB (case 1) in Figs. 6a and 6d). The interpretations of averaging interactions are consistent even when A and B have opposite signs (Fig. 6e) or the system has three genes, A, B, and C (Fig. 6f).

In the case of a 3-gene averaging interaction, $A;B;C = ABC - (AB + AC + BC) / 3 \dots (19)'$ (Fig. 6d). This could be a 3-gene interaction in a 7-gene system, $A;B;C = ABCdefg - (Abcdefg + AbCdefg + aBCdefg) / 3 \dots (19)''$. Thus, the genotype notation not showing the mutant alleles, such as equation (19)', is a more generalized notation.

Fig. 6. Interpretations of interactions in (a-c) additive and (d-f) averaging models. (a, d) Two-gene interactions when both 1-gene effects A and B are positive. Two different cases (cases 1 and 2) of the AB phenotype values are used. (b, e) Two-gene interactions when 1-gene effects have opposite signs. (c, f) Three-gene interactions. “:” and “;” indicate additive and averaging interactions, respectively.

The averaging model-based multi-gene analysis should contain only the genes significantly involved in the phenotype.

Since the averaging interaction is the phenotypic deviation of the corresponding genotype from the average of all genotypes with one gene fewer, it is affected if the analysis includes unnecessary genes. Such unnecessary genes can be detected by comparing all the genotypes containing the gene in question to the corresponding genotypes without the gene. For example, in a 3-gene system with genes A, B, and C, the test for whether gene C should be included is whether any of $ABC - AB$, $AC - A$, $BC - B$, and $C - abc$ have values significantly different from 0. If none of them are significantly different from 0, gene C must be removed from the averaging model.

Reinterpretation of previous results using the averaging model

Using the averaging model, I reinterpreted results from my laboratory of exhaustively combinatorial genotype analysis in a 4-gene system, which were originally analyzed using the NR model shown in Fig. 6 of [2]. The study consisted of four cases of inducible immunity in the model plant *Arabidopsis* against strains of the bacterial pathogen *Pseudomonas syringae*, which are designated as the AvrRpt2-ETI, AvrRpm1-ETI, flg22-PTI, and elf18-PTI cases. ETI is Effector-Triggered Immunity, and AvrRpt2 and AvrRpm1 are triggering effectors [8-12]. PTI is Pattern-Triggered Immunity, and flg22 and elf18 are triggering molecular patterns [13-15]. The inhibition of bacterial growth in the plant leaf, in $\log_{10}(\text{cfu}/\text{cm}^2)$, was the immunity phenotype measure. The hub genes of four major signaling sectors

(subnetworks) in the plant immune signaling network were subjected to mutational analysis. The signaling sectors were the jasmonate, ethylene, PAD4, and salicylate sectors, which are indicated as J, E, P, and S, respectively. I also call their hub genes *J*, *E*, *P*, and *S*, in this context of analysis of the 4-gene system. Biological and experimental details are provided in [2].

Each of the AvrRpt2-ETI, AvrRpm1-ETI, flg22-PTI, and elf18-PTI cases was first tested to determine whether all four genes were significantly involved in the phenotype variation. Except for the elf18-PTI case, all four genes were significant, and the averaging model for the 4-gene system was used. However, the elf18-PTI phenotype was not significantly affected by the *J* gene in any genotype context. Therefore, the averaging model for the 3-gene system with the *E*, *P*, and *S* genes was used.

Fig. 7. The coefficient estimates for the contribution to immunity from averaging model analysis of the data in Tsuda et al. (a) AvrRpt2-ETI, (b) AvrRpm1-ETI, (c) flg22-PTI, and (d) elf18-PTI. The 95% confidence interval is shown as a horizontal bar, with the mean as a point. The Holm-corrected *p*-values are shown in the left part of each plot: red, *p* < 0.05; blue, *p* ≥ 0.05. The dataset used for AvrRpt2-ETI in Fig. 7a is the same as that in used in Fig. 5, and Fig. 7a is the same as the full model (black lines) in Fig. 5c. “:” and “,” indicate additive and averaging interactions, respectively.

