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REVIEW

How chromosomal inversions reorient the evolutionary process

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Abstract

Inversions are structural mutations that reverse the sequence of a chromosome segment and reduce the effective rate of recombination in the heterozygous state. They play a major role in adaptation, as well as in other evolutionary processes such as speciation. Although inversions have been studied since the 1920s, they remain difficult to investigate because the reduced recombination conferred by them strengthens the effects of drift and hitchhiking, which in turn can obscure signatures of selection. Nonetheless, numerous inversions have been found to be under selection. Given recent advances in population genetic theory and empirical study, here we review how different mechanisms of selection affect the evolution of inversions. A key difference between inversions and other mutations, such as single nucleotide variants, is that the fitness of an inversion may be affected by a larger number of frequently interacting processes. This considerably complicates the analysis of the causes underlying the evolution of inversions. We discuss the extent to which these mechanisms can be disentangled, and by which approach.

KEYWORDS
adaptation, balanced polymorphisms, chromosomal rearrangements, inversions, linkage, neutrality, recombination, selection

For author affiliations see page 15

1 | INTRODUCTION

Inversions are structural mutations that reverse the sequence of a chromosomal segment and have been found in numerous organisms (Griffiths et al., 2020; Hoffmann & Rieseberg, 2008; Kirkpatrick, 2010; Stefansson et al., 2005; Sturtevant, 1926; Wellenreuther & Bernatchez, 2018). Their main property is that they reduce the effective rate of crossing over (but not gene conversion) in heterokaryotypes (i.e., diploid individuals carrying two different chromosomal arrangements, an inverted chromosome [I] and a non-inverted, 'standard' chromosome [S]). Because they suppress recombination when heterozygous, inversions introduce an effective barrier to genetic exchange *between* standard and inverted chromosomes at the population level. By contrast, recombination is normal *within* standard and *within* inverted homokaryotypes (Crown et al., 2018; Korunes & Noor, 2019; Navarro et al., 1997; Rozas & Aguadé, 1994).

The dual role of inversions as mutations and as recombination modifiers has major implications for evolutionary processes. Like other mutations, inversions can have direct fitness effects (e.g., at the chromosomal breakpoints). Moreover, as modifiers of recombination, they are subject to indirect selection on the rate of recombination. For example, inversions can promote adaptation by 'capturing' beneficial alleles at multiple loci and keeping them together within arrangements in heterokaryotypes (Charlesworth & Charlesworth, 1973; Dobzhansky, 1948, 1949, 1950; Kirkpatrick & Barton, 2006). They can also play a role in speciation by enabling the accumulation of mutations that cause reproductive isolation (Dagilis & Kirkpatrick, 2016; Fuller et al., 2018; Navarro & Barton, 2003a, 2003b; Noor et al., 2001; Rieseberg, 2001; White, 1978). Moreover, inversions can promote divergence between evolving sex chromosomes (Charlesworth, 1991; Connallon et al., 2018; Kirkpatrick, 2010).

Here, we provide a comprehensive review of how selection affects the evolution of inversions in light of recent progress in population genetic theory, genomic analyses, and empirical studies. Due to limited space, we do not discuss inversions on sex chromosomes or sex linkage in detail; similarly, the role of inversions in speciation is only mentioned in passing.

Section 2 summarizes the theory and gives a systematic overview of the sources of selection affecting inversions. We emphasize that inversions are influenced by many evolutionary processes and that they are unlikely ever to be selectively neutral. In particular, the reduced recombination conferred by inversions can have multiple consequences: decreased effective population size causing increased genetic drift; the 'capture' of beneficial and/or deleterious alleles at multiple loci when a new inversion arises on a specific haplotype; increased genetic hitchhiking; and an accelerated accumulation of weakly deleterious mutations.

Section 3 reviews the empirical evidence and discusses the extent to which different processes affecting the evolution of inversions can be disentangled when confronting data with predictions. Since inversions are affected by a large number of 'partial causes' (cf. Frank, 2022), many of which may co-occur and interact, the analysis of inversions is challenging.

As a counterpoint to some of these challenges, Section 4 discusses promising methodological approaches and opportunities – including state-of-the-art genomic and genetic tools – for identifying adaptive inversions and dissecting the underlying mechanisms.

We end by offering some cautionary conclusions about our understanding of the causal mechanisms affecting the evolution of inversions and the limits of population genetic inference in Section 5.

2 | HOW SELECTION ACTS ON NEW INVERSIONS: A SUMMARY OF THE THEORY

2.1 | Overview

Like all other mutations, inversions are subject to both genetic drift and selection. A long history of theoretical work has developed scenarios for the establishment, spread and long-term fate of inversions (reviewed in Charlesworth, 2023; Charlesworth & Barton, 2018; Charlesworth & Flatt, 2021; Connallon & Olito, 2022; Durmaz et al., 2020; Faria, Johannesson, et al., 2019; Hoffmann et al., 2004; Hoffmann & Rieseberg, 2008; Kapun & Flatt, 2019; Kirkpatrick, 2010; Kirkpatrick & Barton, 2006; Schaal et al., 2022).

The fate of a newly arisen inversion depends on the values of standard population genetic parameters, especially its net fitness relative to the population as a whole (Figure 1). Inversions differ from variants like single nucleotide mutations by the larger variety of processes that affect their fitness. The long-term fate of an inversion may be quite different from what is suggested by its initial characteristics; for example, an initial advantage can be eroded by other processes, notably by the accumulation of deleterious mutations within an arrangement (Berdan, Blanckaert, et al., 2021; Connallon & Olito, 2022; Jay, Tezenas, et al., 2022; Lenormand & Roze, 2022; Nei et al., 1967; Olito et al., 2022).

When a new inversion arises, it will trap a block of the genome, and the inverted arrangement may have a net fitness that is either greater or less than that of the population as a whole. An initial advantage or disadvantage may be due to any of the many processes that generate genetic variance in fitness (Figure 1). Deleterious mutations tend to lead to an ultimate selective disadvantage in a new inversion; other processes, such as gene flow between divergent populations and fluctuating selection, may maintain a higher variance in fitness, and may therefore be more likely to drive the initial increase in frequency of new inversions (discussed in detail below). Note that, given the unavoidable initial association with background mutations, it is unlikely that a new inversion will be selectively neutral (Connallon & Olito, 2022; Jay, Tezenas, et al., 2022; Lenormand & Roze, 2022; Nei et al., 1967; Olito et al., 2022; see also below).

The most obvious consequence of an inversion is that it drastically reduces the effective rate of recombination between the different arrangements. It is therefore influenced by all the processes that mediate indirect selection on recombination. Simple models (e.g., equilibria with multiple loci subject to balancing selection and epistatic fitness interactions) tend to select against modifiers

