Genomic analysis of European *Drosophila* populations reveals major longitudinal structure, continent-wide selection, and unknown DNA viruses

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Abstract

Genetic variation is the fuel of evolution but analysing the spatio-temporal dynamics of genetic changes in natural populations is challenging, comprehensive sampling logistically difficult, and sequencing of entire populations costly. Here we address these issues by performing the first continent-wide genomic analysis of genetic variation in European *Drosophila melanogaster*, based on 48 pool-sequencing samples from 32 populations. Our analyses uncover a novel pattern of major longitudinal population structure; establish previously unknown clines in inversions and transposable elements across Europe; and provide evidence for non-local, continent-wide selective sweeps that are shared among the majority of populations. We also find pronounced variation among populations in the composition of the fly microbiome and identify five new DNA viruses adding to a single example known so far for this species. Our study has important implications for the evolution and demography of *D. melanogaster*, an ancestrally African species that first colonized Europe before becoming cosmopolitan.

Keywords: *P*opulation genomics, demography, selection, clines, SNPs, structural variants, symbionts, viruses.

Introduction

Studying the processes that create and maintain genetic variation in natural populations is fundamental to understanding the process of evolution (Dobzhansky 1970; Lewontin 1974; Kreitman 1983; Kimura 1984; Hudson *et al.* 1987; McDonald & Kreitman 1991; Adrian & Comeron 2013). Until recently, technological constraints have limited studies of natural genetic variation to small genomic regions and small numbers of individuals. With the development of population genomics, we can now analyse patterns of genetic variation on a genome-wide scale for large numbers of individuals, with sampling structured across space and time. As a result, we have gained fundamental new insights into evolutionary dynamics of genetic variation in natural populations (e.g., Hohenlohe *et al.* 2010; Cheng *et al.* 2012; Begun *et al.* 2007; Pool *et al.* 2012; Harpur *et al.* 2014; Zanini *et al.* 2015). Despite this recent technological progress, extensive large-scale sampling and genome sequencing of populations remains prohibitively expensive in terms of cost and labor for any individual research group.

Here, we present the first comprehensive, continent-wide genomic analysis of genetic variation in European *Drosophila melanogaster*, based on 48 pool-sequencing samples from 32 populations collected in 2014 (Figure 1) by the European *Drosophila* Population Genomics Consortium (*DrosEU*; https://droseu.net). *D. melanogaster* offers several advantages for studying the relevant spatio-temporal scales of evolution: a relatively small genome, a broad geographic range, a multivoltine life history that allows sampling across

126 generations over short timescales, ease of sampling natural populations using
127 standardized techniques, and a well-developed context for population genomic analysis
128 (e.g., Powell 1997; Keller 2007; Hales *et al.* 2015). Importantly, this species is studied by
129 an extensive research community, with a long history of developing shared resources
130 (Larracuente & Roberts 2015; Bilder & Irvine 2017).
131
132 The current study complements and extends previous studies of genetic variation in *D.*
133 *melanogaster*, both from its native range in sub-Saharan Africa and from its world-wide
134 expansion as a human commensal into Europe 10–20,000 years ago and into North
135 America and Australia in the last few centuries (e.g., Lachaise *et al.* 1988; David & Capy
136 1988; Li & Stephan 2006; Keller 2007; Sprengelmeyer et al 2018; Arguello *et al.* 2019;
137 also cf. Kapopoulou *et al.* 2018a). The colonization of novel habitats and climate zones on
138 multiple continents makes *D. melanogaster* especially powerful for studying parallel local
139 adaptation. Previous studies of genomic variation have uncovered latitudinal clines in
140 allele frequencies (e.g., Schmidt & Paaby 2008; Turner *et al.* 2008; Kolaczkowski *et al.*
141 2011b; Fabian *et al.* 2012; Bergland *et al.* 2014; Machado *et al.* 2016; Kapun *et al.* 2016a),
142 structural variants such as chromosomal inversions (reviewed in Kapun & Flatt 2019),)
143 transposable elements (TEs) (Boussy *et al.* 1998; González *et al.* 2008; 2010), and
144 complex phenotypes (de Jong & Bochdanovits 2003; Schmidt & Paaby 2008; Schmidt *et*
145 *al.* 2008; Kapun *et al.* 2016b; Behrman *et al.* 2018). Thus far, sampling across these
146 latitudinal gradients has been restricted to single transects on the east coasts of Australia
147 and North America; in addition to parallel local adaptation, clines on these continents may
148 be due to admixture between cohorts of flies with different colonization histories (Caracristi
149 & Schlötterer 2003; Yukilevich & True 2008a; b; Duchen *et al.* 2013; Kao *et al.* 2015;
150 Bergland *et al.* 2016).

