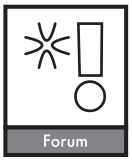

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Molecular substitution rate increases with latitude in butterflies: evidence for a trans-glacial latitudinal layering of populations?

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A well-documented consequence of repeated global ice ages is the negative relationship between latitude and intraspecific genetic diversity. However, little is known about additional effects of such major climatic events on population genetic structure. Here we studied the phylogeographic structure of five lycaenid butterfly species with varied ecological adaptations, sampled across a latitudinal gradient in the Holarctic region. We found a positive correlation between latitude and substitution rate of mitochondrial DNA sequences in all species investigated. We propose that this result is the signal of increased genetic drift and founder effects during post-glacial recolonization of northern populations. Given that phylogenetic branch length is the result of a cumulative process over evolutionary time, we hypothesize that a latitudinal layering of populations has generally been maintained during repeated cycles of glaciation, possibly due to a neutral spatial effect and/or local adaptive advantage. This trans-glacial latitudinal layering could be viewed as a particular case of the more general phenomenon of intraspecific structuring that is created and maintained in a fluctuating environmental gradient.

Climate on Earth has undergone periodic fluctuations during the past ~2 million years (Webb and Bartlein 1992). The Pleistocene ice ages, cycles of extremely cold climate in temperate climatic zones repeated every approximately 10^5 years with shorter warm periods in between, have shaped the distribution of many organisms (Hewitt 1996, Pielou 2008). Numerous species were repeatedly pushed back to warmer areas (glacial refugia) during cold periods, and extended their distribution during interglacial warm periods. Evidence of these past processes can be found in present genetic structure: populations of many species in former glacial refugia are more genetically diverse than those at extreme latitudes, and are genetically distinct from populations in other refugia (Hewitt 1996, 2004, Habel et al. 2008). More generally, colonization processes lead to lower allelic diversity at the edge compared to the centre of distribution ranges, due to the loss of alleles during founder events (bottlenecks) along the expansion front, a phenomenon also called gene surfing (Hallatschek and Nelson 2008). Evidence for this phenomenon has been documented in genetic studies employing rapidly-evolving allozyme or microsatellite DNA-markers for species that recently experienced range expansions (Schmitt et al. 2002, Eckert et al. 2008, Slatkin and Excoffier 2012, Habel et al. 2013, Swaegers et al. 2013).

Small (founder) populations display higher allele fixation rates and thus increased genetic drift and rates of genetic differentiation (Ohta 1973, Dowton and Austin 1995). Molecular substitution rate, which can be quantified as phylogenetic branch length, can therefore be expected to increase in populations near the edge of species' distributions. While genetic diversity can be rapidly lost due to population bottlenecks and founder effects, the generation of new diversity is an evolutionary process with a tempo linked to the mutation rate of each marker. Due to a higher mutation rate and a four times smaller effective population size, the substitution rate mitochondrial DNA is higher than that of nuclear DNA, yet lower than in highly variable markers typically used for population genetic studies, e.g., allozymes or microsatellites (Hung et al. 2016): patterns observed in mitochondrial DNA are usually interpreted as the result of processes happening during the range of at least hundreds of thousands of years. To our knowledge the role of increased genetic drift and founder effects on molecular substitution rates has not been investigated in a comparative analysis in the context of latitude and post-glacial recolonization theory using mtDNA sequences, potentially providing insight into processes happening over very long time, such as the course of multiple glacial cycles.

Here we test the hypothesis that substitution rate (as reflected by intraspecific phylogenetic branch length) in mtDNA increases with latitude in the northern hemisphere,

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presumably because of long-term increased genetic drift and founder effects in more northern populations. We therefore quantify the signal of latitude in a Bayesian Inference haplotype-tree based on mitochondrial DNA sequences (COI) of five lycaenid butterfly species (Lepidoptera: Lycaenidae), and discuss implications of the results for our understanding of species' survival strategies during glaciations. We also test the prediction of a negative correlation between latitude and genetic diversity of populations.