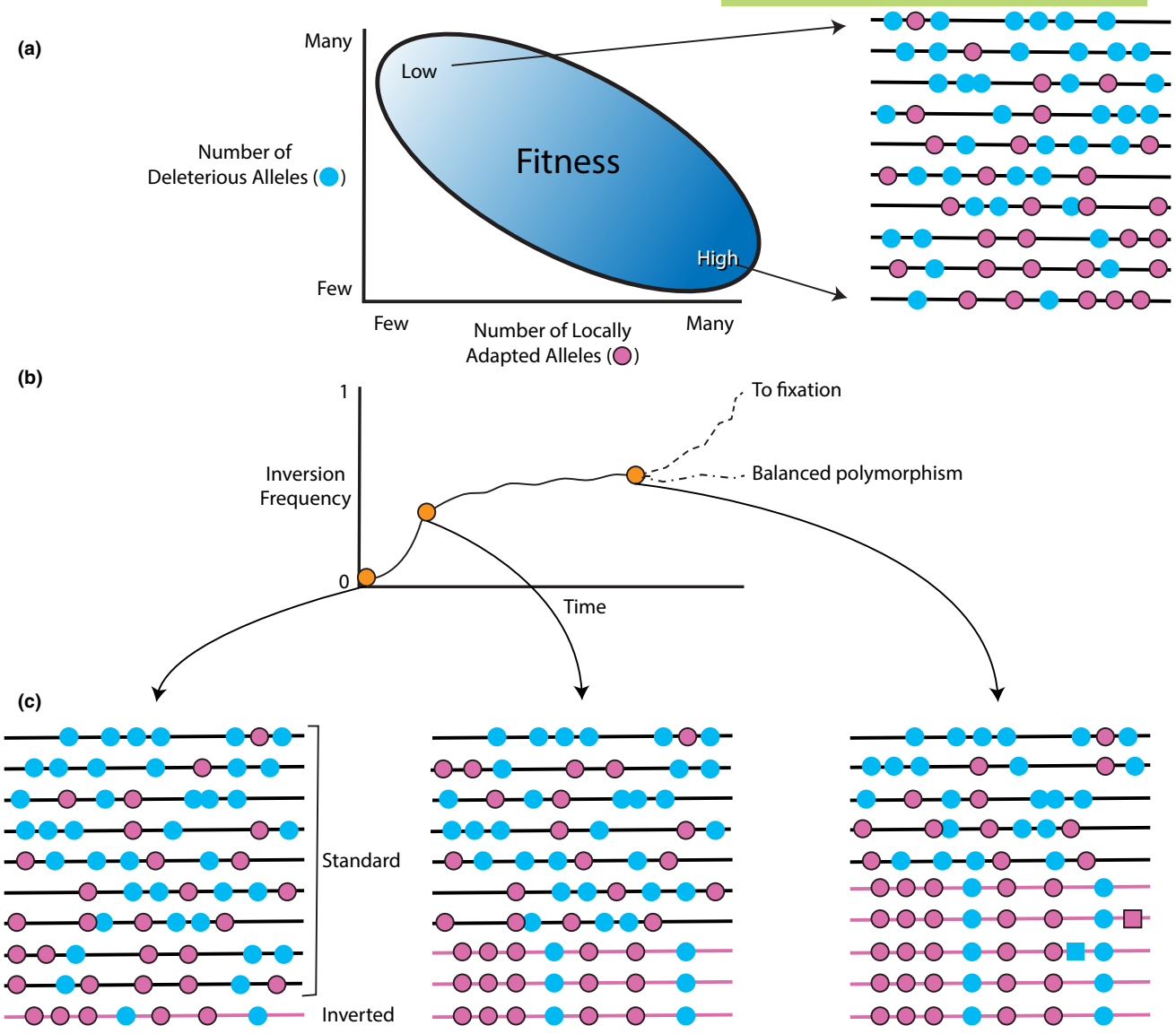

FIGURE 1 An example is the 'life history' of a positively selected inversion. (a) Distribution of haplotypes in a population, with variable numbers of deleterious (blue) and locally adapted (pink) alleles. The fitness variance per unit map length (i.e., per centimorgan) is the key parameter that determines the chance that an inversion establishes and the speed at which it does so. When this variance is high, there is a greater chance for an inversion, especially a large one, to capture a haplotype with unusually high fitness ('large' inversions are those that span a relatively 'large' segment of a chromosome). There is evidence for a substantial variance in fitness in natural populations (Bonnet et al., 2019, 2022; Buffalo & Coop, 2019; Charlesworth, 2015; Gardner et al., 2005). (b) If an inversion captures a block of the genome whose fitness is greater than that of wild-type haplotypes, the new arrangement will increase in frequency under selection (Charlesworth & Charlesworth, 1973; Charlesworth & Flatt, 2021). With free recombination, an unusually fit set of alleles will be broken up, losing its advantage. However, the inversion preserves the fit combination, allowing it to continue to increase in frequency. As the new arrangement becomes common, deleterious recessive mutations or balanced polymorphisms may hold it an at intermediate frequency (Charlesworth & Charlesworth, 1973; Ohta, 1971; Wright & Dobzhansky, 1946). Alternatively, if the inversion carries locally adapted alleles, it might be pushed to high frequency or fixation (Charlesworth & Barton, 2018; Kirkpatrick & Barton, 2006; Mackintosh et al., 2022). Thus, after a rapid initial increase phase, the inversion will equilibrate at a frequency determined by the balance between its initial advantage, the recessive load that it carries, and its intrinsic effects (e.g., on meiosis; see main text). Over the following generations, the karyotypes continue to evolve through the accumulation of deleterious mutations and/or adaptive alleles. (c) Hypothetical samples of standard and inverted chromosomes at three time points. New mutations are shown as squares in the later time point. For details see text (cf. Faria, Johannesson, et al. (2019) for further discussion of inversion 'life history').

that increase recombination and are expected to favour inversions (Charlesworth & Charlesworth, 1973). Yet most eukaryotes have significant levels of recombination in most parts of their genomes, implying that there must be selective processes that favour recombination; these have been widely discussed in the literature (Otto & Lenormand, 2002; Roze, 2021). Inversions, however, have much more drastic effects on effective recombination rates than the recombination modifier alleles postulated in standard models so that they are likely to overwhelm any counter-selection for increased recombination.

2.2 | Sources of selection on newly arisen inversions

2.2.1 | Structural problems with meiosis

Selection can act against an inversion due to the production of duplication/deletion gametes from crossing over in inversion heterokaryotypes, unless female meiosis is ordered in such a way that these gametes fail to pass into the egg nucleus and there is no crossing over in males, as in 'higher' Diptera (Roberts, 1976; Sturtevant & Beadle, 1936). However, two-strand double crossovers or gene conversion (Chovnick, 1973) in heterokaryotypes can occur without generating deleterious products, thereby providing recombinational exchange among arrangements (i.e., so-called 'gene flux'). In most organisms, it remains unclear how the deleterious consequences of crossing over in heterokaryotypes can be avoided, although direct examination of the fertility of heterokaryotypes shows that this occurs in some cases (Coyne et al., 1991, 1993; Stathos & Fishman, 2014; cf. also Koury, 2023 and discussion therein).

2.2.2 | Inversion breakpoint and position effects

The disruption of genes induced by inversion breakpoints, changes in gene expression caused by changes in gene position, order and orientation, or expression level differences due to changes in chromatin architecture, can also lead to direct selection on inversions. In most cases, such effects are expected to be deleterious. More rarely, under the 'adaptive breakpoints' scenario (see below; Figure 2), a new inversion may induce a beneficial mutation at the breakpoints or positively affect gene expression by a 'position effect' (Corbett-Detig, 2016; Krimbas & Powell, 1992; Sperlich & Pfriem, 1986; Villoutreix et al., 2021; Wright & Schaeffer, 2022).

2.2.3 | Interchromosomal effects of inversions on crossing over

The reduction in the rate of crossing over within the inverted region in a heterokaryotype is often associated with an increased rate of crossing over on other chromosomes, and in regions that are sufficiently distant from the inversion on the same chromosome (Lucchesi & Suzuki, 1968; Miller, 2020). Despite numerous examples of this 'interchromosomal effect', the underlying molecular mechanisms remain largely unclear (Miller, 2020). Increases in crossing over associated with heterozygosity for an inversion could create a selective advantage for the inversion due to reduced linkage disequilibrium (LD) among loci under selection, by various mechanisms that have been proposed in models of recombination modifiers (Otto & Lenormand, 2002; Roze, 2021). However, this advantage is likely to be small, since there is free recombination between the inversion and any loci involved in the postulated selective processes. The frequency of nondisjunction for a heterokaryotypic chromosome can also be increased by several percent when *another* chromosome is heterokaryotypic, probably as a result of a non-homologous pairing between chromosomes (Forbes, 1962; Roberts, 1976). Such nondisjunction leads to aneuploidy and hence to a significant fitness loss to the inversion.

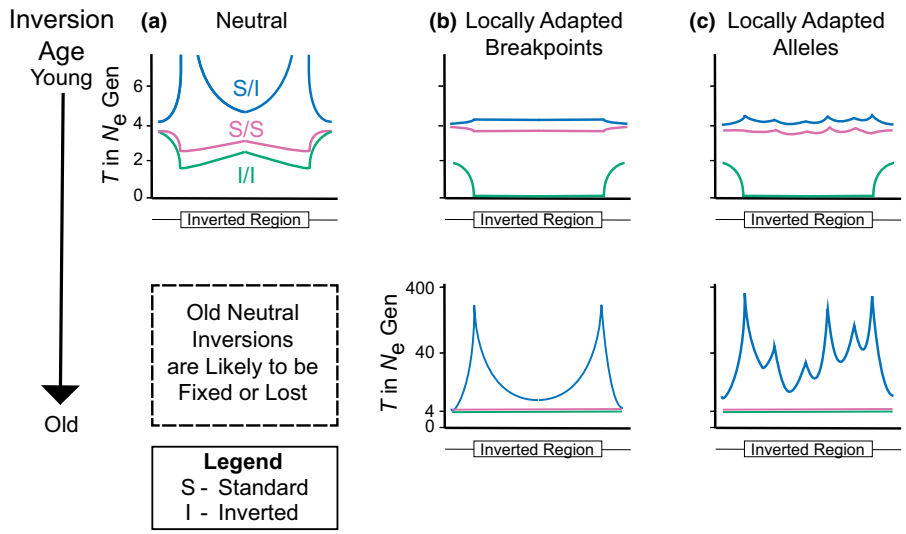

FIGURE 2 Coalescent predictions for the evolution of inversions. Predictions of coalescence times (*T*) for genes sampled across a chromosomal region with an inversion (box on the *x*-axis) under different evolutionary models (redrawn after Guerrero et al., 2012). S/S = standard (non-inverted) homokaryotypes; I/I = inverted homokaryotypes; S/I = heterokaryotypes. The models are neutral (a), 'locally adapted' breakpoints (b), and 'locally adapted' alleles (Kirkpatrick & Barton, 2006) within inverted regions (c) for young (<$4N_e$ generations old; see top) and old (>$4N_e$ generations old; see bottom) inversions. However, other selective mechanisms, e.g., epistatic balancing selection, can lead to adaptive central peaks and generate the pattern shown in (c). In fact, the 'suspension bridge' pattern can be generated under any situation where the inversion is present for a sufficiently long time so that recombinational exchange has affected genetic variability differently in different parts of the inversion. Observing such a pattern can thus not be readily used to distinguish different mechanisms of selection.