The results of applications of the averaging model to these four immunity cases are shown in Fig. 7. The 95% confidence interval is shown as a horizontal black bar with the mean estimate as the point in the middle. In the left part of each plot, the Holm-corrected *p*-values smaller than 0.05, which are considered significant, are shown in red.

There are several differences between the averaging and NR models. The 1-gene effects did not change much since the 1-gene effect definition for the genotype with a single wild-type allele, *J*, *E*, *P*, or *S*, was the same between the two models. The interactions changed substantially as the definitions of the interactions are different. Although the relative differences within the interactions of the same order did not change much between the two models, the interaction values in the averaging model were generally higher than those in the NR models because the 1-gene effects were non-negative and the 1-gene effects in genotypes with multiple wild-type alleles were averaged in the averaging model.

In AvrRpt2-ETI with the averaging model (Fig. 7a), the values for the 1-gene effects were all positive, and *P* had the largest effect. Most 2-gene averaging interactions were not significant, indicating that addition of another gene as the second gene does not change the immunity much from the average of 1-gene effects of the first and the second genes. However, *J*:*S* was significantly positive: while the *J* and *S* effects

are both positive, combining these two genes together (*JS* genotype) increases the immunity from the average of the *J* and *S* genotypes. All the 3-gene and 4-gene averaging interactions were significantly positive, indicating that all the genes increase immunity when added to the system as the 3rd or 4th genes. Note that the averaging model made interpretations of the 3-gene and 4-gene interactions easy and consistent.

In *AvrRpm1*-ETI with the averaging model (Fig. 7b), most immunity was explained by the intercept (i.e., the immunity level in the *jeps* genotype), showing that the quadruple mutant still maintains most of the immunity of wild-type plants. This observation can be explained by the fast kinetics of *AvrRpm1*-ETI signaling compared to *AvrRpt2*-ETI, in respect to the gating timing of the ETI-Mediated and PTI-Inhibited Sector (EMPIS) by PTI signaling [16]. Although all the 1-gene effects and the averaging interactions had lower amplitudes, they generally had a similar trend of up and down as those of *AvrRpt2*-ETI, suggesting that the 4-gene network apart from EMPIS behaves similarly in *AvrRpm1*-ETI and *AvrRpt2* ETI. *J;S* was the only significant averaging interaction with a positive contribution to immunity.

In *flg22*-PTI (Fig. 7c), all the 1-gene effects except *S* were significantly positive with *E* as the highest. The 2-gene averaging interactions were largely low and/or not significant, except *P;S*, which was significantly and strongly positive. The 3-gene and 4-gene averaging interactions were significantly and strongly positive, indicating that all the genes substantially increase the immunity level when added to the system as the 3rd or 4th genes.

In *elf18*-PTI (Fig. 7d), the *J* gene was removed and a 3-gene averaging model including the *E*, *P*, and *S* genes was used. Only *P;S* was significant among all the averaging model terms, except the intercept. The *P;S* averaging interaction was strongly positive, indicating that a single mutation in genes *P* or *S* almost completely abolishes the immunity. The difference in the importance of the *E* gene clearly separated *flg22*-PTI and *elf18*-PTI. Another difference between *flg22*-PTI and *elf18*-PTI was the 3-gene and 4-gene averaging interactions. All were strongly positive in *flg22*-PTI, and none were significant in *elf18*-PTI.

It is noteworthy that the roles of *J;S* and *P;S* were very different in ETI and PTI. A strongly positive *P;S* averaging interaction was observed in PTI (Figs. 7c and 7d). Positive functional interactions between the *P* and *S* genes have been well documented in many aspects of plant immunity [17]. In contrast, this averaging interaction was insignificant in ETI, except for their contributions through higher-order averaging interactions, *J;P;S*, *E;P;S*, and *J;E;P;S*. On the other hand, a strongly positive *J;S* averaging interaction was observed in ETI while it was insignificant in PTI (Fig. 7). Although negative functional interactions between the *J* and *S* genes are often described in plant immunity [17], these two genes positively interact in ETI (Figs. 7a and 7b). In addition, in *flg22*-PTI the 3-gene and 4-gene averaging interactions were strongly positive while they were moderately positive in *AvrRpt2*-ETI. A disadvantage of strong 3-gene and 4-gene averaging interactions is that a mutation(s) in one or two genes results in large loss of immunity. Relatively weak 3-gene and 4-gene averaging interactions in ETI indicates that ETI is more resilient against damage to one or two of these major immune signaling sectors, which could be caused by pathogen effectors [6]. In summary, the averaging model analysis highlighted that while the 4-gene system is important in both ETI and PTI (with *flg22*), how they are used in ETI and PTI is quite

different, and ETI is more resilient than PTI against perturbations to the signaling sectors. It also highlighted substantial differences, particularly in the role of the *J* and *E* genes, in regulation between flg22-PTI and elf18-PTI.

