The effects of GC-biased gene conversion on patterns of genetic diversity among and across butterfly genomes

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GC-biased gene conversion in butterflies

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Abstract
Recombination reshuffles the alleles of a population through crossover and gene conversion. These mechanisms have considerable consequences on the evolution and maintenance of genetic diversity. Crossover, for example, can increase genetic diversity by breaking the linkage between selected and nearby neutral variants. Bias in favor of G or C alleles during gene conversion may instead promote the fixation of one allele over the other, thus decreasing diversity. Mutation bias from G or C to A and T opposes GC-biased gene conversion (gBGC). Less recognized is that these two processes may–when balanced–promote genetic diversity. Here we investigate how gBGC and mutation bias shape genetic diversity patterns in wood white butterflies (*Leptidea* sp.). This constitutes the first in-depth investigation of gBGC in butterflies. Using 60 re-sequenced genomes from six populations of three species, we find substantial variation in the strength of gBGC across lineages. When modeling the balance of gBGC and mutation bias and comparing analytical results with empirical data, we reject gBGC as the main determinant of genetic diversity in these butterfly species. As alternatives, we consider linked selection and GC content. We find evidence that high values of both reduce diversity. We also show that the joint effects of gBGC and mutation bias can give rise to a diversity pattern which resembles the signature of linked selection. Consequently, gBGC should be considered when interpreting the effects of linked selection on levels of genetic diversity.

Significance statement
Recombination is a process which occurs during the formation of egg and sperm and leads to the assembly of novel chromosomes from maternal and paternal copies. This has widespread implications for the evolution of genetic variants by breaking the physical linkage between selected and neutral sites on the same chromosome. Here we show that a balance between the neutral evolutionary processes of gene conversion and variable mutation rate can result in a similar pattern as widespread selection on linked sites. Consequently, this study adds to our understanding of the complex interaction between selective and neutral forces governing genetic diversity patterns.
Introduction

The neutral theory of molecular evolution postulates that the majority of genetic differences within and between species are due to selectively neutral variants (Kimura 1983; Jensen, et al. 2019). Consequently, the level of genetic variation within populations ($\Theta$) is expected to predominantly be determined by the effective population size ($N_e$) and the mutation rate ($\mu$) according to the following relationship: $\Theta = 4N_e\mu$. Indeed, differences in life-history characteristics (as a proxy for $N_e$) have been invoked as explanations for the interspecific variation in genetic diversity among animals (Romiguier, et al. 2014). Also among butterflies, body size is negatively associated with genetic diversity (Mackintosh, et al. 2019).

Lewontin (1974) noted that the range of observed values of $N_e$ estimated from genetic diversity measures is smaller than the range of census population sizes, $N_c$ (Lewontin’s paradox; Lewontin 1974; Kimura 1983; Nevo, et al. 1984; Frankham 1995). Lower $N_e$ compared to $N_c$ may be caused by more efficient selection and subsequently reduced genetic diversity in large compared to small populations (Corbett-Detig, et al. 2015). In particular, selection affects the allele frequency of linked neutral sites (commonly referred to as linked selection or genetic draft) and reduces their diversity (Maynard Smith and Haigh 1974; Charlesworth, et al. 1993).

However, linked selection in itself is not necessarily the solution to Lewontin’s paradox. It has been noted that $N_e = N_c$ is true only for a population in mutation-drift equilibrium (Galtier and Rousselle 2020). Furthermore, changes in population size may amplify the effects of linked selection and the relative importance of selection and demography is an ongoing debate (Corbett-Detig, et al. 2015; Coop 2016; Kern and Hahn 2018; Jensen, et al. 2019). This debate concerns the fate and forces affecting an allele while segregating in a population. While this is important for resolving Lewontin’s paradox, it only addresses variation in $N_e$, which is but a part of the puzzle of genetic diversity. As noted above, variation in the occurrence of mutations also influences genetic diversity. The general pattern observed is a negative relationship between mutation rate and $N_e$ among species (Lynch, et al. 2016). This may be explained by a selective pressure for reducing the overall mutation rate resulting from the distribution effects of new mutations being dominated by deleterious mutations (Eyre-Walker and Keightley 2007; Lynch, et al. 2016). However, mutation rates vary only over roughly one order of magnitude in multicellular eukaryotes (Lynch, et al. 2016) and appear less important than $N_e$ for interspecific differences in genetic diversity.

Genetic diversity can also vary among genomic regions. The determinants of such regional variation are currently debated, but variation in mutation rate (Hodgkinson and Eyre-Walker 2011; Smith, et al. 2018) and linked selection have both been considered (Cutter and Payseur 2013; Corbett-Detig, et al. 2015). Higher rates of recombination are expected to reduce the decline in diversity experienced by sites in the vicinity of a selected locus. Begun and Aquadro (1992) showed for example that genetic diversity was positively correlated with the rate of recombination in Drosophila melanogaster. Their finding validated the impact of selection on linked sites, previously predicted by theoretical work (reviewed in Comeron 2017). Since then, multiple studies have found a positive association between recombination rate and genetic diversity (e.g. Begun and Aquadro 1992; Nachman 1997; Kraft, et al. 1998; Cutter and Payseur...
The positive correlation between diversity and recombination may, however, be caused by factors other than selection on linked sites. Recombination may for instance be mediated towards regions of higher genetic diversity (Cutter and Payseur 2013), or have a direct mutagenic effect (Hellmann, et al. 2005; Arbeithuber, et al. 2015; Halldorsson, et al. 2019). Additionally, analytical evidence suggests that the interplay between mutation bias and a recombination-associated process, GC-biased gene conversion (gBGC), can increase nucleotide diversity (McVean and Charlesworth 1999). GC-biased gene conversion in itself will – like directional selection – reduce diversity of segregating variants. If we additionally consider the long-term effect of gBGC and the concomitant increase in GC content, then genetic diversity may rise as a consequence of gBGC through increased mutational opportunity in the presence of an opposing mutation bias (McVean and Charlesworth 1999). To fully understand the effects of recombination on genetic diversity we must therefore consider both gBGC and opposing mutation bias, in addition to the much more recognized influence of linked selection. In other words, what relationship do we expect between recombination and genetic diversity in the presence of non-adaptive forces such as gBGC and mutation bias?

To understand the mechanistic origins of gBGC we must first consider gene conversion, a process arising from homology directed DNA repair during recombination. Gene conversion is the unilateral exchange of genetic material from a donor to an acceptor sequence (Chen, et al. 2007). A recombination event is initiated by a double-strand break which is repaired by the cellular machinery using the homologous chromosome as template sequence. If there is a sequence mismatch within the recombination tract, gene conversion may occur (Chen, et al. 2007). Mismatches in heteroduplex DNA are repaired by the mismatch-repair machinery (Chen et al. 2007). Importantly, G/C (strong, S, three-hydrogen bonds) to A/T (weak, W, two hydrogen bonds) mismatches can have a resolution bias in favor of S alleles resulting in gBGC, a process that can alter base composition and genetic diversity (Nagylaki 1983; Marais 2003; Duret and Galtier 2009; Mugal, et al. 2015). Direct observations of gBGC are restricted to a small number of taxa, such as human (Arbeithuber, et al. 2015), baker’s yeast (*Saccharomyces cerevisiae*) (Mancera, et al. 2008), collared flycatcher (Smeds, et al. 2016) and honey bees (Kawakami, et al. 2019). Indirect evidence exists for a wider set of species, including arthropods such as brine shrimp (*Artemia franciscana*) and butterflies from the Hesperidae, Pieridae and Nymphalidae families (Eyre-Walker 1999; Perry and Ashworth 1999; Meunier and Duret 2004; Muyle, et al. 2011; Pessia, et al. 2012; Glémin, et al. 2015; Galtier, et al. 2018).

The strength of gBGC can be measured by the population-scaled parameter $B = 4N_e b$, where $b = ncr$ is the conversion bias, which is dependent on the average length of the conversion tract ($n$), the transmission bias ($c$), and the recombination rate per site per generation ($r$) (Glémin, et al. 2015). This means that we can expect a stronger impact of gBGC in larger populations and in genomic regions of high recombination. Nagylaki (1983) showed that we can understand gBGC in terms of directional selection, i.e. the promotion of one allele over another. This leads
to a characteristic derived allele frequency (DAF) spectrum, in which an excess of W→S alleles- and a concomitant lack of S→W alleles, are segregating at high frequencies in the population. Nevertheless, the overall number of S→W polymorphism is expected to be higher in most species because of the widely observed S→W mutation bias, partially caused by the hypermutability of methylated cytosines in the 5’-CpG-3’ dinucleotide context (Lynch 2007). Preventing the fixation of ubiquitous and possibly deleterious S→W mutations have been proposed as one of the ultimate causes for gBGC (Brown and Jiricny 1987; Birdsell 2002; Duret and Galtier 2009). However, while gBGC reduces the mutational load it may also confer a substitutional load by favoring deleterious W→S alleles (Duret and Galtier 2009; Glémin 2010; Mugal, et al. 2015). This effect has led some authors to describe gBGC as an “Achilles heel” of the genome (Duret and Galtier 2009; Mugal, et al. 2015). Detailed analysis of a larger set of taxonomic groups is needed to understand the prevalence and impact of gBGC. There is also limited knowledge about the variation in the strength of gBGC within and between closely related species (Borges, et al. 2019).