2.2.4 | Capture of a haplotype with a fitness effect

An inversion that arises by chance in a haplotype with higher fitness than the mean fitness of the population is expected to have a higher probability of establishment than a neutral mutation, even if the inversion has no direct (positive) effect on fitness (Figure 1). Conversely, an inversion that arises in a haplotype with a substantial fitness disadvantage will have a lower probability of establishment than a neutral mutation and is almost certain to be lost from a large population (Fisher, 1930; Kimura, 1964).

Because of the effective suppression of recombination in a heterozygous individual, strong LD with variants included in the initial inversion haplotype will be maintained over many generations. Since the reduction of recombination often extends beyond the inversion breakpoints, the definition of an inversion haplotype is necessarily somewhat vague, but for population genetic analyses it can be defined as the piece of genome that stays associated with the inverted arrangement until the arrangement is either lost from the population or reaches a sufficiently high frequency so that it behaves deterministically. With random mating, the relevant initial fitness of a new, low-frequency inversion is the mean fitness of the genotypes generated by combining the inversion haplotype with the range of haplotypes present in the population, i.e., its marginal fitness.

An inversion can increase in frequency under selection if the haplotype that it captures has a marginal fitness advantage; by definition, this can occur only in genomic regions where there is variance in the marginal fitnesses of haplotypes (the 'gametic variance' of Ewens, 2004; Figure 1). However, in an isolated randomly mating population at equilibrium for a single locus under balancing selection alone (heterozygote advantage, frequency-dependent selection, etc.), or for multiple such loci that are in linkage equilibrium, the Fundamental Theorem of Natural Selection implies the absence of any additive genetic variance for fitness, and all haplotypes must have the same marginal fitness. No selective effect of an inversion can be generated in this case. Any fitness variance that selects for inversions must therefore result from other sources, such as mutation, gene flow, temporal fluctuations in fitness, or fitness interactions among loci (epistasis). Large gametic fitness variances that could generate a significant initial selective advantage to an inversion can arise from several different processes, described below. These processes all involve capture by the inversion of a haplotype with a fitness effect; they may also involve the subsequent gain of new alleles (Figure 1).

2.3 | Processes generating a selective advantage

2.3.1 | Balancing selection with epistasis

One well-known model of balancing selection, originally due to Dobzhansky, postulates epistatic fitness interactions among polymorphic loci ('epistatic coadaptation'; Charlesworth, 1974; Charlesworth & Charlesworth, 1973; Dobzhansky, 1948, 1949, 1950, 1951). In its simplest form, this mechanism involves two epistatically interacting polymorphic loci, with the double heterozygote having the highest fitness (Charlesworth, 1974; Charlesworth & Charlesworth, 1973; Charlesworth & Flatt, 2021). While epistatic selection induces LD between selected loci, recombination breaks down high-fitness haplotypes, creating gametic fitness variance. A newly arisen inversion that happens to capture such a high-fitness haplotype experiences an indirect advantage because lower recombination in the inversion heterozygotes keeps the inverted arrangement associated with higher fitness. This, in turn, confers an advantage to the inversion heterokaryotype, preventing fixation (Charlesworth, 1974; Charlesworth & Flatt, 2021). This is an instance of the general 'reduction principle': in a randomly mating population multi-locus polymorphisms that are held at equilibrium under any form of balancing selection involving epistatic fitness interactions lead to selection for reduced recombination (Feldman & Liberman, 1986; Zhivotovsky et al., 1994).

The principle of epistatic selection can be extended to include gametic as well as organismal fitness: segregation distorter systems (with their drivers, responders, and drive enhancers or suppressors) select for recombination suppression (Charlesworth & Hartl, 1978; Thomson & Feldman, 1974) and are frequently associated with inversions (Charlesworth & Charlesworth, 2010; see Fuller et al., 2020; Navarro-Dominguez et al., 2022 for recent empirical examples).

2.3.2 | Migration load

Gene flow between populations subject to spatially varying selection can maintain a substantial variance in fitness, at the cost of reduced mean fitness, creating a 'migration load'. By analogy with mutational load, at migration-selection equilibrium, every locally deleterious allele that enters the population by migration must be removed by a selective 'death' (i.e., a failure to reproduce). Thus, mean fitness is reduced by an amount equal to the migration rate. If selection keeps many loci divergent, and if incoming alleles at different loci are eliminated independently, then the migration load is multiplied by the number of divergent loci and can become substantial. Inversions gain an advantage because alleles at multiple loci can be eliminated together (Charlesworth & Barton, 2018; Kirkpatrick & Barton, 2006).

2.3.3 | Mutational load

Mutational load provides another mechanism by which an inversion can acquire a fitness advantage if it captures a haplotype with a lower-than-average number of deleterious mutations and hence a higher-than-average fitness. The effect of mutational load on the fate of new inversions in randomly mating populations has recently been re-examined (Berdan, Blanckaert, et al., 2021; Connallon & Olito, 2022; Jay, Tezenas, et al., 2022; Lenormand & Roze, 2022;

Olito et al., 2022). As originally suggested by Nei et al. (1967), the accumulation of new autosomal deleterious mutations by inversions means that there is little or no increase in net survival probability over neutrality, and there are usually lower than neutral survival probabilities for large autosomal inversions, which are likely to carry several deleterious mutations initially (Connallon & Olito, 2022; Jay, Tezenas, et al., 2022; Olito et al., 2022). This problem is exacerbated by the fact that the suppression of recombination by the inversion means that carriers of the inversion are subject to Hill-Robertson interference (i.e., selection at linked sites reducing the efficacy of selection), thus accelerating the accumulation of slightly deleterious mutations (Berdan, Blanckaert, et al., 2021; Jay, Tezenas, et al., 2022). Overall, therefore, mutational variance in fitness is unlikely to be a significant player favouring the spread of inversions.

A phenomenon related to mutational load that may affect the fate of inversions is associative overdominance (AOD) (Berdan, Blanckaert, et al., 2021; Berdan et al., 2022; Charlesworth & Jensen, 2021; Faria, Johannesson, et al., 2019; Gilbert et al., 2020; Kirkpatrick & Barton, 2006; Ohta, 1971; Ohta & Kimura, 1970; Sturtevant & Mather, 1938; Waller, 2021; Zhao & Charlesworth, 2016). Under AOD, a new inversion captures a haplotype carrying weakly deleterious, partially recessive or recessive mutations at several loci. The deleterious effects of recessive mutations that are absent from the standard arrangement will be masked in the inversion heterokaryotypes (Sturtevant & Mather, 1938). In a sufficiently small population, where mutant alleles at loci other than those at which the inversion is fixed have drifted to high frequencies, a type of 'pseudo-overdominance' or multi-locus heterosis could arise (Pálsson & Pamilo, 1999). In addition, if the inversion had a direct positive fitness effect, or captured beneficial alleles along with the deleterious mutations, and thus reached high frequency, the deleterious alleles would become exposed to selection in the inversion homokaryotypes, preventing the inversion from spreading to fixation (Jay et al., 2021; Kirkpatrick & Barton, 2006). However, it has not yet been shown that AOD can confer sufficient heterotic advantage to a newly arisen inversion for it to establish itself and spread in frequency in a large population.

2.3.4 | Hitchhiking effects

Inversions arising in association with beneficial alleles spreading in the population could be favoured. With an additive fitness model at a single biallelic locus, and selection coefficient of 2*s* for homozygotes for the favoured allele, the total additive variance in fitness over the course of a single gene substitution is 2*s* (Crow & Kimura, 1970). If a substitution takes *T* generations on average, the average variance per generation is 2*s*/*T*. But there are *KT* substitutions in progress at any given time, so the total additive genetic variance contributed by substitutions occurring at rate *K* per genome per generation is approximately 2*Ks*. There is much uncertainty concerning the distribution of selection coefficients involved in adaptive evolution; however, analyses of population genomic data on *Drosophila* suggest that these are mostly very small, of the order of 10^{-3} or less, and that the per genome rate of adaptive substitutions is likely to be approximately 0.01 per generation, and certainly less than 0.1 (Campos et al., 2017; Elyashiv et al., 2016; Sella et al., 2009). If these estimates are approximately correct, the potential contribution of new, beneficial mutations to additive fitness variance is negligible. Of course, there is always a chance that an inversion could capture an advantageous mutation with a sizeable advantage, which results in the inversion being hitchhiked to a high frequency and leading to a loss of linked variation.