In contrast, the population genomics of *D. melanogaster* on the European continent remains largely uncharacterized (Božičević *et al.* 2016; Pool *et al.* 2016; Mateo *et al.* 2018). Because Eurasia was the first continent colonized by *D. melanogaster* as they migrated out of Africa, we sought to understand how this species has adapted to new habitats and climate zones in Europe, where it has been established the longest (Lachaise *et al.* 1988; David & Capy 1988). We analyse our data at three levels: (1) variation at single-nucleotide polymorphisms (SNPs) in nuclear and mitochondrial (mtDNA) genomes (~5.5 x 10^6 SNPs in total); (2) structural variation, including TE insertions and chromosomal inversions; and (3) variation in the microbiota associated with flies, including bacteria, fungi, protists, and viruses.

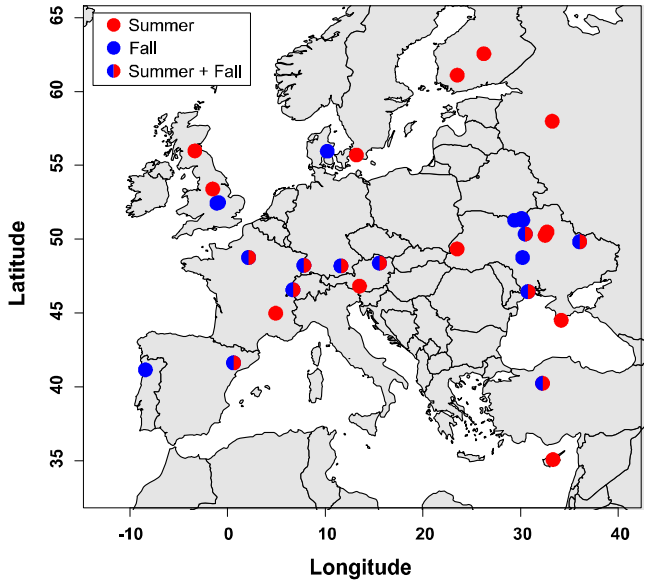

Figure 1. The geographic distribution of population samples. Locations of all samples in the 2014 *DrosEU* data set. The color of the circles indicates the sampling season for each location: ten of the 32 locations were sampled at least twice, once in summer and once in fall (see Table 1 and Supplemental Table 1). Note that some of the 12 Ukrainian locations overlap in the map.

Results

As part of the *DrosEU* consortium, we collected 48 population samples of *D. melanogaster* from 32 geographical locations across Europe in 2014 (Table 1; Figure 1). We performed pooled sequencing (Pool-Seq) of all 48 samples, with an average autosomal coverage ≥50x (Table S1). Of the 32 locations, 10 were sampled at least once in summer and once in fall (Figure 1), allowing a preliminary analysis of seasonal change in allele frequencies on a genome-wide scale.

European and other derived populations exhibit similar amounts of genetic variation

For each sample, we estimated genome-wide levels of nucleotide diversity (π and Watterson's θ, corrected for pooling; Futschik 2010; Kofler *et al.* 2011). We find that most European populations have similar levels of genetic variation (Table S1). Moreover, our estimates of pairwise nucleotide diversity are similar to those from derived non-African (North American and Australian) populations, whether sequenced as individuals or as pools (Figure 2 and Table S2). Thus, although European populations are considerably older than North American and Australian populations, they exhibit similar levels of DNA sequence variability.

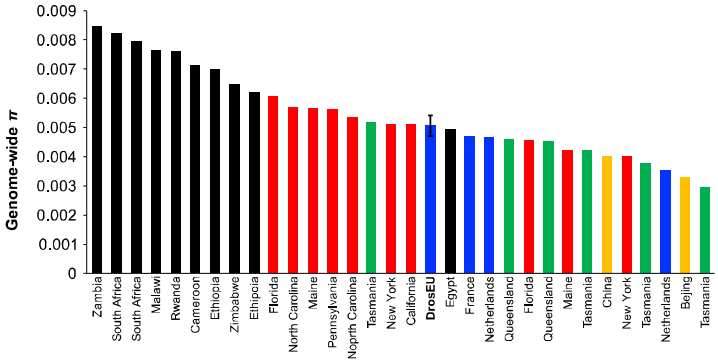

Figure 2. Genetic variation in worldwide samples. Bar plot showing the distribution of genome-wide estimates of Tajima's π of the *DrosEU* and other genomic datasets (also see Table S2 and Materials and Methods) The error bar in the *DrosEU* dataset represent the standard deviation of π across all 48 population samples.