Here, we investigate the dynamics of gBGC in butterflies and characterize the effect of gBGC on genetic diversity. We use whole-genome re-sequencing data from 60 individuals from six populations of three species of wood whites (genus *Leptidea*). Wood whites show distinct karyotype- and demographic differences both within and among species (Dincă, et al. 2011; Lukhtanov, et al. 2011; Dincă, et al. 2013; Lukhtanov, et al. 2018; Talla, Johansson, et al. 2019; Talla, Soler, et al. 2019). This includes, *L. sinapis*, which has the greatest intraspecific variation in diploid chromosome number of any animal, from 2n = 57, 58 in southeastern Sweden to 2n =106-108 in northeastern Spain (Lukhtanov, et al. 2018). Our objectives are threefold. First, we infer the strength and determinants of gBGC variation among *Leptidea* populations. Second, we investigate the patterns of gBGC and mutation bias across the genome, its determinants and association with GC content. Third, we detail the effect of gBGC and opposing mutation bias on genetic diversity across a GC gradient and consider the impact of linked selection and GC content itself as determinants of genetic diversity.

**Results**

**Samples, genome and population resequencing data**

The samples and population resequencing data used in this study were originally presented in Talla, et al. (2017). In brief, 60 male *Leptidea* butterflies from three species and six populations were analyzed. For *L. sinapis*, 30 individuals were sampled: 10 from Kazakhstan (Kaz-sin), 10 from Sweden (Swe-sin) and 10 from Spain (Spa-sin). 10 *L. reali* were sampled in Spain (Spa-re) and 10 *L. juvernica* per population were collected in Ireland (Ire-juv) and Kazakhstan (Kaz-juv), respectively. Reads from all 60 sampled individuals were mapped to a previously available genome assembly of an inbred, male, Swedish *L. sinapis* (scaffold N50 = 857 kb) (Talla, et al. 2017). Detailed information on SNP calling can be found in Talla, Johansson, et al. (2019).
Patterns of gBGC among populations and species

To infer the strength of gBGC in the different Leptidea populations (Figure 1A, B), we calculated separate DAFs per mutation category (GC-conservative/neutral: $S \rightarrow S$ and $W \rightarrow W$, collectively denoted $N \rightarrow N$, GC-changing: $S \rightarrow W$ and $W \rightarrow S$) for segregating non-exonic variants. To polarize alleles, we used invariant sites in one or two outgroup populations (“strict” polarization; Table S1). We used the four basic population genetic models developed by Glemin et al. (2015) to obtain maximum likelihood estimates of the intensity of gBGC ($B = 4N_e b$). Model M0 is a null model with $B$ fixed at 0. In contrast, $B$ is a free parameter in model M1. To correct for polarization errors, we also used extensions of M0 and M1 (M0* and M1*) with one error parameter included per mutation category. The GC content in the ancestral genome was ~0.32. For all populations, the M1 model had a better fit than the M0 model (likelihood-ratio tests (LRT) upper-tailed $\chi^2; \alpha = 0.05; df = 1$), which indicates that gBGC is a significant evolutionary force in Leptidea butterflies (Figure 1B). The quantitative results from the M1 and M1* models were overall congruent, and M1* had a better fit for all populations except Swe-sin (LRT upper-tailed $\chi^2; \alpha = 0.05; df = 3$). When taking all non-exonic sites into consideration and applying model M1*, Spa-rea and Swe-sin had the lowest $B$ (0.21), followed by Kaz-sin ($B = 0.22$). Spa-sin, the population with the largest number of chromosomes (Figure 2B), had a marginally higher $B$ (0.24) compared to the other L. sinapis populations. All these estimates were lower than Irish- (Ire-juv) and Kazakhstani (Kaz-juv) L. juvernica with $B = 0.54$ and $B = 0.79$, respectively (Table S2).

We tried an alternative more “liberal” polarization (only 2 outgroup individuals, see Materials and Methods) to test the impact of the polarization scheme on the estimates from the gBGC model. The results were qualitatively similar but the polarization error rates were inflated compared to the “stricter” polarization scheme (Table S2, Text S1). Thus, we used the “strict” polarization scheme for subsequent analyses unless otherwise stated. We also tested the impact of including and excluding ancestral CpG-prone sites as they may influence the estimation of the $S \rightarrow W$ mutation bias ($\lambda$) and $B$ (Text S1). All populations except Kaz-juv had the highest estimate of $\lambda$ at ancestral CpG-prone sites, followed by all non-exonic sites and lowest when excluding ancestral CpG sites (Table S2). This difference could be caused by hyper-mutagenic methylated cytosines but the level of DNA methylation observed in Lepidopteran taxa is low (Jones, et al. 2018). However, the difference when excluding- and including CpG-prone sites was small (< 0.25) and consequently, we used all non-exonic sites in subsequent analyses.
Figure 1. *Leptidea* butterflies show variation in the genome-wide strength of gBGC. A) Phylogeny of the six *Leptidea* populations included in this study. Node values represent support from 100 bootstrap replicates on sites. The phylogeny in A) is based on a subtree from a maximum-likelihood phylogeny used as a starting tree in Figure 1 of Talla et al. (2017). A mounted specimen of a *Leptidea sinapis* is shown. B) Estimates of the population-scaled coefficient of gBGC (\(B = 4N_e b\)). Circles represent point estimates from the original DAF spectra using model M1*, bars are mean values of \(B\) for the 1,000 bootstrap replicates on segregating sites. Overlaid and opaque violins are bootstrapped values for model M1* and underlain, transparent violins are estimates for model M1.

**Determinants of gBGC intensity variation among populations and species**

The strength of gBGC is dependent on \(N_e\) and the conversion bias \(b = ncr\). Given that transmission bias, \(c\), and conversion tract length, \(n\), require sequencing of pedigrees, we here focus on variation in genome-wide recombination rate, \(r\) to assess variation in \(b\). To understand the relative importance of \(N_e\) and \(r\), we correlated \(B\) with \(\pi\) (as a proxy for \(N_e\)) and diploid chromosome number (as a proxy for genome-wide recombination rate) (Kaback, et al. 1992; Stapley, et al. 2017). Neither genetic diversity, (\(\pi; p \approx 0.13\), adjusted \(R^2 \approx 0.45\)), nor diploid chromosome number (\(p \approx 0.35\), \(R^2 \approx 0.05\)), significantly predicted variation in \(B\) among species in phylogenetically independent contrasts (see insets in Figure 2A, B). Since Spanish *L. sinapis* likely experienced massive chromosomal fission events recently (Lukhtanov, et al. 2011; Talla, Johansson, et al. 2019; Lukhtanov, et al. 2020), it is possible that \(B\) is below its equilibrium value in this population. Excluding Spa-sin yielded a positive relationship between chromosome number and the intensity of gBGC, though above a significance threshold of 0.05 (\(p \approx 0.07\), \(R^2 \approx 0.79\)).
Figure 2 Determinants of variation in the strength of gBGC among populations A) Relationship between $\pi$ and $B$. B) Relationship between diploid chromosome number and $B$ (M1*). Points in B) show lowest and highest estimate of diploid chromosome number for each population. Colors represent the populations shown in Figure 1. Insets in A) and B) show phylogenetically independent contrasts of each respective axis variable based on the phylogeny in Figure 1A. Contrasts for diploid chromosome number were based on midpoint value.