2.3.5 | Factors leading to balanced inversion polymorphism

There are many circumstances in which an inversion might be maintained as a balanced polymorphism within a population, rather than becoming fixed by drift or selection. For example, as already discussed, epistatic coadaptation confers a multi-locus heterozygote advantage to the heterokaryotype. Other forms of balancing selection that could be involved in maintaining inversion polymorphisms include situations where fitnesses are variable, for example when fitnesses depend on the frequencies of the different genotypes (negative frequency-dependent selection) or when they vary in space or time (Faria, Chaube, et al., 2019; Faria, Johannesson, et al., 2019; Haldane, 1948; Kapun et al., 2023; Kapun, Fabian, et al., 2016; Kapun & Flatt, 2019; Kirkpatrick & Barton, 2006; Krimbas & Powell, 1992; Sperlich & Pfriem, 1986; Westram et al., 2021, 2022). Beyond numerous cases of clinically varying inversion polymorphisms maintained by spatially varying selection (references above; cf. Section 3), Dobzhansky famously observed pervasive temporal fluctuations in the frequencies of inversion polymorphisms in natural and laboratory cage populations of *Drosophila pseudoobscura* that are consistent with fluctuating selection (Dobzhansky, 1943; Dobzhansky, 1948; Wright & Dobzhansky, 1946; also cf. Kapun, Fabian, et al., 2016; Kapun & Flatt, 2019; Machado et al., 2021).

Several types of fluctuating selection may maintain inversion polymorphism but the conditions for this to occur can be quite stringent. For instance, genetic variance in fitness is generally greatly increased when a quantitative trait under stabilizing selection responds to a change in environment and experiences directional selection towards a new optimum or a fluctuating optimum (Charlesworth, 1993; Zhang, 2012). These situations can be selected either against or for inversions (or other modifiers that reduce recombination), depending on variables such as the speed at which selective optima change and the periodicity of fluctuations in the optima (Barton, 1995; Charlesworth, 1993).

Under certain conditions, temporal fluctuations in the direction of selection at a single locus can maintain genetic variation (Gillespie, 1973; Haldane & Jayakar, 1963), resulting in additive genetic variance in fitness in a given generation (Eshel & Hamilton, 1984). For biallelic loci subject to such selection, the mean log marginal fitnesses of the two alleles over a long period are the same, so no long-term advantage or disadvantage is expected to accrue for an inversion arising in association with one of the alleles.

However, there could be a temporary advantage to the inversion if the allele in question is currently advantageous and maintains this advantage over several generations.

Fluctuating selection in multi-locus systems with epistatic selection will typically generate selection against inversions or modifiers that reduce recombination, for example, if the sign of linkage disequilibria among the loci fluctuates, or the period of the fluctuations is neither too long nor too short (Barton, 1995; Charlesworth, 1976; Gandon & Otto, 2007). In other cases, suppression (or reduction) of recombination by an inversion could be selected for (Charlesworth, 1976).

2.4 | Effects of inversions on neutral variation

Balancing selection acting on inversions should leave distinctive population genetic signatures at neutral variants associated with the region covered by an inversion, in a similar way to the increase in diversity and LD around a single locus at equilibrium under balancing selection (Hudson & Kaplan, 1988; Kaplan et al., 1988; Zeng et al., 2021; for a recent theoretical treatment see Charlesworth, 2023). If there is some gene flux between arrangements in heterokaryotypes, and if this is more strongly suppressed near the inversion breakpoints (Navarro et al., 1997, 2000), a higher equilibrium degree of divergence between inverted and standard arrangements is expected near the breakpoints as compared with the centre of the inversion (Guerrero et al., 2012; Navarro et al., 1997, 2000; the 'suspension bridge' pattern in Figure 2a,b).

In such cases, selection against alleles that are deleterious when transferred between arrangements might lead to additional peaks of divergence away from the breakpoints, due to reduced flux at linked neutral sites (i.e., a 'suspension bridge' pattern with additional peaks in the centre; Figure 2c). Such a situation might arise, for example, because of epistatic fitness interactions that reduce the fitness of recombinants (Ishii & Charlesworth, 1977), or because of locally adapted alleles associated with the inversion (Guerrero et al., 2012). The resulting peaks of divergence are likely to be centred on the selected loci and maintained despite homogenizing gene flux between the arrangements (Guerrero et al., 2012). The region around the target of selection where one might expect a signal of increased diversity inside the inversion may, however, be very small (Guerrero et al., 2012; Ishii & Charlesworth, 1977).

Even after it has reached its equilibrium frequency under selection, there will be a long period of time during which a new inversion approaches equilibrium with respect to allelic content under mutation, drift and recombination. The initial process of spread eliminates variation within inversion-carrying haplotypes via a partial selective sweep (Charlesworth, 2023; Navarro et al., 2000; Zeng et al., 2021), and the system only slowly approaches the final mutation-drift-gene flux equilibrium, over a time period on the order of the reciprocal of the flux rate. This process is faster for sites that experience higher rates of recombination, as may be the case for sites further away from the breakpoints. Among inversion haplotypes, there will then be much lower variability than among standard haplotypes, especially near the breakpoints. This pattern persists at equilibrium if the inverted arrangement has a lower frequency than the standard arrangement but is reversed if it attains a frequency greater than one-half.

Variability in the population as a whole is thus initially reduced by the spread of the inversion, but gradually increases over time as the two arrangements diverge. If the rate of gene flux is sufficiently low, net variability compared with other regions of the genome is increased as equilibrium is approached, especially at sites where gene flux is low. (Note that both the initial spread of an inversion and the subsequent divergence between arrangements caused by the accumulation of new neutral mutations result in strong LD between SNPs and karyotype.) However, caution should be exercised in taking such patterns as evidence for balancing selection (Guerrero et al., 2012). A neutral inversion that has reached a current frequency as low as 10% has an expected age of approximately N_e generations in a panmictic population with effective population size N_e (Kimura & Ohta, 1973), providing plenty of opportunity for different neutral mutations to accumulate in the two arrangements when the rate of gene flux is low.

The models of how inversions affect patterns of neutral variation discussed above make predictions (cf. Charlesworth, 2023) that are in principle testable using data on sequence variability and divergence (cf. Kapun et al., 2023). However, a major issue is that these models only say something about the age of an inversion, not about the mechanism maintaining it. Moreover, the same patterns can arise from distinct mechanisms that are not easily distinguishable (cf. Charlesworth, 2023; Guerrero et al., 2012; Kapun et al., 2023; for a general discussion see Frank, 2014). Potential clues for distinguishing different processes might come from studies of the distributions of inversion lengths as the lengths of inversions are correlated with their fitness effects and their probability of establishment (Connallon & Olito, 2022; also cf. Cheng & Kirkpatrick, 2019; Corbett-Detig, 2016; Dagilis, 2022; Santos, 1986; Van Valen & Levins, 1968).

3 | CONFRONTING THEORETICAL PREDICTIONS WITH EMPIRICAL OBSERVATIONS

Here we review five common types of empirical observations of inversions and discuss how they match the predictions discussed in Section 2.

3.1 | Variation in fitness between inversion genotypes

We first summarize evidence for three sources of fitness variance between inversion karyotypes: (i) meiotic effects, (ii) breakpoint or position effects, and (iii) capture of haplotypes with fitness effects.

3.1.1 | Meiotic effects

Inversions are expected to disrupt meiosis in heterokaryotypes, thus reducing reproductive success and generating a barrier to gene flow between the two arrangements. This was for many years considered to be their likely contribution to speciation (Rieseberg, 2001; White, 1978), and several examples of underdominant inversions are known (Avelar et al., 2013; Jeffares et al., 2017; Zanders et al., 2014). Such inversions are more likely to be pericentric (pericentric inversions include a centromere; paracentric inversions do not) and, if underdominance is strong, are unlikely to become established, except in very small populations (Coyne et al., 1991; Hoffmann & Rieseberg, 2008; Kirkpatrick, 2010; Kirkpatrick & Barton, 2006). However, if each inversion is only slightly underdominant, several inversions may become fixed between species. In this case one expects that a crossover will occur in at least one of the chromosomes in most meiosis, generating unbalanced gametes and leading to strong underdominance. (Note that this does not apply to *Drosophila* where meiosis is ordered in females and males lack crossing over; cf. Coyne et al., 1991.) Despite some evidence for underdominant inversions, we lack systematic quantification of the frequency of underdominance and the distribution of underdominant effects. Clearly, inversions that spread to fixation, or are maintained as balanced polymorphisms, probably represent a biased subset of all new inversions in which these meiotic effects are weak, but this is difficult to test (Connallon & Olito, 2022; see also Koury, 2023 and discussion therein).