The butterfly species studied represent large parts of the Holarctic region: one from North America (*Feniseca tarquinius*), two from Eurasia (*Glaucopsyche alexis, Plebejus argus*) and two from both continents (*Agriades optilete*, *Plebejus idas*). These taxa were selected because they are widely distributed across latitude, and sufficient samples representing this gradient were available. They also display varied ecological strategies, so that relatively general patterns can be addressed using this set of study species: *G. alexis* is a facultative ant mutualist, *P. argus* and *P. idas* are obligate ant mutualists with different altitudinal preferences and distribution ranges, *F. tarquinius* is an aphytophagous predator of aphids, and *A. optilete* is a boreo-alpine specialist not associated with ants. Importantly, butterflies are among the organisms with the smallest climatic debt (Devictor et al. 2012), so that their distributions are strongly affected by climatic fluctuations, and constitute an ideal system to test the effects of glaciations on genetic structure.

Materials and methods

This investigation is based on mitochondrial DNA sequences of 858 individuals belonging to five species of the butterfly family Lycaenidae. Of these, 546 specimens were obtained and sequenced specifically for this study, while the rest were obtained from GenBank and the 'Barcode of life data systems' (BOLD) taxonomy browser (<www.boldsystems.org/>), originating from a variety of studies (Dincă et al. 2011, 2015, Sielezniew et al. 2011). A detailed overview of samples and their origin as well as GenBank accession numbers are given in Supplementary material Appendix 1 Table A1. *Plebejus idas* was considered in a wide sense (sensu lato) including specimens originating from BOLD that were attributed to the taxa *calliopis* and *christophi*. *Plebejus calliopis* is presently regarded as a synonym of *P. idas* (Tshikolovets 2011, de Jong et al. 2014) and *P. christophi* cannot safely be separated from *P. idas* based on COI (Lukhtanov et al. 2009, Supplementary material Appendix 1 Fig. A1).

Mitochondrial DNA was extracted from wingtips, legs or abdomens of adult butterflies or parts of larvae using an AutoGenprep 965 extraction robot (Bauer Core Laboratory, Harvard Univ., Cambridge, MA). PCR reactions were carried out using the primer pair 'LCO1490' (forward) (Folmer et al. 1994) and 'Nancy' (reverse) (Simon et al. 1994). Each PCR reaction was carried out in a 12.5 µl volume containing 6.25 µl Omega PCR Taq Mixture with dye, 1 µl DNA template, 0.2 mM of each primer as well as ddH_2O added to make up the total volume. PCR conditions, product visualization, purification and cycle sequencing were similar to the conditions described in Kaliszewska et al. (2015). Sanger sequencing was performed on an ABI 3730xl DNA or ABI 3130xl genetic analyzer.

The chromatograms were edited and aligned in Geneious ver. 6.1.2 (created by Biomatters; available at <www.geneious.com/>). The raw sequences were collapsed into haplotypes using the freely available software TCS ver. 1.21 (Clement et al. 2000). For phylogenetic analysis of unique haplotypes, the most suitable model of sequence evolution for the alignment of haplotypes was inferred with jModelTest (Posada 2008) using the corrected Akaike information criterion (AICc) (Sugiura 1978, Hurvich and Tsai 1993). A Bayesian Inference tree was created using the programme MrBayes ver. 3.2.2 (×64) (Ronquist and Huelsenbeck 2003) with the HKY+I+G model of sequence evolution, run for 2×10^7 generations with one cold and three heated chains and an unconstrained branch length prior.

Distances from each species' ancestral node to the tips (considered here as a measure of substitution rate) were calculated from the resulting phylogeny using the packages 'ape' (Paradis et al. 2004) and 'adephylo' (Jombart and Dray 2010) in R ver. 3.2.2 (R Core Team) after rooting the tree to its midpoint. The branch length from the root to the deepest node within each species was subtracted from resulting cumulative branch lengths for better comparison of crown-groups among species.

Nucleotide diversity (Pi) (Nei and Li 1979) of populations consisting of at least four individuals was calculated in Arlequin ver. 3.5.1.2 (Excoffier and Lischer 2010).

The association between distance to the species' ancestral node in the haplotype tree and midpoint latitude $\left(\frac{\min+\max}{2}\right)$ of all unique haplotypes, and latitude and nucleotide diversity (Pi) of populations ($N \geq 4$), was examined using generalized linear mixed models (GLMMs) in the R package 'nlme' (Pinheiro et al. 2016). Midpoint latitude, longitude and their interaction were included as fixed effects and species as a random effect to predict the distances to the common ancestral node within species. Identical analyses were repeated for haplotypes' maximum and minimum latitudes. To investigate whether the increased branch length in northern haplotypes is caused by synonymous or non-synonymous substitutions, we repeated the analysis for midpoint latitude with a sequence alignment excluding sites with non-synonymous changes within species. Nucleotide diversity of populations was assessed in an analogous model including latitude, longitude and their interaction as fixed effects and species as a random effect. Pseudo R-squared values (R_GLMM^2) were calculated for all models using the R package 'MuMIn' (Barton 2016).