Level of mutation bias varies among Leptidea species

The GC content is determined by the relative fixation of S$\rightarrow$W and W$\rightarrow$S mutations (Sueoka 1962), which is governed by the balance of a mutation bias from S$\rightarrow$W over W$\rightarrow$S, and a fixation bias from W$\rightarrow$S over S$\rightarrow$W. The latter may be caused by gBGC only, but may also be observed at synonymous sites due to selection for preferred codons (Galtier, et al. 2018). Protein coding genes make up only 3.7% of the L. sinapis genome (Talla, Soler, et al. 2019) and potential selection on codon usage will hence only affect genome-wide base composition marginally in this species. Using the DAF spectra of different mutation classes allows not only estimation of $B$, but also the mutation bias, $\lambda$ (Muyle, et al. 2011; Glémin, et al. 2015). We found that $\lambda$ (estimated from model M1*) varied from 2.94 (e.g. Spa-sin) to 4.09 (Kaz-juv) (Table S2). Applying the M1 model gave similar results. It is possible that the polarization scheme which only allowed private alleles for the L. juvernica populations, contributes to their high value of $\lambda$. To test this, we used the aforementioned “liberal” polarization. The resulting $\lambda$ were ~3.5 and ~3 for Kaz-juv and Ire-juv respectively and ~3 for the L. reali and L. sinapis populations, with only minor differences in $\lambda$ between the M1 and M1* models for all populations (Table S2). This indicates that the “strict” polarization scheme shapes the DAF spectrum in a way unaccountable for by the demographic $r_i$ parameters of the model. However, the polarization scheme alone cannot explain the higher $\lambda$ observed in Kaz-juv compared to the other populations (see Text S1 for further discussion).

Patterns and determinants of gBGC and GC content across the genome

To understand the effects of gBGC throughout the genome, we partitioned the polarized SNPs into centiles based on their local (1kb) GC content in the ancestral genome. The number of SNPs in each centile ranged from 2,661 in Ire-juv to 21,140 in Spa-sin (Table S1). The models were compared using LRTs on the average difference of all centiles between the reduced (M0)
and full (M1) model and between the models excluding (M1) or including (M1*) polarization error parameters. M0 could not be rejected in favor of M1 for both Ire-juv and Spa-rea. It is possible that the lower number of SNPs per GC centile in these populations increases variance and thus reduces the fit of the M1 model, especially for Spa-rea which had the lowest \( B \) (Figure 1B). However, both of these populations had a genome-wide significant influence of gBGC, and will still be considered in the following analyses. For all populations, M1* was not significantly better than M1, indicating either a lack of power for M1* or that the polarization error was negligible. The strength of gBGC (\( B = 4N_e b \)) varied across GC centiles for all populations with Swe-sin and Kaz-sin showing the lowest standard error of the mean (0.009, Table 1, Figure 3A, B) and Ire-juv the highest (0.026). Because Ire-juv had the lowest number of SNPs per centile, it’s hard to disentangle sample- from biological variance but we note that Kaz-juv showed a similar standard error (0.025). The average value was overall congruent with what we observed in the analysis among populations (Table S2). We saw similar standard errors for the S\( \rightarrow \)W mutation bias, \( \lambda \) (Table 1, Figure 3C, D).

### Table 1. Estimates of \( \lambda, B \) and relevant measures of GC content.

Population specific averages across GC centiles of \( \lambda, B \), equilibrium GC content under mutational equilibrium alone, \( GC(1/[1+\lambda]) \), and when taking \( B \) into account \( GC(1/[1+\lambda e^{-B}]) \), and the observed GC content in the ancestral genome for the centile with the highest average pairwise difference \( GC(\pi_{max}) \) and lowest density of coding sequence (\( GC \ CDS_{min} \)). We also show standard error of the mean for \( \lambda \) and \( B \).

| Population | \( \lambda \)     | \( B \)     | \( GC \) \((1/(1+\lambda))\) | \( GC \) \((1/(1+\lambda e^{-B}))\) | \( GC \pi_{max} \) | \( GC \ CDS_{min} \) |
|------------|------------------|-------------|-------------------------------|--------------------------------|-------------------|-------------------|
| Swe-sin    | 2.99 ± 0.010     | 0.21 ± 0.009| 0.25                          | 0.29                          | 0.27              | 0.35              |
| Spa-sin    | 2.97 ± 0.008     | 0.21 ± 0.010| 0.25                          | 0.29                          | 0.31              | 0.34              |
| Kaz-sin    | 3.00 ± 0.011     | 0.20 ± 0.009| 0.25                          | 0.29                          | 0.28              | 0.34              |
| Kaz-juv    | 4.15 ± 0.019     | 0.79 ± 0.025| 0.19                          | 0.35                          | 0.29              | 0.34              |
| Ire-juv    | 3.54 ± 0.027     | 0.47 ± 0.026| 0.22                          | 0.31                          | 0.29              | 0.34              |
| Spa-rea    | 2.98 ± 0.012     | 0.16 ± 0.011| 0.25                          | 0.28                          | 0.31              | 0.34              |
Figure 3. Relationship between $B$, $\lambda$, and observed GC content in the ancestral genome. A) Association between $B$ and observed GC content in the ancestral genome for the *L. sinapis-L. reali* clade, and B) for the *L. juvernica* populations. Higher GC content was significantly consistent with greater $B$ in all populations except Spa-re and Ire-juv. C) Relationship between $\lambda$ and GC content was negative for all populations in the *L. sinapis-L. reali* clade. D) Shows the same as C) but for the *L. juvernica* populations. Neither Kaz-juv nor Ire-juv showed significant associations between $\lambda$ and GC content. Lines in plots represent significant linear regressions performed separately per population between the X- and Y variables.

To investigate the impact of variation in $N_e$ across the genome we used genetic diversity, as a proxy for $N_e$ and predictor of $B$, in separate linear regressions for each population (Figure S1A). Swe-sin and Kaz-sin showed significant negative relationships ($p < 0.05$), but limited variance explained ($R^2 \approx 0.1$ for both). The regressions were insignificant ($p > 0.05$) for the other populations (Figure S1A). Overall these results suggest that variation among centiles in $B$ could be dominated by differences in conversion bias, $b$, instead of variation in $N_e$. An observation that supported this conclusion is that $B$ significantly ($p < 0.05$; $R^2$: 4-22 %) predicted GC content in four out of six populations (Figure 3A, B). Here GC content may serve as a proxy for recombination rate, assuming that differences in GC content has been caused by historically higher rates of recombination and thus stronger $B$. That two populations lacked a relationship with GC content may be explained partly by a lack of power for Ire-juv, which had the lowest
number of SNPs per centile, while this explanation is less likely for Spa-rea. Nevertheless, for a majority of the populations considered here we saw a relationship between GC content in the ancestral genome and $B$, indicating that gBGC has been influencing the evolution of GC content.

The mutation bias was significantly ($p < 0.05$, separate linear regression per population) negatively associated with observed GC content in the ancestral genome for all populations except Ire-juv and Kaz-juv (Figure 3C, D). To investigate if there was an association between $\lambda$ and $B$, we performed separate linear regressions per population predicting $\lambda$ with $B$. Higher estimates of $\lambda$ across the genomes were consistent with larger values of $B$ for all populations ($p < 0.05$) except Spa-sin and Swe-sin (Figure S1B). This indicates an inability of the model to separately estimate these parameters or increased $B$ in regions more prone to S$\to$W mutations. The former explanation is unlikely given that the most common sign was negative in the regressions between $\lambda$ and GC content.

**Mutation bias and gBGC influence the evolution of GC content**

The equilibrium GC content in the presence of a S$\to$W mutation bias, but in the absence of gBGC, can be calculated as $1/(1+\lambda)$ (Sueoka 1962). The observed mean GC content was 0.32 for all populations, which is higher than expected under mutational equilibrium alone across almost the entire genome for all populations (Figure 4A). When accounting for gBGC ($1/(1+\lambda e^{-B})$ (Li, et al. 1987; Bulmer 1991; Muyle, et al. 2011), the observed mean GC content was higher than the predicted equilibrium GC content in all populations except Kaz-juv (Table 1; Figure 4B). This means that gBGC in general is not strong enough to prevent GC content from decreasing in all the considered *Leptidea* populations except Kaz-juv.

Segregating variants hold information on the evolution of base composition. GC content will decrease if more S$\to$W than W$\to$S mutations reach fixation and vice versa. We can explore the fate of segregating variants by investigating the skewness of the folded site-frequency spectrum (SFS) (Figure 4C) (Glémin, et al. 2015). GC content is at equilibrium if skewness equals zero, evolves to higher GC content if the skew is positive and decreases if its negative. As expected from the relationship between observed and equilibrium GC content (Figure 4A, B), most of the centiles in all populations had a negative skew, which shows that GC content is decreasing in the *Leptidea* genomes (Figure 4C).
Figure 4. Observed GC content, equilibrium GC content and their association with $\lambda$, $B$ and genetic diversity ($\pi$). A) Observed GC content compared to equilibrium GC content determined by mutation bias ($\lambda$) alone. B) Observed GC content compared to equilibrium GC content when accounting for gBGC. Dotted lines in (A) and (B) represent $x = y$. C) The skewness of the folded SFS shows the strong S\rightarrow W bias in the segregating variation which increases with observed GC content in the ancestral genome. Extrapolating from the distribution of skewness values onto the $y=0$ line serves as a validation of the estimated $\lambda$. Dotted vertical lines represent the GC equilibrium under mutation bias alone, $1/(1+\lambda)$, for each population. D) The association between genetic diversity ($\pi$) and observed GC content. Points in all panels represent GC centiles.