3.1.2 | Breakpoint effects and position effects

A striking example of a breakpoint effect occurs when the breakpoints disrupt genes, essentially knocking them out and leading to a 'half-lethal' state where one of the two homokaryotypes is inviable. Evidence consistent with such a mechanism has been reported in the ruff (Küpper et al., 2016; Lamichhaney et al., 2016) and the fire ant *Solenopsis invicta* (DeHeer et al., 1999; Yan et al., 2020). However, demonstrating that gene disruption is causally responsible for lethality is not trivial; moreover, breakpoint effects are not always deleterious. For example, a deletion that co-occurred in association with an inversion breakpoint in stick insects probably altered their colour pattern, a phenotype under strong selection in this group (Villoutreix et al., 2020). Breakpoints occurring within a gene region can also generate new variants, such as new chimeric genes in *Drosophila mojavensis* (Guillén & Ruiz, 2012), or the new lncRNA gene *U3X* at the breakpoint of the *doublesex* mimicry supergene in *Papilio* butterflies (Komata et al., 2022). Finally, breakpoints can have cascading effects by altering patterns of gene expression in adjacent genes (Fuller et al., 2017), or by changing epigenomic patterns such as the structure of topologically associating domains (TADs) which are fundamental units of three-dimensional nuclear organization (Shanta et al., 2020; Wright & Schaeffer, 2022).

3.1.3 | Capture of haplotypes with fitness effects

Within species, epistatic balancing selection might confer fitness overdominance to an inversion polymorphism (cf. section 2; Charlesworth & Flatt, 2021; Fuller et al., 2020); similarly, recessive deleterious mutations may generate associative or pseudo-overdominance (AOD/POD) which confers heterozygote advantage (cf. Section 2; Sturtevant & Mather, 1938). Increased fitness of the heterokaryotype as compared to the homokaryotypes (i.e., heterokaryotype advantage or heterosis) consistent with such mechanisms has been reported in a wide variety of inversion systems including *Coelopa frigida* seaweed flies (Butlin & Day, 1985; Mérot et al., 2020), *Heliconius* butterflies (Jay et al., 2021), and *Drosophila* (Kapun et al., 2023; Kapun & Flatt, 2019; Krimbas & Powell, 1992). Heterosis maintaining a balanced inversion polymorphism can also result from trade-offs between fitness components or from associations with loci experiencing true overdominance. Additionally, heterosis can arise from mutational load when mutations are somewhat recessive and private to each arrangement. Teasing apart such effects requires detailed experimental assays of fitness components, which are challenging and rare (Durmaz et al., 2018; Kapun, Schmidt, et al., 2016; Lowry & Willis, 2010). These studies often find that multiple processes are co-occurring. For example, in *C. frigida* the *α* arrangement of Cf-*Inv(1)* is associated with high reproductive output later in life but lower egg-to-adult survival, while the β arrangement causes lower reproductive success but has a higher egg-to-adult survival rate. Heterokaryotypes seem to balance these effects, and may make the best of both life-history strategies (Mérot et al., 2020). However, this trade-off in fitness is modulated by two additional processes: selection by variable environments and mutational load reducing egg-to-adult survival in homokaryotypes (Butlin & Day, 1985), but the magnitude of fitness effects caused by mutational load remains to be investigated thoroughly.

Several studies have also sought to quantify segregating load and to examine whether recessive mutations might be masked in heterokaryotypes due to AOD/POD. This can be done by comparing the fitnesses of heterokaryotypes and homokaryotypes, for instance by using diallel (or similar) crossing schemes (Crow, 1993; Simmons & Crow, 1977). Experimental crosses are especially informative when they are performed between isolated populations, as accumulated mutations tend to be private to each population (Butlin & Day, 1985; Pegueroles et al., 2010). Theory predicts that rarer arrangements should carry a greater load; some studies in *Drosophila* are broadly consistent with this expectation (Barnes, 1983; Crumpacker & Salceda, 1969; Dobzhansky, 1947a; Yang et al., 2002). However, the evidence is mixed. For example, one study by Mukai and colleagues found an association between inversions and lethal alleles, whereas another study did not, and differences in non-lethal viability between inverted and standard arrangements were found to be small (Mukai & Yamaguchi, 1974; Watanabe et al., 1976). Associations with lethals may instead reflect recent bottleneck effects; they are unlikely to occur in large populations, since selection against them will overcome the effects of drift.

3.2 | Associations between inversion genotype and environment

Selection due to environmental factors often modulates the fate, frequency and distribution of inversion polymorphisms at different spatial and temporal scales. For instance, a polymorphism can be structured geographically and maintained at the scale of the metapopulation under spatially varying selection (cf. Haldane, 1948). In other cases, an inversion polymorphism can be maintained within populations due to various mechanisms of balancing selection (e.g., microhabitat patchiness, seasonal variation, heterozygote advantage), regulating the relative frequency of the different arrangements (cf. Section 2; Faria, Johannesson, et al., 2019; Westram et al., 2022).

Inversion polymorphisms under spatially varying selection are typically detected by a cline of arrangement frequency along an environmental gradient or by a strong association between arrangement frequencies and contrasting environments. Clines can occur at both small and large scales (Dobzhansky, 1944; Kapun et al., 2023; Kapun, Fabian, et al., 2016; Kapun & Flatt, 2019; Krimbas & Powell, 1992; Schaeffer et al., 2003; Schaeffer & Miller, 1992). Many examples focus on the small scale where clines are maintained in the face of extensive gene flow (Christmas et al., 2019; Huang et al., 2020). For example, many inversions in the marine snail *Littorina saxatilis* show frequency clines across replicated environmental gradients in the intertidal occupied by two ecotypes (Faria, Chaube, et al., 2019; Morales et al., 2019; Westram et al., 2021). Clines of inversion frequencies at large scales follow environmental gradients that can be parallel across continents, such as those observed in *Drosophila* (Anderson et al., 2005; Balanyà et al., 2003; Kapun et al., 2023; Kapun, Fabian, et al., 2016; Kapun & Flatt, 2019; Krimbas & Powell, 1992), *Coelopa frigida* (Mérot et al, 2018), and Atlantic Cod (Kess et al., 2020).

3.3 | Associations with adaptive traits

A handful of studies have begun to investigate the architecture of quantitative, complex adaptive traits putatively associated with inversions through mapping experiments. A common observation in these studies is that quantitative trait loci (QTL) for locally adapted traits map to genomic regions spanned by chromosomal inversions or regions with strongly suppressed recombination indicative of the presence of inversions (Koch et al., 2021; Prapas et al., 2022). Examples include flowering time in the plant *Boechera stricta* (Lee et al., 2017), seed and flower production in the monkeyflower *Mimulus guttatus* (Lee et al., 2016), and coat colour and tail length in *Peromyscus maniculatus* (Hager et al., 2022; Harringmeyer & Hoekstra, 2022). Notably, a large QTL for body size in *Drosophila melanogaster*, a trait showing clinal variation along latitudinal gradients on multiple continents, coincides with the genomic position of the *In(3R)P* inversion polymorphism (Calboli et al., 2003), which has subsequently been experimentally confirmed to affect size (Durmaz et al., 2018; Kapun, Schmidt, et al., 2016; Rako et al., 2006).

We do not yet know if such QTLs are composed of one or a small number of large-effect loci or many small-effect alleles that behave as a single large-effect locus (cf. Schaal et al., 2022). However, given that reduced effective recombination is the defining property of inversions and represents a key selective advantage when a new inversion captures a beneficial multi-locus haplotype (cf. Section 2; Charlesworth & Charlesworth, 1973; Charlesworth & Flatt, 2021), an oligo- or polygenic architecture seems most plausible. Indeed, evidence from *B. stricta* (Lee et al., 2017), *M. guttatus* (Lee et al., 2016) and *Heliconius numata* (Jay, Leroy, et al., 2022) suggests the existence of multiple QTL within inversions. Similarly, the proportion of phenotypic variance explained by inversions vs. the collinear genome can be substantial. A recent study in *L. saxatilis* that partitioned additive genetic variance (V_A) for adaptive traits found that approximately half of V_A was inside inversions and half in the collinear genome (Koch et al., 2021, 2022), consistent with the highly polygenic hypothesis (Schaal et al., 2022). Studies in *C. frigida* and *D. melanogaster* also suggest that inversions disproportionately explain variation in quantitative traits (Durmaz et al., 2018; Kapun, Schmidt, et al., 2016; Mérot et al., 2021), which may be consistent with an oligo- or polygenic architecture. Inversions can thus make a major contribution to phenotypic variation.

