Reciprocal sign epistasis and sign epistasis may not be found in multi-peaked landscapes

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Abstract

Fitness landscapes is a mathematical framework for rigorously analyzing evolutionary dynamics including study of epistasis, the main obstacle to predicting phenotype from genotype. In 2011, Poelwijk *et al.* formulated a theorem asserting that in a multi-peaked fitness landscape, "at least two mutations exhibit reciprocal sign epistasis" (Poelwijk *et al.*, *J. Theor. Biol.*, 272:141). However, their proof disregarded neutral mutations – a critical limitation that significantly diminishes both the theoretical value and practical applicability of the theorem.

In this study, we reexamine the relationship between reciprocal sign epistasis (RSE) and multi-peaked fitness landscapes under the more general and realistic condition that neutral mutations are present. Our analysis reveals that when neutral mutations are allowed, the original theorem no longer holds. Instead, in cases when "strictly neutral" mutations could occur, the presence of RSE is guaranteed only if considering composite mutations – that is, groups of one or more mutations considered collectively in all possible combinations. Furthermore, when "near-neutral" mutations could occur – cases where the effect of a mutation is indistinguishable from zero – we cannot ensure statistically significant detection of RSE in the multi-peaked fitness landscapes even if considering composite mutations.

Our findings have profound theoretical and practical implementations. First, – contrary to one of the consequences of Poelwijk *et al.*'s theorem – for *each* mutation in the considered multi-peaked landscape, the compensating mutation(s) do not have to be adjacent in the course of evolution: the compensation can occur after some neutral mutations take place. Second, in real-world scenarios where fitness values are measured with some uncertainty, there may be a fundamental impossibility to detect RSE in a statistically significant manner in multi-peaked fitness landscapes (both at the level of single and composite mutations).

Introduction

Fitness landscapes (Wright, 1931) represent a convenient and straightforward mathematical concept for systematically studying various evolutionary phenomena, including epistasis, the dependence of a mutation's effect on genetic background (Fisher, 1918; Smith, 1970). Epistasis plays a crucial role in evolution (Kondrashov and Kondrashov, 2001; Weinreich *et al.*, 2005; Breen *et al.*, 2011; Weinreich *et al.*, 2013; Weinstein *et al.*, 2023) and is the primary obstacle to accurately predicting fitness or phenotype from genotype (Zhou *et al.*, 2022). It is also critical in pathogenic deviations (Nagel, 2005; Ang *et al.*, 2023) and contributes to diseases such as Alzheimer's disease (Combarros *et al.*, 2009; Lundberg *et al.*, 2023) and autoimmune disorders (Rose and Bell, 2012). Therefore, understanding the theoretical and empirical rules linking epistasis to other evolutionary phenomena is of great importance.

One key type of epistasis is reciprocal sign epistasis (RSE), which occurs when two mutations exhibit compensatory effects in a specific genetic context (Poelwijk *et al.*, 2011). Specifically, each mutation partially, fully, or excessively counteracts the effect (deleterious or beneficial) of the other (Kvitek and Sherlock, 2011). RSE drives compensatory evolution (Mammano *et al.*, 2000; Jucovic and Hartley, 1996; Baresić *et al.*, 2010; Ivankov *et al.*, 2014) and is associated with traversing fitness valleys, making it relatively rare in proteins (Baresić *et al.*, 2010). RNA molecules provide the clearest examples of RSE: Watson-Crick pairings evolve between A:U and G:C via intermediates G:U (more common) or A:C (rarer) (Meer *et al.*, 2010).

In 2011, Poelwijk *et al.* formulated a fundamental theorem stating that "Reciprocal sign epistasis is a necessary condition for multi-peaked fitness landscapes" (Poelwijk *et al.*, 2011). This theorem (referred to throughout this paper as "Poelwijk's theorem" for brevity) guarantees that along the shortest pathways between any two peaks in a multi-peaked landscape, "at least two mutations exhibit reciprocal sign epistasis" (Poelwijk *et al.*, 2011). The mathematical rigor of the statement allows no exceptions – identifying such mutations is merely a matter of thorough analysis and/or experimental efforts.

However, the original proof did not account for the potential presence of genotypes in the landscape with equal or statistically indistinguishable fitness values. This oversight specifically ignores neutral mutations, both strictly neutral (mutations with exactly zero effect) and near-neutral (mutations with effects statistically indistinguishable from zero). Consequently, Poelwijk *et al.* only proved that RSE is necessary for multi-peaked landscapes that lack equal-fitness genotypes, leaving the original statement unproven. Given the critical importance of neutral and near-neutral mutations in evolutionary theory (Kimura, 1968; Ohta, 1973) and their prevalence in real systems (Sarkisyan *et al.*, 2016; Pokusaeva *et al.*, 2019), the applicability of the statement actually proved by Poelwijk *et al.* is significantly limited, while the original statement still awaits validation.