**Pinnacle of genetic diversity close to GC equilibrium**

We found a non-monotonic relationship between GC content and $\pi$ (Figure 4D). The highest genetic diversity was observed close to the predicted genome-wide GC equilibrium, with diversity decreasing in both directions away from equilibrium GC content (Figure 4D). To test if this pattern could result from differential read coverage, we calculated the average read count per base pair in each GC centile per individual (Figure S2). Read coverage was generally even across most of the GC gradient except for two regions around 31 and 35 % GC where the *L. juvernica* populations show a signal consistent with duplications compared to the *L. sinapis* reference genome. Also, the centile with the greatest GC content showed high coverage in all
populations. This is expected given the PCR bias against high and low GC regions in Illumina sequencing (e.g. Browne, et al. 2020). With the exception of \( L. \text{reali} \), the GC content at the centile with the highest \( \pi \), \( GC(\pi_{\text{max}}) \), was at a level between the GC equilibrium defined by \( \lambda \) alone, \( GC(1/(1+\lambda)) \), and equilibrium when accounting for both \( \lambda \) and \( B \), \( GC(1/(1+\lambda e^{B})) \). \( GC(\pi_{\text{max}}) \) was lower for all populations than the GC content of the centile with the lowest density of coding sequence, \( GC(CDS_{\text{min}}) \).

The role of gBGC and mutation bias in shaping genetic diversity

Since gBGC mimics selection, the genetic diversity is directly dependent on the interaction between the strength of gBGC and the potential of an opposing mutation bias (McVean and Charlesworth 1999). To understand how gBGC contributes to genetic diversity in \( \text{Leptidea} \), we estimated the effects of gBGC and opposing mutation bias on genetic diversity by modelling the effect of \( B \) on the SFS (McVean and Charlesworth 1999). In the model, gBGC elevates the relative genetic diversity (\( \pi_{\text{rel}} \)) in the presence of an opposing mutation bias (\( \lambda > 1 \)) by increasing the equilibrium GC content compared to the case when gBGC is absent (\( B = 0 \)). This allows for a greater influx of mutations as long as \( \lambda > 1 \) (Figure 5A). In \( \text{Leptidea} \), genetic diversity (\( \pi \)) showed a non-monotonic relationship along the GC range (Figure 4D). In contrast, given values of \( \lambda \) around 3 and above, relevant for \( \text{Leptidea} \), the model assuming gBGC-mutation-drift equilibrium (GMD) predicts a monotonic increase of \( \pi \) in the 0.2-0.5 GC range (Figure 5A). Using the output from the gBGC inference we could predict \( \pi_{\text{rel}} \) values for each GC centile and population from the GMD model (Figure 5B). The results showed that gBGC and mutation bias has the potential to elevate \( \pi \) compared to \( B = 0 \), by an average of 2.6 % in Spa-rea, 3.3 % in Swe-sin and Kaz-sin, 3.5% in Spa-sin, 8 % in Ire-juv and 14.7 % in Kaz-juv. According to the GMD model this means that at GC equilibrium, gBGC will promote genetic diversity in \( \text{Leptidea} \) butterflies.

We can decompose the GMD model into four spectra standardized by their respective mutational opportunity (Figure 5C) to mimic the empirical data (Figure 5D). For example, the \( S \rightarrow W \) category is standardized by equilibrium GC content. The four spectra include the GC-conservative/neutral spectra (\( W \rightarrow W \) and \( S \rightarrow S \)) and the GC-changing spectra (\( W \rightarrow S \) and \( S \rightarrow W \)) (Figure 5C). The contribution of GC-conservative mutation categories to \( \pi \) are unaffected by equilibrium GC content. In contrast, the influence of \( S \rightarrow W \) on the SFS decreases as \( B \) increases, and vice versa for \( W \rightarrow S \) over the GC range. We also tested the robustness of the model to variation in of \( \lambda \) and \( B \) by drawing values of both parameters from normal distributions in which the standard deviation was determined from observed values (GC centile analysis) from Swedish \( L. \text{sinapis} \) (0.1 and 0.09 for \( \lambda \) and \( B \) respectively, Figure S3). While some variation is evident (especially for the \( S \rightarrow W \) mutation class because of the \( \lambda \) in the numerator, see Materials and Methods), the overall qualitative pattern is unaffected by the estimation error observed in the empirical data.

To understand the role gBGC plays in the variation of \( \pi \) with GC in \( \text{Leptidea} \), we investigated the properties of the DAF spectra separately for all four mutation categories mentioned above.
A majority of the segregating sites were GC-changing and S→W contributed most to $\pi$ across all centiles (Swe-sin: Figure 5D, Others: Figure S4). All mutation classes showed a qualitatively negative quadratic relationship between $\pi$ and GC content (Figure 5D, Figure S4). We therefore suggest that the roughly negative quadratic curves of $\pi$ over GC content is to some degree shaped by factors shared among mutation classes. This means that forces other than gBGC are the main determinants of the relationship between GC content and diversity (c.f. Figure 5C and Figure 5D). Also, we note that observed differences between W→W and S→S diversity ask for a refined GMD model beyond binary states W and S.

Figure 5: A model for genetic diversity under gBGC-mutation-drift equilibrium, predicted $\pi_{rel}$ per population and $\pi$ per mutation category. A) Genetic diversity relative to neutral ($B = 0$) across equilibrium GC content determined by $B$ and $\lambda$. Lines begin at $B = 0$ and end at $B = 8$. The mutation bias is held constant. B) Genetic diversity values predicted from the gBGC-mutation-drift equilibrium model using output from the inference of gBGC. Most of the genome for each population have values of $B$ and $\lambda$ such that their relative strength boosts the long-term genetic diversity compared to $B = 0$. The lower and upper limit of the box correspond
to the first and third quartiles. Upper and lower whiskers extend from the top- and bottom box limits to the largest/smallest value at maximum 1.5 times the inter-quartile range. C) Components of the gBGC mutation drift model. Only results from $\lambda = 3$ are shown. The separate mutation categories were standardized by mutational opportunity while “All” was standardized as in A). The genetic diversity is here assumed to be equal for $N \rightarrow N$ and $W \rightarrow S$ mutations ($\theta_{NN} / \theta_{WS} = 1$). D) Genetic diversity in Swedish $L. sinapis$ measured by average pairwise differences ($\pi$) across genomic GC content for all four mutation categories: $S \rightarrow S$ (SS), $S \rightarrow W$ (SW), $W \rightarrow S$ (WS), $W \rightarrow W$ (WW). The other populations are shown in Figure S4.

The effects of linked selection and GC content on genetic diversity

Having rejected gBGC as a main contributor to $\pi$ along the GC gradient warrants the question: can the pattern be explained by reductions in diversity caused by linked selection? Linked selection has previously been shown to affect genetic diversity in butterfly genomes (Martin, et al. 2016; Talla, Soler, et al. 2019). Selection affecting linked sites will reduce genetic diversity unequally across the genome depending on the density of targets of selection and the rate of recombination. In agreement with this, density of coding sequence (CDS density), which can be used as a proxy for the intensity of linked selection in general but background selection in particular (Lohmueller, et al. 2011), was larger where $\pi$ was lower (Figure 6, Figure S5).

In addition, regional variation in mutation rate will also contribute to a heterogenous diversity landscape. We here suggest that GC content influences mutation rate for three reasons: i) $\pi$ varies conspicuously with GC content (Figure 4D), ii) the $S \rightarrow W$ mutation bias appears to be affected by GC content (Figure 3C), and, iii) GC content has been shown to be a major determinant of the mutation rate at CpG sites in humans (Fryxell and Moon 2005; Tyekucheva, et al. 2008; Schaibley, et al. 2013). Since guanine and cytosine are bound by three hydrogen bonds, one more than for adenine and thymine, it is believed that a higher local GC content reduces the formation of transient single-stranded states (Inman 1966). Cytosine deamination, which leads to $C/G \rightarrow T/A$ mutations, occurs at a higher rate in single-stranded DNA (Frederico, et al. 1993). Thus a higher GC content appears to reduce CpG mutation rates on a local scale of ca 2kb (Elango, et al. 2008). Mutation rate variation determined by local GC content outside the CpG context are less studied but negative correlations have been observed for most mutation classes in humans (Schaibley, et al. 2013).