Results

For the Bayesian haplotype tree inference, the minimum estimated sample size (ESS) for the sum of branch lengths was 12 104 and the potential scale reduction factor (PSRF) (Gelman and Rubin 1992) was 1.000, suggesting convergence of the two independent runs. The resulting tree is shown in Supplementary material Appendix 1 Fig. A1. The midpoint latitude at which haplotypes were found showed a significant positive association with substitution rate

Table 1. Results from maximal GLMMs used in this study. $R(m)^2$ refers to the marginal and $R(c)^2$ to the conditional coefficient of determination (pseudo-R-squared) as implemented in the R package 'MuMIn' (Barton 2016). 'Species' was included as a random effect in all models.

response variable	$R(m)^2$	$R(c)^2$	fixed effect	value	SE	DF	t-value	p-value
substitution rate	0.037	0.649	midpoint latitude	8.382×10^{-5}	3.937×10^{-5}	128	2.129	**0.035**
			midpoint longitude	-3.562×10^{-6}	1.416×10^{-5}	128	–0.252	0.802
			interaction	8.100×10^{-8}	2.545×10^{-7}	128	0.320	0.750
substitution rate	0.021	0.638	maximum latitude	5.159×10^{-5}	2.855×10^{-5}	128	1.807	0.073
			maximum longitude	2.052×10^{-5}	2.351×10^{-5}	128	0.873	0.385
			interaction	-4.230×10^{-7}	4.340×10^{-7}	128	–0.974	0.332
substitution rate	0.066	0.640	minimum latitude	1.103×10^{-4}	3.124×10^{-5}	128	3.532	**<0.001**
			minimum longitude	1.483×10^{-5}	2.330×10^{-5}	128	0.637	0.526
			interaction	-2.250×10^{-7}	4.440×10^{-7}	128	–0.506	0.614
synonymous substitution rate	0.042	0.681	midpoint latitude	7.978×10^{-5}	3.699×10^{-5}	128	2.157	**0.033**
			midpoint longitude	-5.207×10^{-6}	1.334×10^{-5}	128	–0.390	0.697
			interaction	1.260×10^{-7}	2.396×10^{-7}	128	0.527	0.599
nucleotide diversity (Pi) of populations	0.232	0.232	latitude	–0.0770	0.0211	56	–3.650	**<0.001**
			longitude	–0.0106	0.0278	56	–0.383	0.703
			interaction	0.0002	0.0006	56	0.338	0.737

(reflected by the distance to the species' ancestral node in the haplotype tree) (Table 1, Fig. 1), while midpoint longitude did not (Table 1). The same pattern was obtained, even more strongly, for the minimum latitude at which haplotypes were found, and a trend in the same direction was observed for the maximum latitude (Table 1). The association between midpoint latitude and ancestral node distance was even stronger when excluding non-synonymous sites from the alignment (Table 1), suggesting that selection on the COI-gene is not the cause for the observed pattern. Nucleotide diversity (Pi) of populations decreased significantly with latitude (Table1, Fig. 2).

Discussion

The results show that COI substitution rate increases with latitude in the five investigated butterfly species (Fig. 1). However, the question remains: why? Higher temperatures found at near-equatorial latitudes are thought to increase the rate of molecular substitutions (evolutionary speed hypothesis) (Rensch 1959), the opposite of our finding. In insects, this effect could even be reinforced by the often higher number of annual generations in populations near the equator compared to more extreme latitudes (Oppold et al. 2016).