Here, we reexamine the relationship between RSE and multi-peaked fitness landscapes by explicitly considering equal-fitness genotypes, particularly neutral mutations. We demonstrate

that neutral mutations invalidate the original Poelwijk's theorem. Instead, in landscapes containing strictly neutral mutations, only general RSE (uniting conventional RSE and distant RSE) can be guaranteed, i.e. if we consider composite mutations – groups of single mutations analyzed collectively in all possible combinations (Zorin *et al.*, 2022). Furthermore, when near-neutral mutations are included, even consideration of composite mutations cannot ensure statistically significant detection of even general RSE.

Our findings carry significant theoretical and practical implications. First, they restore common sense by showing that compensatory mutations do not need to occur immediately or be directly adjacent during evolutionary pathways – contradicting Poelwijk's theorem, which implies that for at least two mutations in a multi-peaked landscape, mutual compensation must be immediate. Second, in real-world situations where fitness measurements are inevitably subject to uncertainty, it may be fundamentally impossible to detect RSE or sign epistasis with statistical confidence – whether analyzing single or composite mutations – within multi-peaked landscapes. Armed with Poelwijk's theorem in its original form, a researcher might erroneously search for RSE in a multi-peaked landscape where it may not actually exist.

Results

Correct formulation of Poelwijk's theorem

In 2011, Poelwijk *et al.* claimed that "reciprocal sign epistasis (RSE) is a necessary condition for multi-peak landscapes" (Poelwijk *et al.*, 2011). However, their original proof did not account for genotypes with equal or statistically indistinguishable fitness values. Consequently, what Poelwijk *et al.* actually proved in 2011 is a much narrower theorem: RSE is a necessary condition for multi-peaked landscapes that lack genotypes with equal or statistically indistinguishable fitness. To clarify this, we present the corrected formulation of Poelwijk's theorem – matching what was truly proved by Poelwijk *et al.* (2011). For the reader's convenience, the proof of the Corrected Poelwijk's theorem, which is a verbatim copy of the proof given in (Poelwijk *et al.*, 2011) is provided in the Supplementary Materials.

Corrected Poelwijk's theorem. In a bi-allelic *L*-loci system, reciprocal sign epistasis is a necessary condition for the existence of two peaks at a distance *L* in the fitness landscape lacking genotypes with equal or statistically indistinguishable fitness.

Proof:
See (Poelwijk *et al.*, 2011).
End of proof.

Poelwijk *et al.* provided three arguments (see them numbered in Appendix of (Poelwijk *et al.*, 2011)) showing that their statement about two peaks in a bi-allelic *L*-loci system is equivalent to a similar statement about multiple peaks in an *N*-allelic *L*-loci system. Therefore, we have to correct also the general formulation of Poelwijk's theorem:

Corrected Poelwijk's theorem (general formulation). In an *N*-allelic *L*-loci system, reciprocal sign epistasis is a necessary condition for the existence of multiple peaks in the fitness landscape lacking genotypes with equal or statistically indistinguishable fitness.

The identification of exactly one minimum in steps (3) and (5) of the original proof is now justified since the theorem applies only to landscapes lacking genotypes having equal or statistically indistinguishable fitness. An illustration of the proof is provided in Fig. 1.

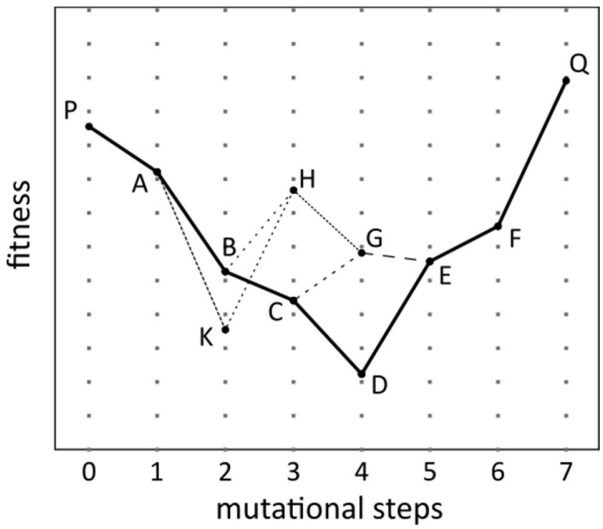

Figure 1. Illustration of the proof of the Corrected Poelwijk's theorem with numbers in parentheses referencing the steps in the proof. Adapted from Poelwijk *et al.* (2011).
– The existence of the two maxima, P and Q, is established (step (1)).
– A random direct path PABCDEFQ of length 7 from P to Q is considered (step (2)).
– This path contains one minimum D between genotypes C and E (step (3)).
– The order of mutations C→D and D→E around the minimum D is reversed, becoming C→G and G→E (mutations C→G and D→E are the same mutation applied in different genetic contexts C and D; mutations G→E and C→D are the same mutation applied in different genetic contexts G and C), and the new path PABCGEFQ is considered (step (4)).
– The minimum in the new path is found between new genotypes B and G, at point C (steps (5) and (5b)), so we proceed to step (4).
– The order of mutations B→C and C→G around the new minimum C is reversed, becoming B→H and H→G, and the new path PABHGEFQ is considered (step (4)).
– The minimum in the new path is found between new genotypes A and H, at point B (steps (5) and (5b)), so we proceed to step (4).
– The order of mutations A→B and B→H around the new minimum B is reversed, becoming A→K and K→H, and the new path PAKHGEFQ is considered (step (4)).
– The minimum in the new path, is found between the same genotypes A and H as previous minimum B, at point K, so the RSE is detected in the group of genotypes A, B, H, and K (steps (5) and (5a)).