To understand the relative contribution of GC content and CDS density on variation in $\pi$, we first used quadratic regression analyses separately for each population and mutation category. We started with a model including both linear and quadratic terms for GC content and CDS density as well as an interaction term and performed step-wise reduction of insignificant predictor terms (Table S4). In general, the best fitting model for all populations and categories included linear terms of GC content and CDS density, both showing negative relationships with $\pi$, where GC content was a stronger predictor in most cases (higher absolute value of $t$).

Since GC content and CDS density were correlated with each other, we separated the data into bins (Figure 6A). The GC centiles were placed in five bins of equidistant GC content and variants were separated by mutation category (Figure 6, Figure S5). This allows an investigation of the impact of CDS density on $\pi$ while keeping the GC range constant, and thereby disentangle the relative contribution of GC content and CDS density on variation in $\pi$.  

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The fifth bin was not considered as it included only a single centile with the highest GC content. First, we studied the association between GC content and CDS density (Figure 6A). GC content was negatively associated with CDS density in bin 1 and 2, while bin 3 showed no relationship and bin 4 a positive correlation (Figure 6A). Second, we considered the relationship between \(\pi\) and CDS density for all mutation categories. Here the general trend was negative, across GC bins, populations and mutation categories. In addition, the slopes got more negative with increasing GC content (Figure 6B, Figure S5).

For the GC-neutral mutation categories we observed the steepest negative slope when CDS density and GC content had a positive relationship (Bin 4, Figure 6A, Figure S5). This may be caused by a joint effect of higher local GC content and CDS density contributing to a strong reduction in genetic diversity (Figure 6A, B). Despite a similar spread in CDS density, most populations showed fewer significant trends for bin 2. For Swe-sin the W\(\rightarrow\)W mutation category even showed a positive slope (Figure 6B). This is possibly a result of the negative relationship between GC content and CDS density causing an antagonistic response on diversity. When only GC content varied, \(\pi\) was also reduced for some but not all mutation categories and populations (Bin 3, Figure 6, Figure S5). When CDS density and GC content had a negative relationship, the slope was shallow but lower \(\pi\) was still consistent with a higher proportion of coding sequence (Bin 1, Figure 6). From these results we conclude that both GC content itself and linked selection affect diversity across the genome in *Leptidea* butterflies.

For the GC-changing mutation categories we observed some evidence that gBGC has affected genetic diversity (Figure 6B, Figure S5). The decomposed GMD model – with separate categories standardized for mutational opportunity – predicts that \(\pi\) will increase and decrease monotonically with GC content for W\(\rightarrow\)S and S\(\rightarrow\)W mutations, respectively (Figure 5C). Our results supported this conclusion for the S\(\rightarrow\)W category which showed a more pronounced negative slope compared to the GC-neutral mutation categories (Figure 6B, Figure S5). However, this pattern is also expected from the relationship between \(\lambda\) and GC content (Figure 3C). The W\(\rightarrow\)S mutation category showed slopes comparable to the GC-neutral categories which means that it did not follow the expectation of the GMD model. Linked selection could interact with the distortion of the S\(\rightarrow\)W and W\(\rightarrow\)S DAF spectra caused by gBGC, which would constitute an indirect effect on \(\pi\) by gBGC. An argument against an indirect effect is that linked selection would be weaker or diminish were recombination is the highest, which most likely occur at greater GC content were \(B\) is stronger (see Discussion, Figure 3A, B) (Pouyet, et al. 2018).
Discussion

The intensity of gBGC varies widely among species

In this study, we used whole-genome re-sequencing data from several populations of *Leptidea* butterflies to estimate gBGC and investigate its impact on rates and patterns of molecular evolution. Our data support previous observations that gBGC is present in butterflies (Galtier,
et al. 2018). The genome-wide level of gBGC ($B$) varied from 0.17 - 0.80 among the investigated Leptidea populations. In general, L. juvernica populations had levels of $B$ in line with previous estimates of gBGC in butterflies (0.69 - 1.16; Galtier, et al. 2018), while the other species had lower $B$, more in agreement with what has been observed in humans (0.38) (Glémin, et al. 2015).

**Determinants of gBGC variation in animals**
Regression analysis suggested that the overall strength of gBGC among the Leptidea butterflies may depend more on interspecific variation in genome-wide recombination rate rather than differences in $N_e$. Galtier et al. (2018) also showed a lack of correlation between $B$ and longevity or propagule size (used as proxies for $N_e$), across a wide sample of animals. We observed that chromosome number (a proxy for genome-wide recombination rate) was positively associated with $B$ after excluding Spa-sin, which has recently experienced a change in karyotype. Galtier, et al. (2018) suggested that $B$ may vary among species due to interspecific differences in transmission bias, c. This observation was supported by a study on honey bees (Apis mellifera) showing a substantial variation in transmission bias at non-crossover gene conversion events (0.10 – 0.15) among different subspecies (Kawakami, et al. 2019). Analyses of non-crossover gene conversion tracts in mice and humans showed that only conversion tracts including a single SNP were GC-biased (Li, et al. 2019). By contrast, in yeast the SNP closest to the end of a conversion tract determines the direction of conversion for all SNPs in a tract (Lesecque, et al. 2013). Both these studies suggest that the impact of conversion tract length may be more complex than the multiplicative effect on conversion bias assumed in the $b = ncr$ equation. The relative importance of recombination rate, transmission bias and conversion tract length, in divergence of $b$ among populations and species remains to be elucidated.

**Butterfly population genomics in light of gBGC**
Linkage maps for butterflies with high enough resolution to establish whether or not recombination is organized in hotspots is currently lacking (Davey, et al. 2016; Davey, et al. 2017; Halldorsson, et al. 2019). Nevertheless, recombination varies marginally (two-fold) between- but substantially within chromosomes in two species of the Heliconius genus (Davey, et al. 2017). Related to this, chromosome length is negatively correlated to both recombination rate and GC content in H. melpomene (Martin, et al. 2016; Davey, et al. 2017; Martin, et al. 2019), which is a pattern typical of gBGC (Pessia, et al. 2012). Shorter chromosomes experience on average more recombination events due to the observation of at least one crossover per chromosome (or chromosome arm) per meiosis in most animals (excluding e.g. butterfly females and Drosophila males) (Baker, et al. 1976; Kaback, et al. 1992). This, in turn, leads to a stronger signature of GC-biased gene conversion on shorter chromosomes. The higher GC content at fourfold degenerate (4D) sites on shorter chromosomes in H. melpomene was interpreted to be a consequence of stronger codon usage bias on short chromosomes (Martin, et al. 2016). An alternative explanation is that the higher recombination rate per base pair observed on smaller chromosomes leads to an increased intensity of gBGC and consequently a greater GC content. Galtier et al. (2018) showed significant positive correlation ($r = 0.18-0.39$) between GC content of the untranslated region and the third codon position in genes of three butterflies. This supports the conclusion that gBGC and possibly variation in
mutation bias across the genome, affects codon usage evolution in butterflies. The degree of mutation bias in *H. melpomene* is unknown (as far as we know), but a $\lambda \approx 3$ is possible given that *H. melpomene* has a genome-wide GC content of 32.8 % (Challis, et al. 2017), which is similar to the ancestral *Leptidea* genome and the *L. sinapis* reference assembly (Talla, et al. 2017; Talla, Johansson, et al. 2019). We conclude that assessment of natural selection using sequence data should also include disentangling the effects of potential confounding factors like gBGC, especially in taxa where this mechanism is prevalent (Bolivar, et al. 2016; Pouyet, et al. 2018).

**GC-biased gene conversion, mutation bias and genetic diversity**

Many studies have in the recent decades investigated the association between genetic diversity and recombination rate and have in general found a positive relationship (e.g. Begun and Aquadro 1992; Nachman 1997; Kraft, et al. 1998; Cutter and Payseur 2003; Lohmueller, et al. 2011; Langley, et al. 2012; Cutter and Payseur 2013; Mugal, et al. 2013; Corbett-Detig, et al. 2015; Wallberg, et al. 2015; Martin, et al. 2016; Pouyet, et al. 2018; Castellano, et al. 2019; Rettelbach, et al. 2019; Talla, Soler, et al. 2019). Somewhat later, debates on the determinants of so-called GC isochores in mammalian genomes gave rise to much research on the impact of gBGC on sequence evolution (Eyre-Walker 1999; Eyre-Walker and Hurst 2001; Meunier and Duret 2004; reviewed in Duret and Galtier 2009). In this study we emphasize that gBGC and the widespread opposing mutation bias may also influence variation in genetic diversity across the genome. This can be considered as an extended neutral null model to which the importance of selective forces can be compared.