In some cases, inversions have been inferred to have captured QTLs, whereas in others the inversions might have arisen first and then gained QTLs over time. A handful of studies have found tentative evidence for both mechanisms: Lee et al. (2017), for example, examined an evolutionarily young inversion that controls ecologically important traits in *B. stricta*. To test if existing linked QTLs were captured by the inversion, the authors crossed standard collinear haplotypes from a hybrid zone and found multiple linked QTLs that mapped to the inverted region. These findings are compatible with a scenario whereby the QTL associated with adaptive traits were already segregating in the population before being captured by the inverted haplotype (but cf. Charlesworth & Barton, 2018). Additional evidence for the capture hypothesis comes from a recent detailed study on the *In(3R)P* inversion in *D. melanogaster* (Kapun et al., 2023).

3.4 | Associations with mating patterns

In some situations, selection for non-random mating might favour reduced recombination and drive the spread of a new inversion. In particular, heterosis is predicted to favour the evolution of disassortative mating (Jay et al., 2021; Maisonneuve et al., 2021). In line with this, disassortative mating relative to inversion genotypes has been observed for the strongly heterotic inversion complex Cf-*Inv(1)* in the seaweed fly *C. frigida* (Day & Butlin, 1987; Enge et al., 2023) and *H. numata* (Chouteau et al., 2017). Similarly, the white-crowned sparrow *Zonotrichia albicollis* exhibits two reproductive morphs which are determined by inversion polymorphism whose alternative karyotypes are subject to nearly complete disassortative mating (Tuttle et al., 2016).

While assortative mating is generally much more widespread than disassortative mating (Jiang et al., 2013), there are only a few

examples where it is associated with inversions and more work is needed to confirm these connections. In *Formica* ants, mating in relation to the Sm/Sp supergene is mostly assortative (Avril et al., 2019) which seems to be necessary for the maintenance of the monogyne/polygyne polymorphism (Tafreshi et al., 2022). However, the behavioural basis is unclear since mating in the laboratory is random with respect to karyotype (Avril et al., 2019). Similarly, mating in redpolls, *Acanthis*, is assortative by phenotype, and there is a deficiency of heterokaryotypes, suggesting (but not confirming) assortment by karyotype (Funk et al., 2021).

Inversions may also link mating traits and one or more other traits that are likely to be involved in local adaptation. In *M. guttatus*, an inversion appears to have suppressed recombination between loci influencing life-history traits that contribute to both local adaptation as well as prezygotic isolation (Coughlan & Willis, 2019; Lowry & Willis, 2010). A particularly clear example is the European corn borer, *Ostrinia nubilalis*, where an inversion spanning about 40% of the Z chromosome (~10 Mb) contains both a pheromone receptor locus and a locus contributing to seasonal adaptation of different ecotypes (Kozak et al., 2017; Kunerth et al., 2022).

3.5 | Genomic patterns

3.5.1 | Inversion breakpoints

Inversion breakpoints are often enriched for duplications and repetitive elements, including transposable elements (TEs; Catacchio et al., 2018; Corbett-Detig et al., 2019; Ranz et al., 2007; Richards et al., 2005), and their distribution across the genome seems far from random (Corbett-Detig, 2016; Pevzner & Tesler, 2003). TEs, for example, may accumulate within inversions because of reduced rates of ectopic exchange, lower effective sizes, or increased Hill-Robertson interference due to reduced recombination (Sniegowski & Charlesworth, 1994). (However, note that TEs can cause an inversion in the first place [Aulard et al., 2004; Cáceres et al., 1999; Kent et al., 2017], so there are two distinct effects of TEs – inversion origin and TE accumulation.) Overlapping inversions generally occur due to breakpoint reuse, usually in genomic regions that are prone to mutational bias (Calvete et al., 2012; Guillén & Ruiz, 2012) caused by repetitive sequences and unstable secondary structure or local chromatin environment. Breakpoint reuse is high between several *Drosophila* species (Bhutkar et al., 2008; Fuller et al., 2017; González et al., 2007; Orengo et al., 2019; Puerma et al., 2016), and in mosquitoes (Corbett-Detig et al., 2019) but it has also been found in humans and other great apes (Maggiolini et al., 2020; Porubsky et al., 2021).

3.5.2 | Signatures of mutational load

Mutational load in inversions can be assessed by looking at population genetic signatures in protein-coding genes (e.g., ratios of nonsynonymous to synonymous mutations), or by the frequency of potentially deleterious elements such as TEs. Although these signatures cannot distinguish relaxed purifying selection (and thus increased load) from increased positive selection, an higher ratio of nonsynonymous to synonymous variance or an increased abundance of TEs may be indicative of higher load. (However, we note that the accumulation of TEs might often not be a good indicator of increased load: most insertions are in intergenic regions, and there is evidence that the main reason for their elimination by selection is the production of deleterious arrangements by ectopic exchange; if there is less recombination, there are more TEs Sniegowski & Charlesworth, 1994).

Increased load has been documented for inversions in *Heliconius* (Jay et al., 2021), the fire ant (Stolle et al., 2019), and the white throated sparrow (Jeong et al., 2022; Tuttle et al., 2016). As mutational load should be tied to rates of recombination and to effective population size, polymorphic inversions should carry a larger load than monomorphic ones. More specifically, only rare segregating inversions should accumulate more deleterious mutations. In agreement with this expectation, Huang et al. (2022) found evidence that the same inversions in *Helianthus* sunflowers had a higher load when they were polymorphic vs. locally fixed. Stenløkk et al. (2022), on the other hand, did not find any evidence for accumulation of mutations in Atlantic salmon inversions but attributed this to their young age.

3.5.3 | Patterns of divergence across inversions

Major central peaks of divergence that may be indicative of balancing selection (cf. Section 2) have been identified, for instance, for the clinally varying *D. melanogaster* inversion *In(3R)P* (Fabian et al., 2012; Kapun et al., 2023; Kapun, Fabian, et al., 2016). Similar patterns have been documented for the *2La* and *2Rb* inversions of *Anopheles gambiae* (Cheng et al., 2012; Kirkpatrick, 2017; White et al., 2007), with association mapping revealing several SNPs within these inversions associated with desiccation resistance (Ayala et al., 2019).

However, a major problem with such patterns is that they can arise from processes other than selection (see Section 2; cf. Figure 2). Moreover, if really due to selection, they can be caused by distinct forms of balancing or divergent selection that cannot be readily distinguished (see Kapun et al., 2023). Similarly, peaks at the breakpoints can result from direct selection at the breakpoints or from neutrality, with these processes being difficult to tell apart (Charlesworth, 2023; Guerrero et al., 2012). Thus, despite being informative, these cases illustrate the difficulties in distinguishing different evolutionary processes on the basis of patterns that are subject to multiple, partial causes.

3.6 | Multiple patterns and processes per system

As several of the case studies above indicate, the various patterns and processes affecting the evolution of inversions do not act in

isolation. In particular, since large inversions can contain hundreds of genes and affect a wide range of phenotypes (cf. Crow et al., 2020; Durmaz et al., 2018; Kapun, Schmidt, et al., 2016; Wellenreuther & Bernatchez, 2018), multiple processes influencing the evolution of inversions are likely to be occurring simultaneously and/or to be interacting (cf. Nosil et al., 2023).

An excellent example of how evolutionary processes interact in affecting the evolution of inversions comes from *Heliconius*. In *H. numata*, the *P* locus, made up of three overlapping inversions, harbours adaptive QTL for wing colour pattern (Jay et al., 2021; Joron et al., 2011). As the *P* locus shows strict dominance between arrangements (Le Poul et al., 2014), we expect that arrangements would locally fix via positive frequency-dependent selection to mimic the most common warning pattern. However, the dominant mimetic haplotypes exhibit a substantial genetic load (Jay et al., 2021). Due to strongly reduced survival of the homokaryotypes, these haplotypes show fitness benefits only as heterokaryotypes and have highest fitness when rare, i.e., they are subject to negative frequency-dependent selection (Jay et al., 2021). Moreover, individuals carrying inversions at the *P* locus have disassortative mating preferences which further contributes to balancing the inversion frequencies (Chouteau et al., 2017). Thus, only when considering multiple interacting processes does the evolutionary trajectory of this inversion system become clear.

While the issue of multiple causation clearly complicates the analysis of inversions, there are now many promising genetic and genomic approaches that can be used to study how inversions are affected by selection, as we discuss next.

4 | HOW CAN WE DISENTANGLE THE SELECTIVE PROCESSES AFFECTING INVERSIONS?

4.1 | Detection of inversions

The recent switch from unbiased cytogenetic methods to genomic sequencing approaches has improved the detection of inversions, the characterization of their genomic positions, the mapping of their breakpoints, and the determination of breakpoint sequences in a range of model and non-model species (Box S1). This has brought important information about the age, gene content, history, and evolution of specific inversions, mostly large ones with phenotypic and selective effects (Wellenreuther & Bernatchez, 2018). However, detecting inversions with clusters of SNPs in LD will be biased towards the detection of large inversions that have persisted for $>>4N_e$ generations (Guerrero et al., 2012). It is still not clear how often inversion mutations occur in the germline and never increase to appreciable frequency. Answering this question would require high-throughput methods for screening for new chromosomal rearrangements in the germline. Consequently, the focus here is on detecting inversions that have increased to appreciable frequencies in populations.