Scenarios of fitness equality

After presenting the formulation of Corrected Poelwijk's theorem, the next question arises: does RSE exist in multi-peaked landscapes that include genotypes with equal fitness? One might

wonder if including genotypes with equal fitness leaves the original proof largely unchanged, implying that Poelwijk *et al.*'s statement remains valid for multi-peaked landscapes containing equal-fitness genotypes, and that the omission of these genotypes in the original paper (Poelwijk *et al.*, 2011) can be considered simply as an innocuous oversight.

The occurrence of equal-fitness genotypes can be divided into two distinct scenarios: (1) when equal-fitness genotypes are distant in the landscape, and (2) when they are neighbors, i.e., when neutral mutation(s) exist. Additionally, equal fitness can arise in two forms: (1) when fitness values are strictly identical, and (2) when fitness values differ but the difference is statistically insignificant due to experimental uncertainty. Altogether, this yields four distinct situations.

We will analyze these four cases separately, beginning with the least problematic situation of distant genotypes with strictly equal and statistically indistinguishable fitness values. Next, we will examine landscapes with strictly neutral mutations. Finally, we will address the remaining case involving near-neutral mutations.

Reciprocal sign epistasis does exist in multi-peaked landscapes having distant genotypes with equal fitness

When two distant genotypes along the pathway between two peaks have equal fitness and serve as minima, a common mathematical solution is to slightly modify the proof (Poelwijk *et al.*, 2011) of the Corrected Poelwijk's theorem by randomly selecting one of these minima. This typically resolves the issue. To incorporate this, steps (3) and (5) of the proof should include an instruction to randomly choose a minimum if multiple minima are found along the path. However, randomly selecting the next minimum can make detecting RSE take an arbitrarily long time.

For example, consider the hypothetical landscape in Fig. 2A, which modifies the landscape from Fig. 1 so that genotypes B, K, D, and G have identical fitness. Starting again from path PABCDEFQ and randomly choosing between minima B and D, suppose B is selected. Transitioning to path PAKCDEFQ, the next minimum must be chosen between K and D. If K is chosen, RSE is correctly identified involving genotypes A, B, K, and C. However, if D is chosen instead, we switch to the next path PAKCGEFQ. In this manner, the process could continue indefinitely travelling through four paths, as illustrated by two graph cycles in the center of Fig. 2B.

The existence of RSE in a multi-peaked landscape having equal-fitness genotypes but lacking neutral mutations can be proved as follows. We modify the proof of Corrected Poelwijk's theorem (Poelwijk *et al.*, 2011 or Supplementary Materials) to randomly select one of the equal-fitness minima. Additionally, at step (5a) of the proof, instead of searching for a new minimum along the entire path, we simply check for RSE immediately after reversing the order of the two mutations surrounding the current minimum on the path. For the landscape in Fig. 2A this approach identifies RSE involving genotypes A, B, K, and C in two steps. Notably, this is

equivalent to always choosing the next minimum with the same number of mutational steps as the current minimum when multiple minima are present.

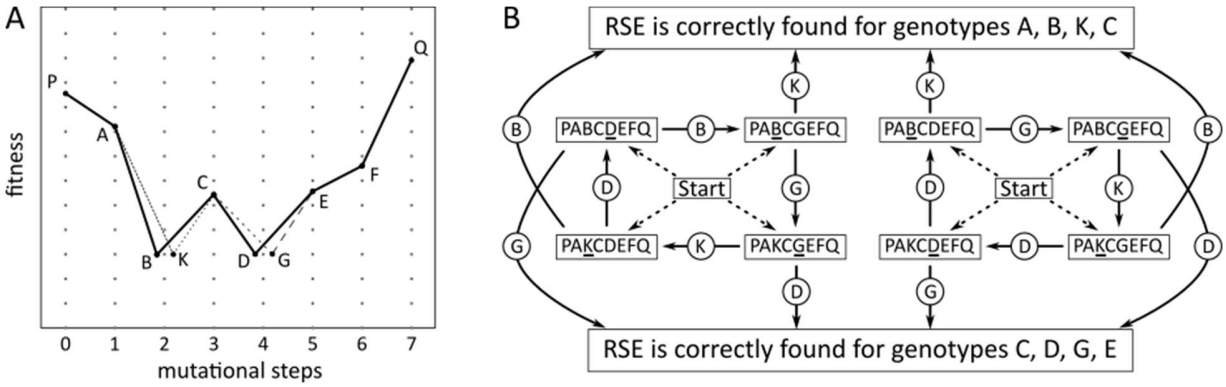

Figure 2. (A) Example of a multi-peaked landscape with distant genotypes having equal fitness. Genotypes B, K, D, and G share the same fitness. Genotypes B and K are two mutational steps from genotype P, while genotypes D and G are four mutational steps away from P. To avoid overlapping genotypes in the picture, B, K, D, and G are slightly shifted from their exact vertical positions. (B) Graph of states (rectangles) illustrating the proof of Corrected Poelwijk's theorem modified so that the next minimum is chosen randomly from two or more equal-fitness minima along the current pathway. 'Start' refers to the state at step (2) of the proof (Poelwijk *et al.*, 2011 or Supplementary Materials), where the first path is selected randomly. The process concludes at the two rectangles beginning with "RSE is correctly found." Other rectangles represent the currently considered paths, with the chosen minima underlined. Arrows indicate transitions between paths, with symbols on the arrows denoting the next randomly selected minimum.