Several empirical studies have noted the impact of gBGC on genetic diversity. Castellano, et al. (2019) observed that the $\pi$ of GC-changing mutations had a stronger positive correlation with recombination than GC-conservative mutations. Pouyet, et al. (2018) observed that in genomic regions with sufficiently high recombination to escape background selection, GC-neutral mutations were evolving neutrally while $S \rightarrow W$ mutations were disfavored and $W \rightarrow S$ mutations favored. This illustrates an important point that genomic regions where the diversity-reducing effects of background selection may be weakest or absent, are the same regions in which gBGC affects the SFS the most. Consequently, we suggest that future studies on the impact of linked selection also consider the impact of gBGC. A simple solution would for example be to compare observed data with predictions from the GMD model and consider GC-neutral and GC-changing mutations separately (Castellano, et al. 2019).

The impact of gBGC on genetic diversity is dependent on the evolutionary timescale considered. For segregating variants, gBGC can only decrease diversity. If we also consider substitutions and model the evolution over longer timescales, gBGC may indirectly increase genetic diversity. In the GMD equilibrium model, gBGC raises genetic diversity indirectly by increasing GC content, which in turn allows greater mutational opportunity for $S \rightarrow W$ mutations. This can only be achieved when there is a $S \rightarrow W$ mutation bias greater than one and the intensity of gBGC is not too strong. Under identical conditions, gBGC may produce a positive correlation between recombination rate and genetic diversity through an increase in GC content. The impact of this effect will depend on the relative proportion of GC-neutral- and
GC-changing variants. In the GMD model, the diversity of GC-neutral variants is unaffected by GC content. While this is a reasonable null model, it is also a simplistic view in light of the diversity-reducing effects on GC-neutral variants imposed by high GC content observed in our study. GC-neutral variants are only independent of gBGC on the timescale of segregating variation. Over longer timescales gBGC and mutation bias will cause GC-content to evolve towards an equilibrium which may or may not be conducive for GC-neutral mutations.

**Determinants of genetic diversity across the genome**

Identifying determinants of genetic diversity and evaluating their relative importance remains a challenging task. First, we usually lack information on the relationship between GC content and mutation rate due to the sizable sequencing effort required to establish reliable estimates (Messer 2009). Divergence at synonymous sites have been used as a proxy for mutation rate (Martin, et al. 2016; Talla, Soler, et al. 2019), but synonymous divergence is a biased estimator of mutation rate in systems where $B \neq 0$ (Bolivar, et al. 2016). In model organisms, such as humans, it has become feasible to study mutation rates using singletons in massive samples (>14,000 individuals; Schaibley, et al. 2013), or through large-scale sequencing of trios (Jónsson, et al. 2017). Second, the predictor variables of interest are often correlated (e.g. GC content and recombination rate in the presence of gBGC) which complicates interpretation for conventional multiple linear regression approaches (Talla, Soler, et al. 2019). A solution to this problem has been to use principal component regression (PCR) in which the PCs of predictor variables are used as regressors (Mugal, et al. 2013; Martin, et al. 2016; Dutoit, et al. 2017). Using this method, Dutoit, et al. (2017) found that the PC which explained most variation of $\pi$ among 200 kb windows in the collared flycatcher genome was mainly composed of a negative correlation with GC. Martin, et al. (2016) considered 4D sites in *H. melpomene* and found that GC content was less important than gene density. It is likely that synonymous variants show greater impact of background selection compared to non-exonic variants, given the tight linkage between synonymous sites and nonsynonymous sites putatively under (purifying) selection. Instead of PCR we opted for an alternative approach in which the quadratic relationship between GC content and CDS density was binned into separate categories showing differential correlations. For example, in one bin GC content and CDS density showed a clear negative correlation ($k = -0.96$, Bin 2, Figure 6A), and in this bin the genetic diversity was almost invariant. This would suggest that the concordance between the $GC(\pi_{\text{max}})$ and $GC(1/[1+\lambda e^{-B}])$ is a byproduct of the interaction between mutation and linked selection. However, given that $GC(1/[1+\lambda e^{-B}])$ is close to $GC(\pi_{\text{max}})$, the balance between gBGC and opposing mutation bias in these populations is driving GC content to values which favor diversity. In addition, by investigating the GC-neutral and GC-changing mutation categories separately, we could to some extent distinguish the effects of linked selection, from the effects of gBGC. The effects of GC content in itself is harder to separate from gBGC as it may differ between mutation categories resulting in patterns congruent with predictions from the GMD model. For example, the S$\rightarrow$W category showed stronger negative slopes across bins (especially evident in bin 4, Figure 6B) compared to the GC-neutral categories in line with both the GMD model and the observed negative relationship between $\lambda$ and GC content for a majority of the populations (Figure 3C). Nevertheless, the effects of gBGC and mutation bias
on $\pi$ within the GMD model should be interpreted with caution given that it describes $\pi$ at GC content equilibrium while in reality many centiles are some distance away from equilibrium.

Conclusion
In this study, we highlight that gBGC is a pervasive force, influencing rates and patterns of molecular evolution both among and across the genomes of *Leptidea* butterflies. We further emphasize that gBGC shapes genetic diversity and may — through fixation of $W \rightarrow S$ mutations — lead to a concomitant increase in diversity if opposed by a $S \rightarrow W$ mutation bias. This means that positive correlations between genetic diversity and recombination does not necessarily imply that selection is affecting diversity in the genome. Especially if the recombination rate is correlated with GC content, a pattern typical of gBGC. Here, we reject gBGC as a main determinant of diversity in *Leptidea* butterflies but recognizes its impact on diversity along with linked selection and GC content. Our model of how mutation bias and gBGC affect segregating variation provides a part of the puzzle linking the evolution of GC content to genetic diversity.

Materials and methods
Data set
We used 60 male *Leptidea* butterflies from three species and six populations ranging from Kazakhstan in the east to Spain in the west. Further information on parameters used for genotype calling can be found in Talla, Johansson, et al. (2019). Chromosome numbers for each population (if available) or species were obtained from the literature (Dincă, et al. 2011; Lukhtanov, et al. 2011; Šíchová, et al. 2015; Lukhtanov, et al. 2018).

Filtering and polarization of SNPs
Allele counts for each population were obtained using VCFtools v. 0.1.15 (Danecek, et al. 2011). Only non-exonic, biallelic SNPs with no missing data for any individual, and in regions not masked by RepeatMasker in the *L. sinapis* reference assembly (Talla, et al. 2017; Talla, Johansson, et al. 2019), were kept for downstream analyses. The rationale behind excluding exonic SNPs was to minimize the impact of selection on the allele frequencies, and SNPs in repetitive regions were excluded because of the reduced ability for unique read mapping (Sexton and Han 2019), and their higher potential for ectopic gene conversion, which deserve a separate treatment (Roy, et al. 2000; Chen, et al. 2007). Sex-chromosome linked SNPs were considered like any other SNP. The lack of recombination in female meiosis in butterflies (Maeda 1939; Suomalainen, et al. 1973; Turner and Sheppard 1975) and the reduced effective population size ($N_e$, three Z chromosomes per four autosomes [$A$]) cancel out (Charlesworth 2012). This leaves only their relative recombination rate ($r$) affecting intensity of gBGC ($B$), assuming that effective sex ratios are equal, and that conversion tract length ($n$) and transmission bias ($c$) are identical for $Z$ and $A$,

$$B_Z = \frac{3N_en_{CRZ}^2}{3} = \frac{r_Z}{r_A}$$

$$B_A = \frac{4N_en_{CR_A}^1}{2}$$
SNPs were polarized using invariant sites in one or two outgroup populations, again allowing no missing data (Table S1). We denote this polarization scheme “strict”. We also tested a more “liberal” polarization approach where only the individual with highest average read depth per outgroup population was used to polarize SNPs, allowing for one missing allele per individual. Mean read depth per individual was obtained using VCFtools v. 0.1.15 (Danecek, et al. 2011). The liberal polarization scheme was mainly used to test the impact of polarization on estimation of the mutation bias ($\lambda$) of S$\rightarrow$W mutations over W$\rightarrow$S mutations (Table S1). The “strict” polarization was used for all analyses unless otherwise stated. We considered alternative (i.e. not in the reference genome) alleles as the ancestral allele if all outgroup individual(s) were homozygous for that allele (“strict” polarization and “liberal” polarization).