We next tested for associations between geographic variables and genome-wide average levels of genetic variation. We found that neither π nor θ was correlated with latitude or longitude, but both strongly decreased with altitude (Table 2). This contrasts with previous studies of flies collected from a broader range of altitudes, which found increased genetic diversity in high-elevation populations (Lian *et al.* 2018). Finally, we tested for a correlation between genome-wide variation and the season of collection, finding no relationship (Table 2). Together, these results suggest that there is little spatio-temporal variation among European populations in overall levels of sequence variability.

For all populations, the ratio of *X*-linked to autosomal variation (π_X/π_A) was well below the value of 0.75 expected under neutrality with equal sex ratios (ranging from 0.53 to 0.66, one-sample Wilcoxon rank test, $p < 0.001$). These estimates are broadly consistent with those from previous studies of European and other non-African populations (e.g. Andolfatto 2001; Kauer *et al.* 2002; Hutter *et al.* 2007; Betancourt *et al.* 2004; Mackay *et al.* 2012; Langley *et al.* 2012). Surprisingly, the π_X/π_A ratio increased significantly, significantly, albeit weakly, with latitude (Spearman's = 0.315, p = 0.0289). This observation is at odds with a the predictions of a simple model of periodic bottlenecks leading to a lower X/A ratio in northern populations (Hutter *et al.* 2007; Pool & Nielsen 2007), but might be consistent with stronger selection or more male-biased sex-ratios in the south as compared to the north (Charlesworth 2001; Hutter *et al*. 2007).

Genetic variation was heterogeneous across the genome, as has been previously reported (Begun & Aquadro 1992; Mackay *et al.* 2012; Langley *et al.* 2012; Huang *et al.* 2014). Both π and θ were markedly reduced close to centromeric and telomeric regions (Figure 3), and strongly positively correlated with recombination rate (linear regression against fine-scale

recombination rate estimates from Comeron *et al.* (2012), $p < 0.001$; not accounting for autocorrelation; Table S3). Recombination rate explained 41–47% and 31–38% of the variation in π, for the autosomes and X chromosome, respectively. Using broad-scale recombination rate estimates (Fiston-Lavier *et al.* 2010) yielded a qualitatively similar, but slightly stronger correlation in autosomes and weaker in the X chromosome (Figure 3, Table S3, Figure 3 - figure supplement 1).

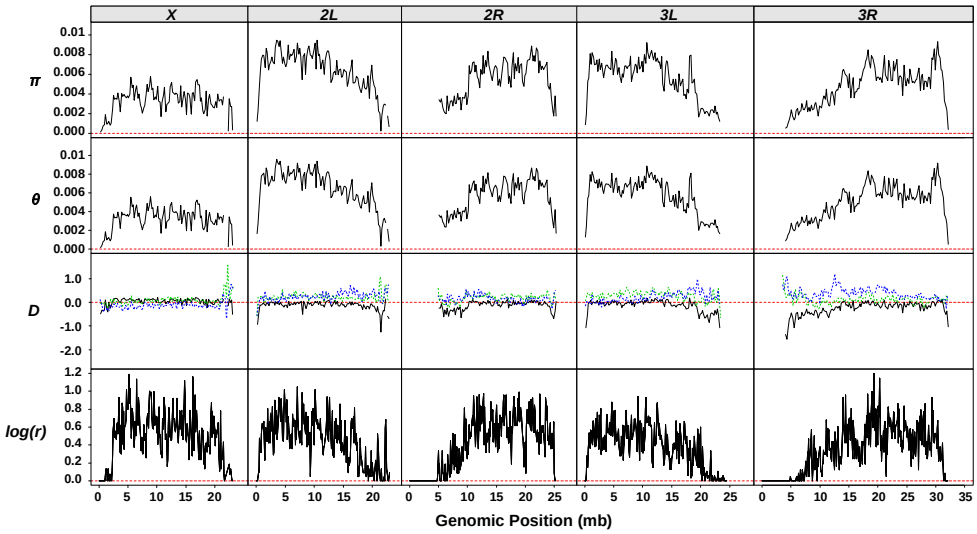

Figure 3 with 1 supplement**. Genome-wide estimates of genetic diversity and recombination rates**. The distribution of Tajima's π, Watterson's θ and Tajima's D (from top to bottom) in 200 kb non-overlapping windows plotted for each chromosomal arm separately. The dashed blue and green lines show estimates for 14 individuals from Rwanda and Zambia, respectively. Bold black lines depict statistics, that were averaged across all 48 samples and the upper and lower grey areas show the corresponding standard deviations for each window. Red dashed lines highlight the vertical position of a zero value. The bottom row shows log-transformed recombination rates (r) in 100 kb non-overlapping windows as obtained from Comeron et al. (2010).