With this adjustment, we can prove the following theorem, restricting ourselves to multi-peaked landscapes lacking neutral mutations (which are excluded from consideration here):

Theorem 1. In a bi-allelic *L*-loci system, reciprocal sign epistasis is a necessary condition for the existence of two peaks at a distance *L* in a fitness landscape lacking neutral mutations.

Proof.

(1) Assume there are two maxima at distance *L* in a bi-allelic *L*-loci system.

(2) Consider a random direct path (necessarily of length *L*) between the two maxima.

(3) This path necessarily contains at least one minimum. If multiple minima exist (either having strictly equal or statistically indistinguishable fitness values), choose one at random.

(4) Modify the path by reversing the order of the two mutations leading to and away from this minimum.

(5) Two cases arise:

(a) If the genotype replacing the current minimum has lower fitness than its neighboring genotypes on the path, reciprocal sign epistasis is detected. Note that neighboring genotypes always have different fitness values as neutral mutations are absent.

(b) Otherwise, find the new minimum along the path. If multiple minima exist, randomly choose one. Then return to step (4) for the mutations surrounding this new minimum.

(6) Repeating steps (4) and (5) must eventually break the loop via case (5a), since the minimum fitness along the path is bounded above by the value of the lowest peak.

End of proof.

Taking into account the three numbered arguments given in Appendix of (Poelwijk *et al.*, 2011), we can provide general formulation of Theorem 1:

Theorem 1 (general formulation). In an *N*-allelic *L*-loci system, reciprocal sign epistasis is a necessary condition for the existence of multiple peaks at a distance *L* in a fitness landscape lacking neutral mutations.

The modification added in the proof of Theorem 1 also accelerates the identification of RSE in the proof of Corrected Poelwijk's theorem, when equal-fitness genotypes are absent. For example, by slightly altering the landscape from Fig. 1 so that fitness of genotype K is lower than that of A and H but higher than G (see Fig. S1), RSE involving genotypes A, B, K, and H is detected immediately. In contrast, the original proof (Poelwijk *et al.*, 2011 and Supplementary Materials) proceeds to the path PAKHGEFQ with minimum E and continues searching for RSE.

Alternatively, we can prove Theorem 1 by always choosing the leftmost (or rightmost) minimum. The corresponding proof is provided in Supplementary Materials.

It should be stressed explicitly that Theorem 1 is valid for both strictly equal and statistically indistinguishable phenotypes of distant genotypes. This is because as soon as we do not have neutral mutations, the two adjacent genotypes in the path have distinct phenotypes, thus, making the proof of Theorem 1 be equally applicable for the cases of both strictly equal and statistically indistinguishable phenotypes.

Reciprocal sign epistasis and sign epistasis may be absent in multi-peaked landscapes with strictly neutral mutations

When genotypes with strictly equal fitness are neighbors in sequence space, we have strictly neutral mutations. To be concise, let us consider the minimal fitness landscape that invalidates the original Poelwijk's theorem. This landscape represents a combinatorially complete dataset for three loci, each with two alleles, where local maxima occur at the wild-type P and the triple mutant Q. All intermediate single and double mutants A, B, C, D, E, and F have strictly equal low fitness (Fig. 3A). As can be easily seen, the landscape does not contain RSE: manual inspection of all six 2D combinatorially complete datasets (PACB, PAED, PCEF, ABDQ, CBFQ, and EDFQ) reveals no instances of both reciprocal sign and sign epistasis.