Derived allele frequency spectra of segregating variants were computed for the following categories of mutations; GC-conservative/neutral (S$\rightarrow$S and W$\rightarrow$W, collectively denoted N$\rightarrow$N), strong to weak (S$\rightarrow$W) and weak to strong (W$\rightarrow$S). All alternative alleles inferred as ancestral alleles were used to replace the inferred derived reference allele to make a model of an ancestral genome using BEDTools v. 2.27.1 maskfasta (Quinlan & Hall 2010). This method leverages the information from invariant sites in all sequenced individuals to decrease the reference bias when calculating GC content. However, the ancestral genome was biased towards L. sinapis given that it both served as a reference genome and had more polarizable SNPs than the L. reali and L. juvernica populations (Table S1).

**Inferring GC-biased gene conversion from the DAF spectrum**

To estimate the strength of gBGC, we utilized a population genetic maximum likelihood model (Muyle, et al. 2011; Glémin, et al. 2015), implemented as a notebook in Mathematica v. 12.0 (Wolfram Research 2019). The model jointly estimates the S$\rightarrow$W mutation bias ($\lambda$) and the population-scaled coefficient of gBGC ($B = 4Ne_b$), in which $b$ is the conversion bias. To account for demography, the model introduces a nuisance parameter ($r_i$) per derived allele frequency class ($i$), except singletons, following Eyre-Walker, et al. (2006). The model also estimates the genetic diversity of N$\rightarrow$N and W$\rightarrow$S spectra ($\theta_N$ and $\theta_{WS}$ respectively) and computes an estimate of the skewness of S$\rightarrow$W and W$\rightarrow$S alleles in the folded site frequency spectrum. We applied four of the implemented models, i.e. M0, M0*, M1 and M1*, as the more extended models have large variance without prior information on heterogeneity of recombination intensity at a fine scale (Glémin, et al. 2015), which is currently lacking for Lepidoptera. The M0 model is a null model that evaluates the likelihood of the observed DAF spectrum for a population genetic model without gBGC (i.e. $B = 0$). M1 extends this model by including gBGC via the parameter $B$. M0* and M1* are extensions of M0 and M1, respectively, where one additional parameter per mutation class is incorporated, to account for polarization errors. We analyzed separately all non-exonic sites, and excluding- or including ancestral CpG-prone sites, meaning trinucleotides including the following dinucleotides: CG, TG, CA, NG, TN, CN, NA centered on the polarized variant. N here means either a masked or unknown base. Following Glémin, et al. (2015), we used GC content as a fixed parameter in the maximum likelihood estimation. GC content in the repeat- and gene-masked ancestral genome model was determined by the nuc program in the BEDTools v.2.27.1 suite. Coordinates of
repeats and exons (including introns and UTR regions if available) were obtained from Talla, et al (2017) and Leal, et al (2018), respectively. The number of G and C bases at ancestral CpG-prone sites were computed using a custom script and subtracted from the GC of all non-exonic sites to obtain the GC content for the set excluding ancestral CpG-prone sites.

GC centiles
The polarized non-repetitive, non-exonic SNPs of each population were divided into 100 ranked bins based on local GC content (GC centiles) in the repeat- and exon-masked ancestral genome. This means, all GC centiles represented unequally sized chunks of the genome with equal numbers of polarizable SNPs. The GC content was estimated in 1 kb windows of the reconstructed and repeat- and exon-masked ancestral genome (described above) using BEDTools v. 2.27.1 nuc (Quinlan and Hall 2010), correcting for the number of N bases. To calculate the overall GC content of a centile, we summed the GC content of each 1 kb window. Separate DAFs were created per centile and parameters of gBGC and mutation bias were estimated with the models previously described. We also estimated the genetic diversity per GC centile and population using the average pairwise differences (nucleotide diversity, π), and excluded masked bases when averaging. We calculated π for all sites without any missing data, separately for each population, using 1 as value for the max missing (-mm) parameter in the --site-pi function of VCFtools v. 0.1.15 (Danecek, et al. 2011). We also calculated separate π for polarized sites belonging to the following mutation categories (S→S), (W→W), (S→W) and (W→S) for each population and centile, using a custom function in R (R Core Team 2020), based on the following parameterization,

\[ \pi_{XY}^{obs} = \frac{\sum_{i=1}^{n-1} i(n-i)x_{i}^{XY}}{(n\choose 2)L_X} \]

where \( n \) is the sample size and \( x_{i}^{XY} \) is the number of sites with the \( i \):th derived allele frequency for mutations from \( X \rightarrow Y \) with \( X, Y \in \{W, S\} \); \( L_X \) is the number of AT or GC sites in a certain centile for alleles of W or S origin respectively.

To average \( π \), we used the number of unmasked bases within the range of GC values defined by each centile. The proportion of coding bases (CDS density) was used as a proxy for the impact of linked selection in general, and background selection in particular. CDS density was estimated separately for each population and centile by aggregating the CDS content across all 1 kb windows for a particular centile. A custom-made script was used to assess the impact of read depth on the pattern of \( π \) across GC centiles. This script combined BEDTools v. 2.27.1 (Quinlan and Hall 2010) complement, genomecov and intersect to calculate the read depth per non-masked base pair. Average read-depth per individual and centile was then plotted against GC content to qualitatively assess if the population-specific patterns followed what was observed for the association between \( π \) and GC.

Model of the effect of gBGC and mutation bias on genetic diversity
We consider a model in which the effect of gBGC ($B$) and mutation bias ($\lambda$) determines the level of $\pi$ relative to a reference case where $B = 0$ (McVean and Charlesworth 1999). For this purpose, we first define diversity $\pi$ as the weighted sum of the following mutation categories ($S \rightarrow W$), ($W \rightarrow S$), ($S \rightarrow S$) and ($W \rightarrow W$),

$$\pi = x_{GC}\pi^{SW} + (1 - x_{GC})\pi^{WS} + x_{GC}\pi^{SS} + (1 - x_{GC})\pi^{WW}.$$ 

Then, under the assumption that GC-conservative/neutral mutations are equal and can be summarized by $N \rightarrow N$ diversity,

$$\pi = x_{GC}\pi^{SW} + (1 - x_{GC})\pi^{WS} + \pi^{NN}.$$ 

Next, we let $x_{GC}$ represent the equilibrium GC content determined by gBGC ($B$) and opposing mutation bias ($\lambda$) (Li, et al. 1987; Bulmer 1991; Muyle, et al. 2011),

$$x_{GC} = \frac{1}{1 + \lambda e^{-B}},$$

and introduce relative diversity $\pi_{rel}$ that is standardized for the reference case ($B = 0$).

$$\pi_{rel} = \frac{2\lambda x_{GC} \left( \frac{1}{1 - e^{-B}} + \frac{1}{B} \right) + 2(1 - x_{GC}) \left( \frac{1}{1 - e^{-B}} - \frac{1}{B} \right) + \frac{\theta_N}{\theta_{WS}}}{\frac{2\lambda}{1 + \lambda} + \frac{\theta_N}{\theta_{WS}}}.$$ 

From an empirical perspective this means that $\pi_{rel}$ is the predicted $\pi$ relative to the reference case ($B = 0$) when the observed GC content is at a value determined by gBGC and mutation bias ($1/1+\lambda e^{-B}$). Here, the numerator of the equation for $\pi_{rel}$ consists of three terms each describing the relative contributions of $S \rightarrow W$, $W \rightarrow S$ and $N \rightarrow N$ mutations. GC-changing mutations have a diversity determined by $\lambda$ and $B$, while the contribution of GC-conservative/neutral mutations are affected by the ratio of $N \rightarrow N$ diversity ($\theta_N$) over $W \rightarrow S$ diversity ($\theta_{WS}$). The model assumes gBGC-mutation-drift (GMD) equilibrium.

Fitting the GMD model to data relies on obtaining a neutral reference $\pi$ value unaffected by demographic fluctuations in population size, selection or gBGC. Such a value is unattainable, except for the most well-studied model organisms (Pouyet, et al. 2018). Maximum observed genetic diversity, $\pi_{max}$, could be used as a proxy for neutral diversity which should be reasonable if the entire genome is reduced below the neutral value through linked selection (Torres, et al. 2020). Another approach, which we employ here, is to fit the model without estimating a neutral reference $\pi$. This allows us to estimate how $B$, $\lambda$ and the relative amount of GC-changing mutations affect $\pi_{rel}$ when GC content equilibrium is reached.
For the GMD model, we can also define $\pi_{rel}$ values for the separate mutation categories.

$$\pi_{rel} = x_{GC}\pi_{rel}^{SW} + (1 - x_{GC})\pi_{rel}^{WS} + x_{GC}\pi_{rel}^{SS} + (1 - x_{GC})\pi_{rel}^{WW}$$

This means,

$$\pi_{rel}^{SW} = \frac{2\lambda\left(\frac{1}{1 - e^{-B}} + \frac{1}{B}\right)}{1 + \lambda + \theta_{WS}}, \quad \pi_{rel}^{WS} = \frac{2\left(\frac{1}{1 - e^{-B}} - \frac{1}{B}\right)}{1 + \lambda + \theta_{WS}},$$

$$\pi_{rel}^{SS} = \frac{\theta_{SS}}{2\lambda + \theta_{WS}}, \quad \pi_{rel}^{WW} = \frac{\theta_{WW}}{2\lambda + \theta_{WS}}$$

These equations provide expectations for how the $\pi_{rel}$ of $S \rightarrow W$, $W \rightarrow S$, $S \rightarrow S$ and $W \rightarrow W$ mutations vary with GC content.