Limitations of using the general linear model platform

The goal of this study is to propose a standard statistical model that works reasonably well with most multi-gene systems to gain mechanistic information about the systems. Among the models discussed here, the additive, NR, and averaging models, the averaging model is the most versatile, consistent, scalable, and interpretable general linear model. Fundamentally all models are linear models, so of course they have limitations in applications to non-linear systems. I assumed the associative law for the addition operator “+”, which may not be true for every biological system. I also used the averaging principle, in which the arithmetic mean of multiple different expressions for the same quantity was taken as the true value of the quantity. This principle was used to practically accommodate the non-associativity of the interaction operators within the general linear model framework, which in principle does not allow non-associativity for the interaction operators. Although the averaging principle is probably the best compromise for the purpose of accommodating the non-associativity of interactions in the model framework, whether it truly provides a good approximation in the averaging model depends on the type of non-linearity. Since the highest order of averaging interaction is defined as the deviation of the corresponding genotype from the arithmetic mean of all involved genotypes with one gene fewer (equations (16), (19), and (20) and Fig. 6f), if the system is strongly non-linear in the phenotypic range of these genotypes with one gene fewer, the averaging principle fails and, consequently, the averaging model fails. However, with a range-limiting non-linearity, which does not have strong non-linearity in the middle of the phenotypic range, the chance that such major failure of the averaging principle and the averaging model occurs is not very high. Therefore, the averaging model should work well in systems with range-limiting non-linearity.

Concluding Remarks

I have demonstrated that multi-gene systems subjected to exhaustively combinatorial mutation analysis typically violate the distributive law and that therefore, the additive model is not appropriate for analysis of such systems consisting of more than two genes. In contrast, an averaging model conforms to non-distributivity and maintains consistency from the 1-gene effects to the highest order of averaging interactions. Furthermore, averaging model results are consistently and intuitively interpretable from the 1-gene effects to the highest order of averaging interactions. I propose the averaging model as a standard general linear model for combinatorial mutation analysis of multi-gene systems.

METHODS

Data sets

Biological data sets used in this study are the same data sets used in Figs. 6A and 6B in Tsuda et al. (2009). Each data set consists of bacterial counts ($\log_{10}(\text{colony forming units/cm}^2)$) for 16 exhaustively combinatorial genotypes for a 4-gene system, with or without treatment, with replication. Since the raw bacterial count data were not published previously, they are provided as Supplemental Dataset 1.

Random simulation with three models

The simulation was performed with a 7-gene system. The phenotype values for $2^7 = 128$ genotypes were randomly sampled from a uniform distribution ranging from 1 to 10. The 128 phenotype values were solved for the coefficients (gene effects and interactions) in each of the additive, NR, and averaging models. To solve the 128 equations per model, the 7-gene system matrix equivalent of the 3-gene system matrix in Fig. 2 was used (the matrices are provided in an R workspace file in Supplemental Dataset 2). This procedure was repeated 10,000 times for each model, and the distributions of each coefficient (except the intercept) across the repeats are shown by a box-and-whiskers in Fig. 4.