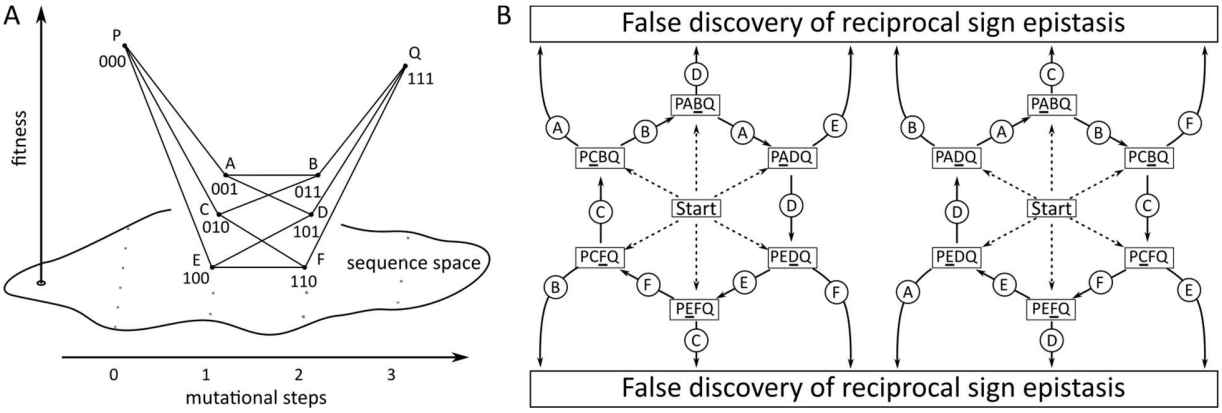

Figure 3. (A) The minimal landscape that invalidates the original Poelwijk's theorem. This landscape is a combinatorially complete dataset for three loci, each with two alleles. The fitness values of the single and double mutants, that is, of genotypes A, B, C, D, E, and F, are strictly equal. (B) Graph of states (rectangles) illustrating the application of the proof of Theorem 1 to the landscape from Fig. 3A. 'Start' refers to the state at step (2) of the proof, where the first path is selected randomly. The end of the process is indicated by two rectangles labeled 'False discovery of reciprocal sign epistasis.' Other rectangles represent currently considered paths, with the chosen minima underlined. Arrows indicate transitions between paths, with symbols on the arrows denoting the next randomly chosen minimum.

The practical application of the original proof (Poelwijk *et al.*, 2011) to the landscape in Fig. 3A is impossible because it does not provide instructions for handling equal-fitness genotypes, and consequently, neutral mutations. Therefore, we should try to apply the proof of Theorem 1 to address equal-fitness genotypes. Choosing a minimum randomly from two minima is illustrated in Fig. 3B. In this scenario, only two outcomes are possible: either the process continues indefinitely through the graph cycles, or it leads to the false conclusion that the landscape contains RSE. If we apply the alternative proof of Theorem 1 from Supplementary Materials, we are immediately forced to incorrectly conclude that PACB, PAED, or PCEF contain RSE.

Thus, Poelwijk's theorem is invalid in the general case when multi-peaked landscapes include strictly neutral mutations. Therefore, only the narrower Theorem 1 remains valid.

Only general RSE is guaranteed for multi-peaked landscapes having strictly neutral mutations

Consider two fitness peaks, P and Q, separated by lower-fitness genotypes (Fig. 3A). Along the pathway between them, fitness initially decreases before being compensated upon reaching genotype Q. In the landscape from Fig. 3A, this compensation is delayed. For example, on the path PEFQ, the first mutation occurs at the first locus (P: 000 to E: 100), followed by a neutral second mutation at the second locus (E: 100 to F: 110). The final mutation at the third locus

compensates for the initial fitness decrease, but only in the context of genotype F (110). Importantly, this does not mean that the mutation at the third locus immediately compensates for the first mutation, since the step from E (100) to D (101) is neutral. Thus, the compensation happens through a "composite" mutation (Zorin *et al.*, 2022) in both the second and third loci together. Reciprocal sign epistasis here is observed only when considering combinations of mutations rather than individually.

For the landscape in Fig. 3A and similar landscapes where two peaks are separated by a valley of other low-fitness peaks, the following logic applies. After selecting any mutant in the valley, we find the complementary composite mutation with respect to the two peaks. The two peaks, this initial mutant, and the mutant with its complementary composite mutation form a group of four genotypes exhibiting distant RSE. Since the initial genotype can be chosen in $2^L - 2$ ways, such landscapes contain $(2^L - 2)/2$ distinct groups of four genotypes showing distant RSE.

However, this logic does not hold generally. For example, fitness values f could be organized as follows: $f(A) = f(C) = f(E) < f(P) < f(B) = f(D) = f(F) < f(Q)$. The general proof strategy is to select a genotype neighboring the lower of the two peaks, then find its complementary mutant. If this mutant has fitness lower than the lowest peak, we have distant RSE. Otherwise, we focus on the sublandscape defined by this complementary mutant as the second peak and repeat the process iteratively. This reasoning leads to the following theorem:

Theorem 2. In a bi-allelic *L*-loci system, general RSE (uniting conventional RSE and distant RSE) is a necessary condition for the existence of two peaks separated by distance *L* in the fitness landscape lacking near-neutral mutations.

Proof.

(1) Assume two maxima at distance *L* in a bi-allelic *L*-loci system. Denote the lower maximum as P and the higher as Q. If the two maxima have equal fitness, arbitrarily label them P and Q. For the proof, only the condition $f(P) < f(Q)$ is required. By redefining Q as M, we have $f(P) < f(M)$.

(2) Take P as the reference genotype. Since P is a peak, all its single mutants have lower fitness than P.

(3) Select genotype A with a single mutation in the first locus relative to P. Note that $f(A) < f(P)$, see (2).

(4) Identify the loci differing between genotypes P and M. Define genotype B as having mutations at all loci except the first one. These mutations form a complementary composite mutation to the mutation in A.

(5) Two cases arise:

(a) If $f(B) < f(P)$ then distant RSE occurs, because:

$$f(A) < f(P) \leq f(M), \text{ therefore, } f(A) < f(M);$$

$$f(B) < f(P) \leq f(M), \text{ therefore, } f(B) < f(M);$$

combined with $f(A) < f(P)$ and $f(B) < f(P)$, genotypes P, A, B, and M form distant RSE.