**Statistical analyses**

All statistical analyses were performed using R v. 3.5.0-4.0.2 (R Core Team 2020). Linear models and correlations were performed using default packages in R. We analyzed the relative contribution of GC content and CDS density to variation in $\pi$ per mutation category using quadratic regressions:

$$\pi_{oobs}^{XX} \sim \text{GC content} + [\text{GC content}]^2 + \text{CDS density} + [\text{CDS density}]^2 + \text{GC content: CDS density}$$

We performed model reduction such that insignificant predictor terms where dropped until only significant terms remained. Phylogenetic independent contrasts (Felsenstein 1985) were performed using the pic() function in the package ape (Paradis and Schliep 2018). This package was also used to depict the phylogeny in Figure 1A. Other plots were either made using base R or the ggplot2 package (Wickham 2016).

**Data availability**

Raw sequence reads and binary alignment map files (.bam) have been deposited in the European Nucleotide Archive (ENA) under accession number PRJEB21838. Variant-call format file (.vcf) and other essential analyses files will be uploaded to DataDryad and in-house developed scripts and pipelines will be available as a repository on GitHub, (user name: JesperBoman) upon acceptance or earlier per request.
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Author contributions

NB and JB designed research. JB performed data analysis with input from NB and CFM. CFM and JB conducted the modelling work. All authors approved the final version of the manuscript before submission.
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Figure 1. Leptidea butterflies show variation in the genome-wide strength of gBGC. A) Phylogeny of the six Leptidea populations included in this study. Node values represent support from 100 bootstrap replicates on sites. The phylogeny in A) is based on a subtree from a maximum-likelihood phylogeny used as a starting tree in Figure 1 of Talla et al. (2017). A mounted specimen of a Leptidea sinapis is shown. B) Estimates of the population-scaled coefficient of gBGC ($B = 4N_{eb}$). Circles represent point estimates from the original DAF spectra using model M1*, bars are mean values of $B$ for the 1,000 bootstrap replicates on segregating sites. Overlaid and opaque violins are bootstrapped values for model M1* and underlain, transparent violins are estimates for model M1.
Figure 2 Determinants of variation in the strength of gBGC among populations A) Relationship between $\pi$ and $B$. B) Relationship between diploid chromosome number and $B$ ($M_1^*$). Points in B) show lowest and highest estimate of diploid chromosome number for each population. Colors represent the populations shown in Figure 1. Insets in A) and B) show phylogenetically independent contrasts of each respective axis variable based on the phylogeny in Figure 1A. Contrasts for diploid chromosome number were based on midpoint value.
Figure 3. Relationship between $B$, $\lambda$ and observed GC content in the ancestral genome. A) Association between $B$ and observed GC content in the ancestral genome for the *L. sinapis*-*L. reali* clade, and B) for the *L. juvernica* populations. Higher GC content was significantly consistent with greater $B$ in all populations except Spa-rea and Ire-juv. C) Relationship between $\lambda$ and GC content was negative for all populations in the *L. sinapis*-*L. reali* clade. D) Shows the same as C) but for the *L. juvernica* populations. Neither Kaz-juv nor Ire-juv showed significant associations between $\lambda$ and GC content. Lines in plots represent significant linear regressions performed separately per population between the X- and Y variables.
Figure 4. Observed GC content, equilibrium GC content and their association with λ, B and genetic diversity (π). A) Observed GC content compared to equilibrium GC content determined by mutation bias (λ) alone. B) Observed GC content compared to equilibrium GC content when accounting for gBGC. Dotted lines in (A) and (B) represent x = y. C) The skewness of the folded SFS shows the strong S⇒W bias in the segregating variation which increases with observed GC content in the ancestral genome. Extrapolating from the distribution of skewness values onto the y=0 line serves as a validation of the estimated λ. Dotted vertical lines represent the GC equilibrium under mutation bias alone, 1/(1+λ), for each population. D) The association between genetic diversity (π) and observed GC content. Points in all panels represent GC centiles.
Figure 5: A model for genetic diversity under gBGC-mutation-drift equilibrium, predicted \( \pi_{rel} \) per population and \( \pi \) per mutation category. A) Genetic diversity relative to neutral \( (B = 0) \) across equilibrium GC content determined by \( B \) and \( \lambda \). Lines begin at \( B = 0 \) and end at \( B = 8 \). The mutation bias is held constant. B) Genetic diversity values predicted from the gBGC-mutation-drift equilibrium model using output from the inference of gBGC. Most of the genome for each population have values of \( B \) and \( \lambda \) such that their relative strength boosts the long-term genetic diversity compared to \( B = 0 \). The lower and upper limit of the box correspond to the first and third quartiles. Upper and lower whiskers extend from the top- and bottom box limits to the largest/smallest value at maximum 1.5 times the inter-quartile range. C) Components of the gBGC mutation drift model. Only results from \( \lambda = 3 \) are shown. The separate mutation categories were standardized by mutational opportunity while “All” was standardized as in A). The genetic diversity is here assumed to be equal for \( N \Rightarrow N \) and \( W \Rightarrow S \) mutations \( (\theta_N / \theta_{WS} = 1) \). D) Genetic diversity in Swedish L. sinapis measured by average pairwise differences \( (n) \) across genomic GC content for all four mutation categories: \( S \Rightarrow S \) (SS), \( S \Rightarrow W \) (SW), \( W \Rightarrow S \) (WS), \( W \Rightarrow W \) (WW). The other populations are shown in Figure S4.
Figure 6: Relationship between π, CDS density and GC content. A) shows the relationship between CDS density and GC content for Swe-sin in four nonoverlapping equidistant intervals of GC content. B) instead shows the relationship between π and CDS density in the same bins separately for: S⇒S, W⇒W, S⇒W and W⇒S mutations. The fifth GC content bin is not shown because it includes only one centile. See Figure S5 for the other populations. $R^2$ = proportion of variation explained, $k$ = slope of regression (times $10^3$ for readability in B). GC bins 1-4 shown left to right. Mutation categories from top to bottom row: S⇒S, W⇒W, S⇒W and W⇒S.

190x215mm (300 x 300 DPI)
Table 1. Estimates of $\lambda$, $B$ and relevant measures of GC content. Population specific averages across GC centiles of $\lambda$, $B$, equilibrium GC content under mutational equilibrium alone, $GC(1/[1+\lambda])$, and when taking $B$ into account $GC(1/[1+\lambda e^{-B}])$, and the observed GC content in the ancestral genome for the centile with the highest average pairwise difference $GC(\pi_{max})$ and lowest density of coding sequence ($GC_{CDS_{min}}$). We also show standard error of the mean for $\lambda$ and $B$.

| Population | $\lambda$ | $B$ | $GC_{1/(1+\lambda)}$ | $GC_{1/(1+\lambda e^{-B})}$ | $GC_{\pi_{max}}$ | $GC_{CDS_{min}}$ |
|------------|-----------|-----|----------------------|-----------------------------|------------------|------------------|
| Swe-sin    | 2.99 ± 0.010 | 0.21 ± 0.009 | 0.25 | 0.29 | 0.27 | 0.35 |
| Spa-sin    | 2.97 ± 0.008 | 0.21 ± 0.010 | 0.25 | 0.29 | 0.31 | 0.34 |
| Kaz-sin    | 3.00 ± 0.011 | 0.20 ± 0.009 | 0.25 | 0.29 | 0.28 | 0.34 |
| Kaz-juv    | 4.15 ± 0.019 | 0.79 ± 0.025 | 0.19 | 0.35 | 0.29 | 0.34 |
| Ire-juv    | 3.54 ± 0.027 | 0.47 ± 0.026 | 0.22 | 0.31 | 0.29 | 0.34 |
| Spa-rea    | 2.98 ± 0.012 | 0.16 ± 0.011 | 0.25 | 0.28 | 0.31 | 0.34 |