Fitting averaging models to the data

A linear mixed-effect model (the lme function in the nlme R package [18]) was used. This was because (i) each data set has factors regarding the experimental design, which were included as random effects in the model and (ii) the numbers of replicates were not the same across the genotype x treatment combinations. First, a linear mixed-effect model with the genotype x treatment interactions was fit to each of the data sets for “AvrRpt2-ETI”, “AvrRpm1-ETI”, “flg22-PTI”, and “elf18-PTI”. The formula for the fixed effects was “~ genotype/treatment -1”. The random effects for the data sets were “~ 1|replicate/flat/pot”. The interaction coefficients of the linear mixed-effect model were used to test whether each gene is significant. For example, to test the significance of the *J* gene, the estimate differences, *JEPS* – *EPS*, *JEP* – *EP*, *JPS* – *PS*, *JES* – *ES*, *JE* – *E*, *JP* – *P*, *JS* – *S*, and *J* – *jeps* were subjected to *t*-tests using the associated standard errors calculated from the variance/covariance matrix and the residual degree of freedom. If none of the *p*-values from the *t*-tests were smaller than 0.05, the gene was designated insignificant and omitted from the following averaging model analysis. To avoid overly stringent tests, multiple tests correction was not used for selection of significant genes. Only the *J* gene in “elf18-PTI” was found insignificant. In this case, the data were bundled by ignoring the *J* gene. For example, the *JEPS* data were considered as part of the *EPS* data.

Second, the averaging model using the significant genes was fit. The 4-gene system equivalent matrix of the 3-gene system matrix in Fig. 2c or the 3-gene system matrix was used (the matrices are provided in an R workspace file in Supplemental Dataset 2). The rows were replicated according to the genotypes of the observations (the design matrix for the averaging model coefficients, denoted as “m.”). Using the

design matrix m , the fixed effects were, “~ m . -1 + genotype” and the random effects were, “~ 1|replicate/flat/pot” in the lme function. The averaging model coefficient estimates, their standard errors, and the p -values were extracted from the coefficient table of the lme model. The estimates, the standard errors, and the residual degree of freedom of the lme model were used to calculate the 95% confidence intervals. The p -values were subjected to the Holm multiple tests correction. The R script used to generate Fig. 7 from the raw bacterial count data sets is provided as Supplemental Dataset 3.

ACKNOWLEDGEMENTS

I thank Dave Mackey and Alex Turo for exposing me to their unpublished data from a 7-gene system, which led me to realization of the averaging model. I also thank Yaniv Brandvain, Ruth Shaw, Kenichi Tsuda, and Linda Jeanguenin for critical reading of the manuscript and Jane Glazebrook for editing. This work was supported by grants from National Science Foundation (MCB-1518058 and IOS-1645460).

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FIGURE LEGENDS

Fig. 1. A simple network behavior can be well described by the wild-type allele effects of a multi-gene system but not by the mutant allele effects. (a) A mechanistic model of a network containing 3 nodes that can be mutationally manipulated (a 3-gene system). The network consists of 6 nodes, among which nA, nB, and nC are mutationally manipulable and nX, nY, and nZ are not. The output of each node is given either as a value or an equation. The output of nZ is the quantitative phenotype of the system. (b) The phenotype values of all 8 combinatorial genotypes. (c) The values for the mutant allele effects and interactions. (d) The values for the wild-type allele effects and interactions.

Fig. 2. Matrices for the linear equations to obtain model coefficients from the genotype values in a 3-gene system for (a) additive, (b) NR, and (c) averaging models. The rows are genotypes and the columns are model variables. “:” and “,” indicate the additive and averaging interactions, respectively.

Fig. 3. Non-linearity in a system violates the distributive law. (a) a 3-gene system, in which signals from nA and nB feed into nC . The output of nC is defined as $f_1(nA + nB)$. (b) When $f_1(x) = \frac{10}{1+\frac{7}{x}}$, the input-output relationships at nC are shown. If the input is nA , the output is expressed as $A:C$ in the additive model. This plot clearly shows that $(A + B):C \neq A:C + B:C$ (Y-axis values in orange and green, respectively), a violation of the distributive law. (the y-axis values in orange and green)

Fig. 4. Distributions of the gene effects and interaction values when the phenotype values were randomly sampled from a uniform distribution with (a) additive, (b) NR, and (c) averaging models. Each order of interactions is color-coded separately, and the color coding is shown in the bottom of (a). Note that the scales in the y-axes are very different in (a) compared to (b) and (c).