(b) If $f(B) \geq f(P)$, redefine B as M, reset B, and repeat step (4) on the smaller sublandscape between P and M having one fewer locus.

(6) Repeating steps (4) and (5) must terminate in case (5a) because each iteration reduces the landscape by one locus. Eventually, either (i) distant RSE is found or (ii) the system reduces to two loci, where the complementary mutant is a neighbor of peak P with lower fitness. In the latter case, conditions $f(A) < f(P) \leq f(M)$ and $f(B) < f(P) \leq f(M)$ (see (2)) guarantee conventional RSE.

End of proof.

Analogously to previous theorems, we can provide general formulation of Theorem 2:

Theorem 2 (general formulation). In an *N*-allelic *L*-loci system, general RSE (uniting conventional RSE and distant RSE) is a necessary condition for the existence of multiple peaks separated by distance *L* in the fitness landscape lacking near-neutral mutations.

In landscapes with near-neutral mutations both conventional RSE and distant RSE may not exist

Strictly neutral mutations are not the only challenge because fitness and its proxies are often estimated through measurements that include measurement error. Consequently, each fitness value is associated with, or should be reported with, some standard deviation. Consider the hypothetical landscape shown in Fig. 4, representing a bi-allelic three-loci combinatorially complete dataset. Suppose the fitness values were measured with uncertainties such that the single mutants A, C, and E are statistically indistinguishable from the double mutants B, D, and F, which themselves are statistically indistinguishable from the fitness of the triple mutant Q.

It is necessary to reconsider the concept of a peak since the previous definition (Orr, 2009) applies only to single genotypes defining peak as a ‘sink’ in an adaptive evolution directed graph, where edges point from lower- to higher-fitness neighboring genotypes. Genotype P is clearly a peak, as all its neighbors (A, C, E) have significantly lower fitness, making it a ‘sink’ by this definition. However, Q cannot be definitively identified as a peak because its fitness is statistically indistinguishable from its neighbors (B, D, F). Intuitively, we can only conclude that the second peak lies somewhere within the group Q, B, D, and F (genotypes A, C, E are not included since they have statistically significantly lower fitness than Q). The ‘sink’ concept does not help because movement in the graph from Q to B, D, and F and further, to A, C, and E cannot be prohibited. Therefore, the peak concept must be redefined for landscapes with fitness measurement uncertainty:

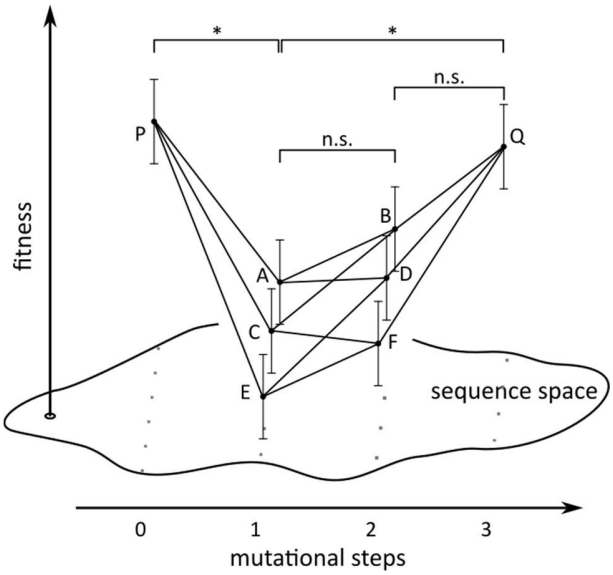

Figure 4. Example of a bi-allelic three-loci landscape where fitness values are measured with some error, indicated by vertical bars. Single mutants A, C, and E have identical fitness that is significantly lower (denoted by '*') than the fitness of genotypes P and Q, but statistically indistinguishable (denoted by 'n.s.') from the fitness of double mutants B, D, and F, which in turn are statistically indistinguishable from the fitness of genotype Q.

Definition: Sort genotypes by fitness from lowest to highest. For each genotype, considered in the sorted order, remove neighboring genotypes with statistically significantly lower fitness. The remaining connected components in the genotype graph define peaks. The number of peaks equals the number of such components.

Applying this definition to our example, removing genotypes A, C, and E (which have significantly lower fitness than P) leaves two connected components, or peaks: {P} and {B, D, F, Q}. Possible recognizing several genotypes as a single peak is an inevitable consequence of measurement uncertainty.

Now that we redefined the concept of peak, we could check for RSE in the two-peak landscape from Fig. 4. If we manually check all six 2D complete datasets (PACB, PAED, PCEF, ABDQ, CBFQ, and EDFQ), we do not observe statistically significant RSE or sign epistasis since mutations between single (A, C, E) and double (B, D, F) mutants are statistically indistinguishable from neutral. If we use concept of composite mutations and check three remaining datasets (PAFQ, PCDQ, and PEBQ), then we do not observe statistically significantly distant RSE or sign epistasis since all mutations from double mutants (B, D, F) to triple mutant Q are statistically indistinguishable from neutral.