4.1.1 | Forward genetics: Phenotypes to genotypes

Forward genetics begins with phenotypes that are known or suspected to be under selection and then maps the genomic location of quantitative trait loci (QTL) that contribute to variation in those phenotypes using either controlled crosses between individuals that differ in the phenotypes of interest (i.e., QTL mapping) or samples from wild populations that harbour variation in the phenotypes (i.e., association mapping). Normally this is done by genotyping large numbers of individuals, but a cheaper and faster alternative is to use bulk segregant analysis where pools of individuals with similar (often extreme) phenotypes from crosses or populations are sequenced and compared to pools with the alternative (extreme) phenotypes (Arunkumar et al., 2017; Benowitz et al., 2019; Cheng et al., 2012; Hu et al., 2015).

Forward genetic mapping provides a relatively unbiased approach to asking how often inversions are associated with phenotypic variation because it does not assume a priori that QTL are associated with inversions. Furthermore, it provides information on the amount of variation in a trait that can be explained by association with an inversion. Several forward genetic studies have now shown that loci within inversions contribute substantially to variation in putatively selected phenotypes (cf. Section 3). Directly testing this hypothesis requires identifying the causal variants that underlie the QTLs within the inversions, which is quite difficult, precisely because the rarity of recombinants within inversion heterokaryotypes prevents fine mapping approaches. Depending on the age of the inversion polymorphism and sufficient gene flux, causal QTLs may be in LD with the arrangement while non-causal variants may not. New technologies like CRISPR/Cas may provide possible ways to test if inversion-specific QTLs are functionally significant with respect to selection or just the consequence of the accumulation of neutral SNP variants (see below and Box S1).

4.1.2 | Reverse genetics: Genotypes to phenotypes

Reverse genetics starts with known polymorphic inversions detected with direct methods and uses data on nucleotide variation of inverted and non-inverted chromosomes to test loci for signatures of selection (Fuller et al., 2017; Kapun et al., 2023). The genus *Drosophila* is the classic case where inversions were initially discovered as factors reducing genetic map distances (Sturtevant, 1917) and later were confirmed with polytene chromosome squashes (Dobzhansky & Sturtevant, 1938; Painter, 1934; Tan, 1935). Evolutionary genomic approaches are now being applied to understand which loci may be important in generating phenotypic variation.

4.1.3 | Inferring the ancestry of arrangements

Some species have multiple inversions segregating on a single chromosome, which raises questions about the evolutionary history of

the different arrangements (Dobzhansky & Sturtevant, 1938; cf. table II in Sperlich & Pfriem, 1986 for a comprehensive list). The sequence of inversion events may be important for understanding whether an arrangement is neutral or selected. One can use phylogenetic analysis of genes within the inverted segment to infer the evolutionary history, although selection on some genes and not others can obscure the true history of the arrangements. An alternative and complementary approach is to use the conserved linkage information at the breakpoints within species compared to that of an outgroup species to polarize inversion events (Bhutkar et al., 2008; Fuller et al., 2017; Ma et al., 2006).

4.2 | Identifying locally selected inversions in clines

Inversion polymorphisms often form geographic clines that could be due to mutation and random genetic drift in subdivided populations if the scaled migration parameter Nm is <1, where N is the local effective population size and m is the migration rate. Alternatively, a value of Nm >1 is sufficient to substantially homogenize allele frequencies among geographic populations. Spatially varying selection is implicated when inverted segments of the genome are differentiated among populations, but gene flow homogenizes allele frequencies genome-wide (Berry & Kreitman, 1993; Slatkin, 1985). This approach was used to support selection operating on the inversions in *D. pseudoobscura* (Schaeffer, 2008; Schaeffer et al., 2003; Schaeffer & Miller, 1992). However, estimates of Nm based on F_{ST} are often biased and rarely accurate (Meirmans & Hedrick, 2011; Nei & Maruyama, 1975; Whitlock & McCauley, 1999; also cf. Hoban et al., 2016).

4.3 | Identifying selected loci and testing models of inversion establishment

Resequencing studies including standard and inverted arrangements are needed to test whether data on an inversion polymorphism reject a neutral model (note, however, that inversions are unlikely to be fully neutral; cf. Section 2). The reverse genetics approach identifies inversions but does not tell us whether any genes within the inverted regions affect phenotypes or have been targets of selection. The forward genetics approach identifies QTLs that map within an inverted region but does not identify genes associated with the QTL or whether such genes coincide with signatures of selection.

Tests to identify non-neutral outlier loci have the common feature that they contrast levels of heterozygosity or divergence within and between arrangements in genome-wide screens either in gene regions or in sliding windows. These include test statistics such as F_{ST} (White et al., 2007), elevated frequencies of derived alleles (Fuller et al., 2017), long population-specific branch lengths (Fuller et al., 2017; Yi et al., 2010), parallel divergence (Westram et al., 2016, 2018), SNP associations with clines (Cheng et al., 2012; Kapun et al., 2023; Kapun, Fabian, et al., 2016), Tajima's D/D_{min} ratio (Fuller et al., 2017; Tajima, 1989), or clusters of nucleotides in LD (Box S1). Outlier loci that are selected would be expected to have fixed amino acid differences or be differentially expressed among arrangements. RNA-Seq analyses allow one to test whether loci are differentially expressed among arrangements (Berdan et al., 2021; Crow et al., 2020; Fuller et al., 2016; Kapun et al., 2023; Lavington & Kern, 2017; Said et al., 2018). Differential expression along with SNP divergence in the region provides evidence that a gene might be the target of selection. The expression of genes within the inverted region may have trans-effects whereby loci within the inversion chromosome may alter transcript levels of genes on other non-homologous chromosomes (Berdan, Mérot, et al., 2021; Fuller et al., 2016; Said et al., 2018). This could occur if a differentiated transcription factor on an inverted chromosome alters the expression of a gene on a non-inverted chromosome.

Significant genetic differentiation or LD among the genome sequences of an inversion polymorphism is expected. If differentiation and LD are observed despite gene flux, then this adds support for selection acting on genes within inversions. Estimates of the recombination parameter $4Nr$ for genes or windows along the inverted regions (Chan et al., 2012) are problematic, however, because they assume complete random mating, which may not be the case. If gene flux is low uniformly across the inverted region, then one is unlikely to identify genes associated with selection on the arrangement. On the other hand, if some genes are differentiated and others are not, due to a combination of selection and gene flux in the form of gene conversion, then the differentiated genes may be inferred to represent candidate-selected genes.

The tests described above can in principle identify candidate-selected loci. Coalescent models (Charlesworth, 2023; Guerrero et al., 2012; Navarro et al., 1997, 2000) can provide some guidance for interpreting patterns of divergence within and between inverted chromosomes depending on the age of the polymorphism (see Figure 2). The pattern and organization of genetic differentiation may provide clues that selection may have acted on inversions, but it does not provide insights into causal mechanisms. In addition, detecting reduced levels of variation within an inverted chromosome could be interpreted as either a recent sweep or that the inversion has been maintained at a low effective population size.

4.3.1 | Interpreting data from genome-wide screens

The appropriateness of genome-wide screens for detecting selected loci within inversions has been evaluated with simulation studies (Lotterhos, 2019; Schaal et al., 2022). Schaal et al. (2022) used forward simulations to examine conditions for new locally adapted inversions to establish. The results of their model show that selected inversions tended to be old and large, and the associated traits under selection were polygenic with many genes of small effect contributing to their establishment. These evaluations showed that commonly used methods to detect outliers for genetic differentiation

were able to discriminate between neutral and adaptive inversions, as long as the set of SNPs used for neutral parameterization in the methods was filtered appropriately for LD (Lotterhos, 2019; Schaal et al., 2022). However, these models did not consider gene flux between arrangements, so they could not evaluate the power of methods to uncover selected loci within the inversion.

4.3.2 | The nature of variation in selected genes

The model in Figure 1 suggests that successful inversions capture a majority of alleles that are selectively favourable and a minority of alleles that are deleterious. It is tempting to use tests such as the one of McDonald and Kreitman (1991) or the elevated nonsynonymous to synonymous polymorphism ratio approach of Hughes and Nei (1988). However, the assumptions of these tests are unlikely to hold for inversions and a new inversion will capture random variants with a spectrum of fitness effects. The identification of deleterious alleles is more problematic. There are several approaches that are designed to predict the functionality of amino acid variants based on the chemical properties of ancestral versus derived alleles (Adzhubei et al., 2010; Kircher et al., 2014; Ng & Henikoff, 2001; Pejaver et al., 2020). These methods assume that drastic changes in amino acid properties will be deleterious, which may not be valid. The results of this type of analysis should be interpreted with caution.