Fig. 5. The coefficient estimates for the contribution to immunity using the data from Tsuda et al. in (a) additive, (b) NR, and (c) averaging models. The 95% confidence interval is shown as a horizontal bar, with the mean as a point. Different levels of model reduction (omitting higher order interactions from the model) are color-coded according to the color code in (a). Different shades of gray background are used to show different orders of interactions. “:” and “;” indicate additive and averaging interactions, respectively.

Fig. 6. Interpretations of interactions in (a-c) additive and (d-f) averaging models. (a, d) Two-gene interactions when both 1-gene effects A and B are positive. Two different cases (cases 1 and 2) of the AB phenotype values are used. (b, e) Two-gene interactions when 1-gene effects have opposite signs. (c, f) Three-gene interactions. “:” and “;” indicate additive and averaging interactions, respectively.

Fig. 7. The coefficient estimates for the contribution to immunity from averaging model analysis of the data in Tsuda et al. (a) $AvrRpt2$ -ETI, (b) $AvrRpm1$ -ETI, (c) $flg22$ -PTI, and (d) $elf18$ -PTI. The 95% confidence interval is shown as a horizontal bar, with the mean as a point. The Holm-corrected p -values are shown in the left part of each plot: red, $p < 0.05$; blue, $p \geq 0.05$. The dataset used for $AvrRpt2$ -ETI in Fig. 7a is the same as that in used in Fig. 5, and Fig. 7a is the same as the full model (black lines) in Fig. 5c. “:” and “;” indicate additive and averaging interactions, respectively.

SUPPLEMENTAL DATASETS

Supplemental Dataset 1. A .zip file containing four bacterial count data files (tab-delimited text) for “ $AvrRpt2_ETI$ ”, “ $AvrRpm1_ETI$ ”, “ $flg22_PTI$ ”, and “ $elf18_PTI$ ”. Each has columns of genotype, treatment, replicate, flat, pot, and colony. The colony column has log₁₀-transformed colony counts (colony forming unit/cm²). Although the data were originally reported in [2], these raw data were not published.

738 Supplemental Dataset 2. A .RData file (R workspace file) containing a list object, “ave.model.mats”,
 739 which contains the matrices for the averaging model for 2- to 7-gene systems (equivalents of matrix in
 740 Fig. 2c for different order gene systems).
 741
 742 Supplemental Dataset 3. An R script file (.r file), which is used to generate Fig. 7 from the data sets in
 743 Supplemental Dataset 1. It includes algorithms for selecting significant genes for the analysis and the
 744 averaging model. In the script, the object of a 3-gene or 4-gene matrix included in Supplemental Dataset
 745 2 is called “rec.mx”, which is generated by a function, “make.rec.mx”.

Fig. 1

Fig. 2

a Additive model

	Intercept	A	B	C	A:B	A:C	B:C	A:B:C
triple.mut	1	0	0	0	0	0	0	0
A	1	1	0	0	0	0	0	0
B	1	0	1	0	0	0	0	0
C	1	0	0	1	0	0	0	0
AB	1	1	1	0	1	0	0	0
AC	1	1	0	1	0	1	0	0
BC	1	0	1	1	0	0	1	0
ABC	1	1	1	1	1	1	1	1

b NR model

	Intercept	A	B	C	A:B	A:C	B:C	A;B;C
triple.mut	1	0	0	0	0	0	0	0
A	1	1	0	0	0	0	0	0
B	1	0	1	0	0	0	0	0
C	1	0	0	1	0	0	0	0
AB	1	1	1	0	1	0	0	0
AC	1	1	0	1	0	1	0	0
BC	1	0	1	1	0	0	1	0
ABC	1	1	1	1	1/3	1/3	1/3	1

c Averaging model

	Intercept	A	B	C	A;B	A;C	B;C	A;B;C
triple.mut	1	0	0	0	0	0	0	0
A	1	1	0	0	0	0	0	0
B	1	0	1	0	0	0	0	0
C	1	0	0	1	0	0	0	0
AB	1	1/2	1/2	0	1	0	0	0
AC	1	1/2	0	1/2	0	1	0	0
BC	1	0	1/2	1/2	0	0	1	0
ABC	1	1/3	1/3	1/3	1/3	1/3	1/3	1

Fig. 3

a

b

Fig. 4

Fig. 5

Fig. 6

Fig. 7