Equal-fitness genotypes and neutral mutations are abundant in experimental landscapes

Having theoretically analyzed all relevant cases, we now turn to their practical significance in real biological systems. A key question is how prevalent equal-fitness genotypes are in actual fitness landscapes, particularly the frequency of strictly neutral or near-neutral mutations.

To investigate the abundance of strictly equal-fitness genotypes and, in particular, strictly neutral mutations, we examined a randomly chosen landscape with strongly discrete fitness levels (Adkar *et al.*, 2012). It includes the wild type and most single mutants. In this landscape, the activity of the bacterial toxin CcdB serves as a proxy for fitness. The fitness span eight discrete phenotype levels, from 2 to 9, with the wild-type being at level 2 (Adkar *et al.*, 2012).

To explore statistically indistinguishable genotypes and near-neutral mutations, it is important to use a landscape where experimental measurement error is well characterized. For this, we selected the GFP landscape, where measurement error was reliably estimated by averaging 3645 replicates of the wild type. GFP fluorescence, a proxy for fitness, ranged from 1.28 to 4.12, with the wild-type level at 3.72 and a measurement error of 0.11. This landscape includes the wild type and random mutants carrying between one and fifteen mutations (Sarkisyan *et al.*, 2016).

From these datasets, we calculated (1) the fraction of cases when two genotypes have equal fitness, either strictly equal (CcdB toxin) or statistically indistinguishable (GFP), and (2) the fraction of single mutations being strictly neutral (CcdB toxin) or near-neutral (GFP). We found that 48% of genotype pairs in the CcdB toxin landscape have strictly equal fitness values, while 62% of genotype pairs in the GFP landscape have statistically indistinguishable phenotypes. Regarding neutral mutations, 50% of single mutations are strictly neutral in the CcdB dataset, and 84% are near-neutral in the GFP dataset (Table 1).

Table 1. Number of genotypes with equal or statistically indistinguishable phenotypes.

Landscape	# genotypes	# genotype pairs	# equal-fitness genotype pairs	# single mutations	# neutral mutations
CcdB (Adkar *et al.*, 2012)	1,177	692,076	331,505 (48%)	7,916	3,934 (50%)
GFP (Sarkisyan *et al.*, 2016)	51,715	1,337,194,755	831,518,558 (62%)	34,008	28,450 (84%)

These results reveal that equal-fitness genotypes and neutral mutations are highly abundant in empirical fitness landscapes and ignoring them in any analysis drastically diminishes the value of the obtained results.

Discussion

The relationship between types of epistasis and fitness landscape properties is a key question in evolutionary biology. The deterministically unavoidable presence of RSE in multi-peaked landscapes, proclaimed by Poelwijk *et al.* (2011), has been the most notable example. However, Poelwijk's proof considered only landscapes lacking equal-fitness genotypes, particularly neutral mutations. This significantly limits the relevance of Poelwijk's theorem given the abundance of neutral mutations in experimental landscapes. Indeed, for the CcdB and GFP landscapes analyzed here, equal-fitness phenotypes accounted for 48% and 62%, with neutral mutations being even more prevalent at 50% and 84%. We revisited Poelwijk's statement and demonstrated that when distant equal-fitness genotypes occur in the landscape, RSE still occurs but requires modification of the original proof of Poelwijk's theorem. For strictly neutral mutations in multi-peaked landscapes, only general RSE (uniting conventional RSE and distant RSE) can be guaranteed, which requires application of the composite mutation approach (Zorin *et al.*, 2022). In contrast, near-neutral mutations in such landscapes may make it principally impossible to detect any type of RSE or sign epistasis.

Why did Poelwijk *et al.* not address the possibility of identical phenotypes arising from different genotypes in their paper? It is unlikely they were unaware of neutral mutations, as a previous study noted that "neutral mutations do not play an essential role …" when simulating pathways using mutation data (Poelwijk *et al.*, 2006). More likely, their original proof aligns with the paradigm that identical phenotypes cannot exist and that phenotypes can be measured with arbitrary high precision. From an idealized perspective, this paradigm can be justified: with infinite resources, experiments could be repeated enough times to detect statistically significant differences even between initially identical phenotypes, let alone very similar but distinct ones. From the same perspective, the discreteness of experimental fitness landscapes can be seen as artificial, often resulting from technical limitations that force experimenters classify protein variants into a few bins. However, not all discrete fitness values stem from these limitations; for instance, fitness defined as the number of offspring in a generation naturally produces a highly discrete range of values, and estimating it for humans cannot be replicated, at least for ethical reasons. In practice, many phenotypes in experimental landscapes have been measured only a few times, often just once. This highlights that scientific research often proceeds under conditions where phenotypic measurement accuracy is far from perfect. Consequently, if an experimenter relies on Poelwijk's theorem to search for RSE in a landscape where it may not be found in principle, their efforts may be futile.

The framework of considering single mutations in different combinations at once as one "mutation", which was so useful here, is called composite mutations (Zorin *et al.*, 2022). It does not bring much from the evolutionary point of view, since in the course of evolution the probability of multiple mutations in particular loci during one generation is prohibitively low (Ivankov *et al.*, 2014). However, from the chemical point of view both single and multiple mutations are just changes in the chemical structure of proteins. Composite mutations give opportunity to analyze

adaptive landscapes at any distance, discovering distant epistasis along with conventional epistasis, thus maximizing the information about epistasis (Zorin *et al.*, 2022). Based on the effective algorithm for finding combinatorially complete datasets (Esteban *et al.*, 2019) it is possible to show that composite mutations approach dramatically expands the repertoire of combinatorially complete datasets and allows detection of distant epistasis (Zorin *et al.*, 2022).