On the other hand, the nearly neutral theory (Ohta 1973) proposes more rapid evolution in small populations because of random genetic drift. Those predictions

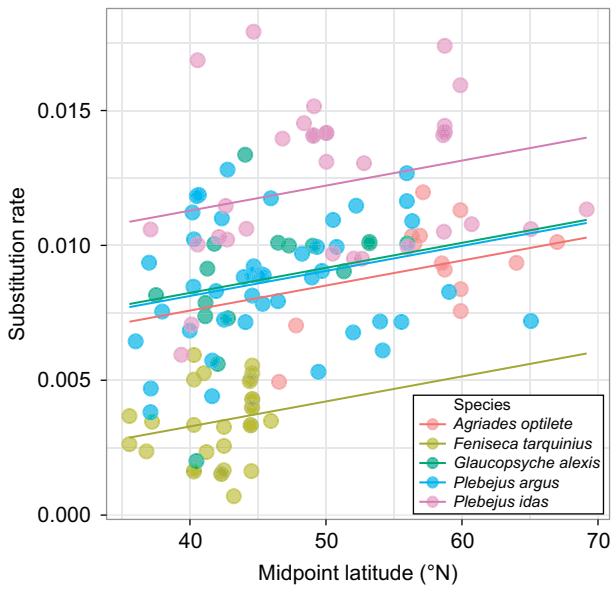

Figure 1. Partial GLMM response values and fitted lines for the fixed effect 'midpoint latitude of haplotype distribution' as a predictor of 'substitution rate' (reflected by branch length in a haplotype tree). The different colours illustrate the random effect 'species'. The model is based on 136 COI-haplotypes belonging to five species of the butterfly family Lycaenidae. This plot was created using the R package 'ggplot2' (Wickham 2009).

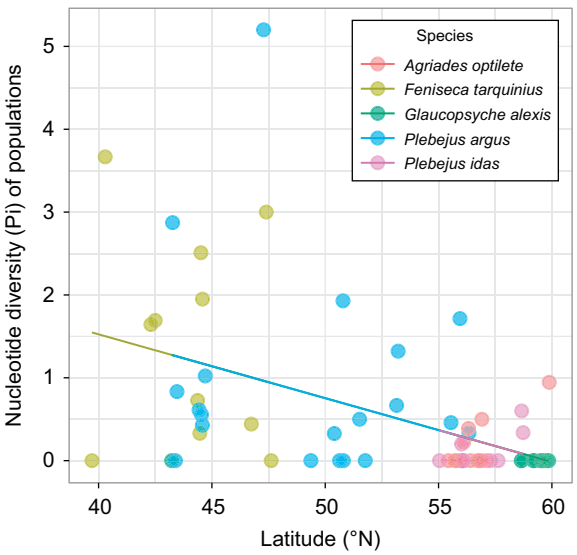

Figure 2. Partial GLMM response values and fitted lines for the fixed effect 'latitude of haplotype distribution' as a predictor of 'nucleotide diversity (Pi)'. The different colours illustrate the random effect 'species'. The model is based on 64 populations (defined as sampling locations with $N \geq 4$) of five species of the butterfly family Lycaenidae. This plot was created using the R package 'ggplot2' (Wickham 2009).

have been supported by studies on species living on islands (DeSalle and Templeton 1988) and with life history strategies that are associated with small population sizes (Dowton and Austin 1995, Pellissier et al. 2012). A similar effect has been documented in edge populations of species undergoing range expansion (Eckert et al. 2008, Swaegers et al. 2013), especially via 'stepping-stone' type colonization (the migration of a single or few individuals bridging large distances, leading to bottlenecks) (Hewitt 1996), which is known from several insect species (Habel et al. 2013). Thus, postglacial range expansions could explain the observed increase of substitution rate with latitude, as has already been proposed for decreased genetic diversity (Hewitt 1996, Fig. 1). However, while genetic diversity may be lost quickly (particularly when measuring this in loss of individual alleles) and could merely reflect the last postglacial recolonization (several thousands of years from now), phylogenetic branch length is the outcome of a long-term increased substitution rate. Given published substitution rates for invertebrate mitochondrial genomes of 1.5–2.3% uncorrected pairwise distance per million years (Brower 1994, Quek et al. 2004), it is difficult to imagine how a process occurring in the last post-glacial recolonization period since ~15 000 years (Thompson et al. 1998) could fully explain our results. We therefore propose that this phenomenon was repeated during multiple cycles of glaciation and that a latitudinal layering of populations was generally maintained during multiple glacial cycles. This scenario, which we term 'trans-glacial latitudinal layering' (Fig. 3), would lead to more probable resampling of particular populations as founders in the northern range limits that would accumulate mutations through drift during evolutionary time. A current latitudinal structuring of populations, lineages and closely related species has been documented for several organisms, for example in what has been called 'refugia within refugia' (Gómez and Lunt 2007). Thus, latitudinal layering could potentially lead to speciation in the long term, a hypothesis also supported by the typically parapatric distributions of closely related cryptic species (Vodă et al. 2015a, b). However, a latitudinal layering does not exclude the existence of a simultaneous longitudinal structuring as the result of diverse glacial refugia (Hewitt 1996, 1999). The hypothesis proposed here could be viewed as a particular case of a more general phenomenon: an intraspecific structuring that is created and maintained in a fluctuating environmental gradient. For example, altitudinal structuring in mountains across climatic fluctuations, or coastal areas repeatedly exposed because of sea level fluctuation.