4.4 | Mutational analysis of identified candidate loci within inverted regions

Once candidate loci are identified, gene annotations may provide clues about the functions of candidate loci, which need to be tested with functional analyses. Mutational analyses allow one to understand how the genes contribute to phenotypic differences and enable the study of their fitness effects. CRISPR/Cas technology allows precise genetic manipulation of identified selective targets. CRISPR/Cas can alter candidate genes within inverted regions either by knocking them out, switching alleles between ancestral and derived arrangements, or reverting derived inversions to the ancestral form. With all CRISPR/Cas approaches, one must be aware of off-target effects (Zhang et al., 2015). This approach allows one to understand the potential phenotypic and fitness effects of the mutated genes if effect sizes are large enough, but may not help to understand the ecological forces acting on the inversions.

4.4.1 | CRISPR/Cas knockouts

The first application of CRISPR/Cas is to knock out candidate alleles. This approach only shows what happens if one knocks out a gene's function, which may or may not be relevant to allelic differences between inversion types, but is still a good start for finding the possible function of a gene. The effectiveness of using the knockout approach depends on the number of candidate loci. If the number of putative selected genes is small, this approach is feasible, however, as the number of loci needed to be tested increases this approach is untenable.

4.4.2 | CRISPR/Cas knockins

The goal of a knockin mutation is to swap one functional candidate allele for another. While the knockout approach generates a non-functional allele, the knockin approach replaces one functional allele for another (Auer & Del Bene, 2014). Assume that two putatively selected loci A and B have alleles A1 and B1 in the ancestral arrangement and A2 and B2 in the derived arrangement. A knockin experiment might be used to generate the A1 B2 haplotype in the ancestral arrangement and the A2 B1 in the derived arrangement, but this may be very hard to accomplish. The identity of candidate genes may provide clues about the mechanism of selection and inform the kinds of assays used in testing functional significance. For instance, odorant and gustatory receptors are associated with *D. pseudoobscura* inversions (Fuller et al., 2016, 2017) so functional assays could ask whether replacing odorant receptors from one arrangement background to another alters binding of odorants (Hallem & Carlson, 2004).

4.4.3 | Revert inversions to allow the generation of recombinants

Often many loci are likely to be involved in the establishment (or maintenance) of inversions, so that targeting individual loci or combinations of loci with CRISPR/Cas would be time- and cost-intensive. For example, 127 putative-selected loci have been implicated within the 12 Mb Pikes Peak arrangement of *D. pseudoobscura* (Fuller et al., 2019). Dissecting the targets of selection is prohibitive with many genes because of the vast number of knockouts or knockins one would need to screen. In addition, a large number of genes suggests that effect sizes of individual loci are likely to be small making it difficult to detect their effects experimentally. An alternative strategy of CRISPR/Cas technology is to introduce elements such as flippase/flippase recognition target (FLP/FRT) elements that would allow an inversion to be reverted to its ancestral state (Cox, 1988). Said et al. (2018) used available *D. melanogaster* strains with FRT sites near inversion breakpoints of naturally occurring inversions to create synthetic inversions. Alternatively, guide RNAs can be designed to cut on both sides of the inversion so that the repaired chromosome is in the opposite orientation (Schmidt et al., 2020; Schwartz et al., 2020). A recent preprint describes new plasmids capable of inverting segments up to 3 Mb (Stern et al., 2023). Although this approach is not yet possible in many systems, we suggest that this approach offers great promise for the characterization of genes involved in selection on inversions.

In organisms where genetic crosses can be performed, one could then cross the ancestral form with the reverted derived arrangement to generate shuffled combinations of candidate loci. This would enable the fine mapping of loci within the inversions that contribute to phenotypes associated with inversions. Ultimately, this approach would allow one to overlay this fine-mapping data with the original genome scan data to see whether the loci within the inversions that contribute to phenotypic variation are actually the loci under selection.

4.5 | Phenotype-to-fitness methods to test functional significance of candidate loci

A major goal in examining neutral versus selective forces acting on genetic polymorphisms is to link genotypes, phenotypes, and fitness in the wild (Barrett & Hoekstra, 2011). Such links have been made in a few rare cases that do not involve inversions; for example, the genotype–phenotype-fitness map is relatively well understood for pigmentation in deer mice (Barrett et al., 2019) and armour traits in sticklebacks (Barrett et al., 2008; Colosimo et al., 2005; Rennison et al., 2019). With inversions, there are two forms of fitness to consider, that of the karyotypes and that of the allelic genotypes within the arrangements. Two-locus models predict that the establishment and maintenance of an inversion polymorphism is based on the combined fitness contributions of alleles held in LD with arrangements (Charlesworth, 1974; Charlesworth & Hartl, 1978). Teasing apart how the many arrangement-specific alleles interact additively or epistatically to generate overall karyotypic fitness will depend on our ability to interconvert arrangements.

With CRISPR/Cas technology, it may be possible in the future to generate recombinant haplotypes to test whether loci interact additively or epistatically. Synthetic reversion chromosomes that convert a derived arrangement back to its ancestral state will allow the estimation of the fitnesses of recombinant chromosomes similar to the recombinant analysis of three non-overlapping inversions on the sex-ratio meiotic drive chromosome of *D. pseudoobscura* (Fuller et al., 2020). Creating several of these mutated inversions (with small differences) and quantifying phenotypic and fitness changes in relation to the ancestral and derived alleles would allow us to create a map between genotype–phenotype-fitness and directly test whether additive or epistatic interactions (or both) were involved in the inversion's impact on fitness. However, this type of setup will be hard to implement, so an initial step might be to quantify changes in gene expression within the inversion between homo- and heterokaryotypic individuals to test for candidate genes that can be explored later (Fuller et al., 2016; Said et al., 2018). A similar approach was used by Crow et al. (2020) in maize, which allowed them to pinpoint changes in the expression of genes involved in photosynthesis and chloroplast physiology between inverted and non-inverted segments.

Finally, deciding what environmental conditions should be used to determine the fitness effects of karyotypes and their allelic contents will also be challenging. Direct measures of selection and/or the fitness effects of inversions have been conducted in the lab in *C. frigida* seaweed flies (Butlin, Collins, et al., 1982; Butlin & Day, 1984; Butlin, Read, et al., 1982; Mérot et al., 2020) and *D. pseudoobscura* (Beardmore et al., 1960; Dobzhansky, 1947a, 1947b; Wright & Dobzhansky, 1946), under semi-natural conditions in *H. numata* butterflies (Chouteau et al., 2017), and in the wild in *M. guttatus* (Lowry & Willis, 2010). Laboratory studies can provide valuable clues about whether and how selection operates on genic and inversion variation, but understanding how selection works in the wild is the ultimate goal.

5 | CONCLUSIONS

> … in the explanation of a specific case they [the population geneticists] regard it as a defeat to be able to say only that "x could not have been the cause of the observations, y could have been a significant factor, and z certainly played an important causal role." Yet for evolutionary phenomena, with so many weakly determining and interacting causal pathways and with a dependence on historical contingency, that is the best that can be done'.
>
> (Lewontin, 2000, p. 194)

As we have emphasized, the most challenging problem in studying inversions is that multiple, often interacting processes affect their evolution (e.g., Nosil et al., 2023). This multiplicity of influences ('partial causes'; cf. Frank, 2022) makes it difficult to distinguish between different mechanisms acting on inversions, especially since distinct processes might lead to similar or identical patterns (Frank, 2014). Population genomic patterns might in many cases be 'overdetermined' in the sense that several processes can generate the same pattern, for example as may be the case for divergence between inverted and standard karyotypes (see Figure 2 and discussion in Kapun et al., 2023; also cf. Cotto & Day, 2023).

Although this phenomenon of multiple causation is common in evolutionary biology (Frank, 2014, 2022; Lewontin, 2000), it may be exacerbated for inversions as their fitness is affected by a greater number of factors as compared to single nucleotide variants, especially as the suppression of recombination in heterokaryotypes causes extreme levels of non-random associations among variants. And while it is in principle possible to fit different models for the evolution of inversions to genomic data, for instance using Approximate Bayesian Computation (Guerrero et al., 2012; Peischl et al., 2013; Rousset et al., 2014; cf. Nosil et al., 2023 for a recent example), model fitting has major limitations (Frank, 2007, p.87; also cf. Dyson, 2004).

Ultimately, therefore, understanding the causes underlying the evolution of inversions requires careful investigation, ideally combining experiments, field studies, genomics and modelling; even more importantly, it necessitates cautious interpretation and an understanding of the limits of evolutionary inference.

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