Obviously, Poelwijk *et al.* did not mean composite mutations in their study (Poelwijk *et al.*, 2011), as they explicitly refer to “single mutations” throughout the paper. Moreover, we did not find a way to put composite mutations into the original proof instead of single mutations. For example, it is completely unlikely to mean composite mutations when making “changing the order of mutations leading to the current minimum” (Poelwijk *et al.*, 2011).

It is also important to comment on the case where the landscape is analyzed at the gene level, that is, where the locus corresponds not to a single position in the protein sequence but to an entire gene. In this context, a single mutation represents a transition from one gene variant to another, which may differ by numerous amino acid substitutions as well as insertions and deletions. However, despite these multiple changes within the gene, such a mutation is not considered composite in a conventional analysis. Indeed, the compositeness of a mutation is determined not by the nature or extent of the changes it involves, but by the method of analysis – specifically, when single mutations in all different combinations are probed to find all combinatorially complete datasets in the protein sequence space.

It should be noted that we are not the first to recognize that Poelwijk’s proof overlooks neutral mutations and equal-fitness phenotypes. Indeed, in a 2022 study, Saona *et al.* extended Poelwijk’s work by analyzing multi-peaked landscapes using Morse theory, explicitly stating that their investigation is valid only in case of strictly neutral mutations (Saona *et al.*, 2022).

At first glance, the landscape in Fig. 3A may appear to exhibit compensation because fitness decreases after the first mutation but returns to a high level as the trajectory approaches the second peak. However, this pattern can occur even in the absence of epistasis, for example, when one of the two single mutations is deleterious and the double mutant has fitness equal to the wild type. According to standard evolutionary terminology, the term “compensation” is reserved exclusively for cases of reciprocal sign epistasis and is not applied in this context.

It is important to note influence of the symmetry between single and double mutants in the landscapes presented in Figs. 2A and 3A: all single mutants have identical fitness values, as do all double mutants. Due to this symmetry, the absence of reciprocal sign epistasis automatically implies the absence of sign epistasis, since sign epistasis that is not reciprocal is an asymmetric phenomenon.

The abundance of neutral mutations in experimental fitness landscapes highlights the practical relevance of our work, rather than it being a purely theoretical exercise in mathematical rigor for a hypothetical case. Though, given the mathematically strict level of analysis set by Poelwijk *et al.* (2011), our findings are worth sharing even in a “would-be” hypothetical context.

By the way, the fact that neutral mutations outnumber equal-fitness phenotypes suggests some degree of correlation within the landscapes (Kaufmann and Levin, 1987) and indicates that the landscapes do not follow the "house-of-cards" model (Gillespie, 1983).

To summarize, while Poelwijk's theorem established an important link between RSE and multi-peaked fitness landscapes, its original proof applies only to landscapes lacking equal-fitness genotypes and particularly neutral mutations. Given the widespread presence of neutral mutations and equal-fitness phenotypes in empirical landscapes, the theorem's direct applicability is drastically limited. However, with modifications to the proof, conventional RSE can still be demonstrated when distant equal-fitness genotypes exist. At the same time, in the presence of strictly neutral mutations only general RSE (conventional RSE plus distant RSE) can be guaranteed and it may become even impossible to find any type of RSE and sign epistasis for landscapes containing near-neutral mutations. This highlights the practical importance of revisiting and refining the theorem for real-world experimental contexts where measurement precision is limited and phenotypic replication may be infeasible. Furthermore, the concept of composite mutations offers a broader analytical framework for epistasis. Overall, our findings emphasize the need for careful interpretation of epistasis in complex, realistic fitness landscapes and caution against uncritical application of Poelwijk's theorem in empirical studies.

Materials and Methods

Two landscapes were downloaded from the Supplementary Materials of the corresponding papers (Adkar *et al.*, 2012; Sarkisyan *et al.*, 2016). The code for getting numbers in Table 1 is available at https://github.com/ivankovlab/neutral_mutations.

The number of possible pairs of genotypes was calculated as $n \cdot (n-1)/2$, where n is the number of genotypes in a landscape.

For estimating the fraction of genotype pairs statistically indistinguishable at 0.05 level in GFP landscape (Sarkisyan *et al.*, 2016) we assumed that all measured phenotypes have one and the same standard deviation σ. This standard deviation equaling $\sigma \approx 0.11$ we took from the measurement of 3645 wild-type replicates. Then, for any two phenotypes f_1 and f_2 we calculated absolute value of their difference $d = |f_2 - f_1|$ with its standard deviation $\sigma_d = \sigma\sqrt{2}$ and concluded that the phenotypes are statistically indistinguishable if $d/\sigma \leq 1.96$.

Data availability

Python code for processing landscapes and generating numbers in Table 1 is available at https://github.com/ivankovlab/neutral_mutations.

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