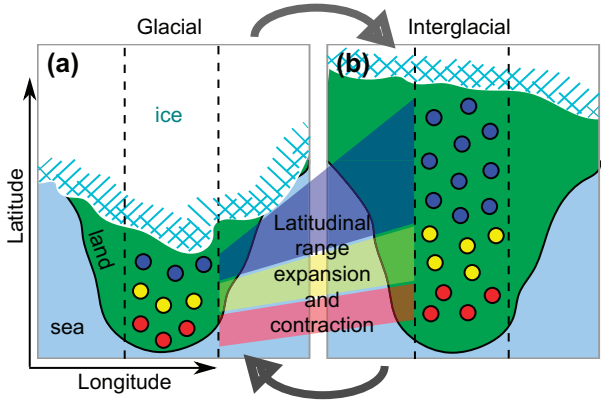

Figure 3. Schematic representation of the trans-glacial latitudinal layering hypothesis, defined as a latitudinal layering of populations (groups of circles with the same colour) that is maintained through range contractions (a) and expansions (b) of serial glacial cycles. Note that the layers of populations at higher latitudes experience more intense range expansions and contractions, which results in higher substitution rates.

While distributional shifts and fragmentation of populations may also occur in the near-equatorial boundaries because of climate fluctuations, they are likely not comparable in scale to the range shifts at more extreme latitudes and their effects. The fact that both the minimum and maximum latitudes for haplotypes display the same gradual pattern across latitude suggests that populations behave as 'layers' that move towards the poles in an orderly fashion, shifting both the southern and northern limits. Populations could maintain their relative latitudinal positions because of positional advantage at the onset of dispersal (the result of a neutral process: those already closer to the poles are more likely the new founders in a new recolonization event). Additionally, the hypothesised layering of populations could reflect their different degree of adaptation to cold climate, but the fact that the observed increase in branch length in northern COI haplotypes is caused by neutral (synonymous) DNA substitutions indicates that any potential advantage would involve other genes.

Because all five species studied displayed a similar trend in substitution rate versus latitude (Fig. 1), the trans-glacial latitudinal layering hypothesis seems to be applicable to species across a wide range of ecological specialization. Nonetheless, the results here reported may not be reflected in other species of butterflies or in other groups of organisms. In fact, the phenomenon proposed here is but one of many factors linking the biogeographical history of species to their genetic structure (Avise 2000). It is likely that the signal of the trans-glacial latitudinal layering is often overshadowed by phenomena such as the differentiation between populations in different glacial refugia (Seddon et al. 2001, Hewitt 2004), genetic sweeps (Narita et al. 2006), introgressive events (Talavera et al. 2013), the decrease in metabolism and annual number of generations with latitude (Oppold et al. 2016), etc. Indeed, the low marginal R-squared values in the models testing the association between substitution rate and latitude (Table 1) suggest that the effect of latitude is only one out of several factors influencing substitution rate. Moreover, even though the COI-gene has been shown to be quite robust in detecting general phylogeographic patterns (Hung et al. 2016), the pattern displayed by a single mtDNA marker may or may not be representative of that of the whole genome. For the reasons mentioned above (taxa and genetic marker studied), this study should be considered as a proof-of-concept. An ideal dataset to reject or confirm the proposed hypothesis would include a high number of species and multiple genetic markers that are variable enough to show considerable inter- and intraspecific variation, and for which substitution rate can be inferred. Thus, future studies may show how general this phenomenon is across genetic markers and taxa, and whether it is generated primarily by neutral or adaptive processes.

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Supplementary material (Appendix ECOG-02487 at <www.ecography.org/appendix/ecog-02487>). Appendix 1.