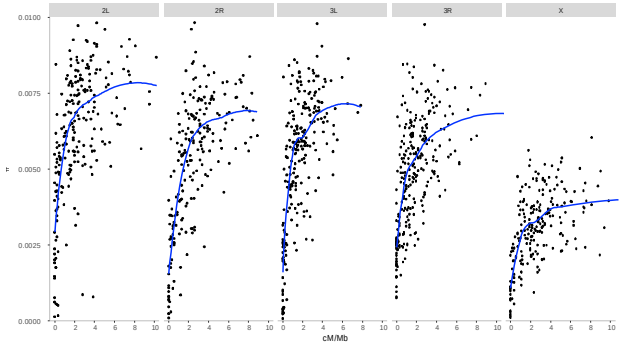

Figure 3 - figure supplement 1. Correlation between recombination and genetic diversity. Smooth local regression (LOESS) between recombination rate in cM/Mb (Comeron et al. 2012) and the average of the 48 samples' genetic diversity (π) in 100 kb non-overlapping windows by chromosome arm.

In contrast to π and θ, the European populations showed major differences in mean Tajima's *D* (Table S1). Tajima's *D* measures deviations from neutral expectations in allele frequencies, which can be due either to selection or complex demography, with negative *D* indicating an excess of low-frequency variants (Tajima 1983). Approximately half of the European samples have negative *D*, It is possible that this result is artefactual, caused by heterogeneity in the proportion of sequencing errors among multiplexed sequencing runs. However, this is unlikely, because including sequence run as a covariate in the statistical model did not improve its fit (Supplementary File 2; Table S4). In all of these analyses, we controlled for confounding effects of spatio-temporal autocorrelations between samples by accounting for similarity among spatial neighbors (Moran's $I \approx 0$, $p > 0.05$ for all tests). When comparing *D* in European samples with ancestral African populations from Zambia and Rwanda, the values were generally lower in the European populations, possibly due to the recent range and population size expansion (Figure 3 and Table S5). Similar to genetic diversity, *D* was also heterogeneous across the genome. Tajima's *D* was broadly reduced in the vicinity of telomeric and centromeric regions, possibly reflecting extended purifying selection or selective sweeps close to heterochromatic regions, and due to reduced recombination.

Several genomic regions show signatures of continent-wide selective sweeps

Genomic regions that show localized reductions in Tajima's *D* are attractive candidates for having undergone recent selective sweeps. To identify such genomic regions, we used *Pool-hmm* (Boitard *et al.* 2013; Table S6A), which – like Tajima's *D* – identifies candidate sweep regions *via* distortions in the allele frequency spectrum. Several genomic regions identified in this way coincide with previously identified, well-supported sweeps in the proximity of *Hen1* (Kolaczkowski *et al.* 2011b), *Cyp6g1* (Daborn *et al.* 2002), *wapl*

(Beisswanger *et al.* 2006), and around the chimeric gene *CR18217* (Rogers & Hartl 2012), among others (Table S6B). These regions also showed local reductions in Tajima's *D* and genetic variation, again consistent with selection (Figure 4 and Figure 4-figure supplement 1 and 2). The putative sweep regions included 145 of the 232 genes previously identified using *Pool-hmm* in an Austrian population (Boitard et al 2012; Table S6C). Other regions identified have not previously been described as harboring sweeps; these represent potential novel targets of positive selection deserving of further investigation (Table S6A). Overall, we identified 64 genes that showed signatures of selection across all European populations analysed (Table S6D); thirty-five of them were located in regions with low Tajima's *D*. This pattern suggests the existence of continent-wide sweeps that either predate the colonization of Europe (e.g., Beisswanger *et al.* 2006), or that have swept across the majority of European populations more recently (Table S6D). Finally, we classified the populations according to the Köppen-Geiger climate classification (Peel *et al.* 2007) and identified several candidate sweeps exclusive to arid, temperate or cold regions; Table S6A). For temperate climates, candidate sweep regions were enriched for functions such as 'response to stimulus', 'transport', and 'nervous system development'; for cold climates, they were enriched for 'vitamin and co-factor metabolic processes' (Table S6E). In contrast, we did not find any significant GO enrichment for arid candidate sweep regions. In summary, this dataset represents a rich genomic resource for future in-depth studies of selective sweeps and adaptation to different climates in *Drosophila*.

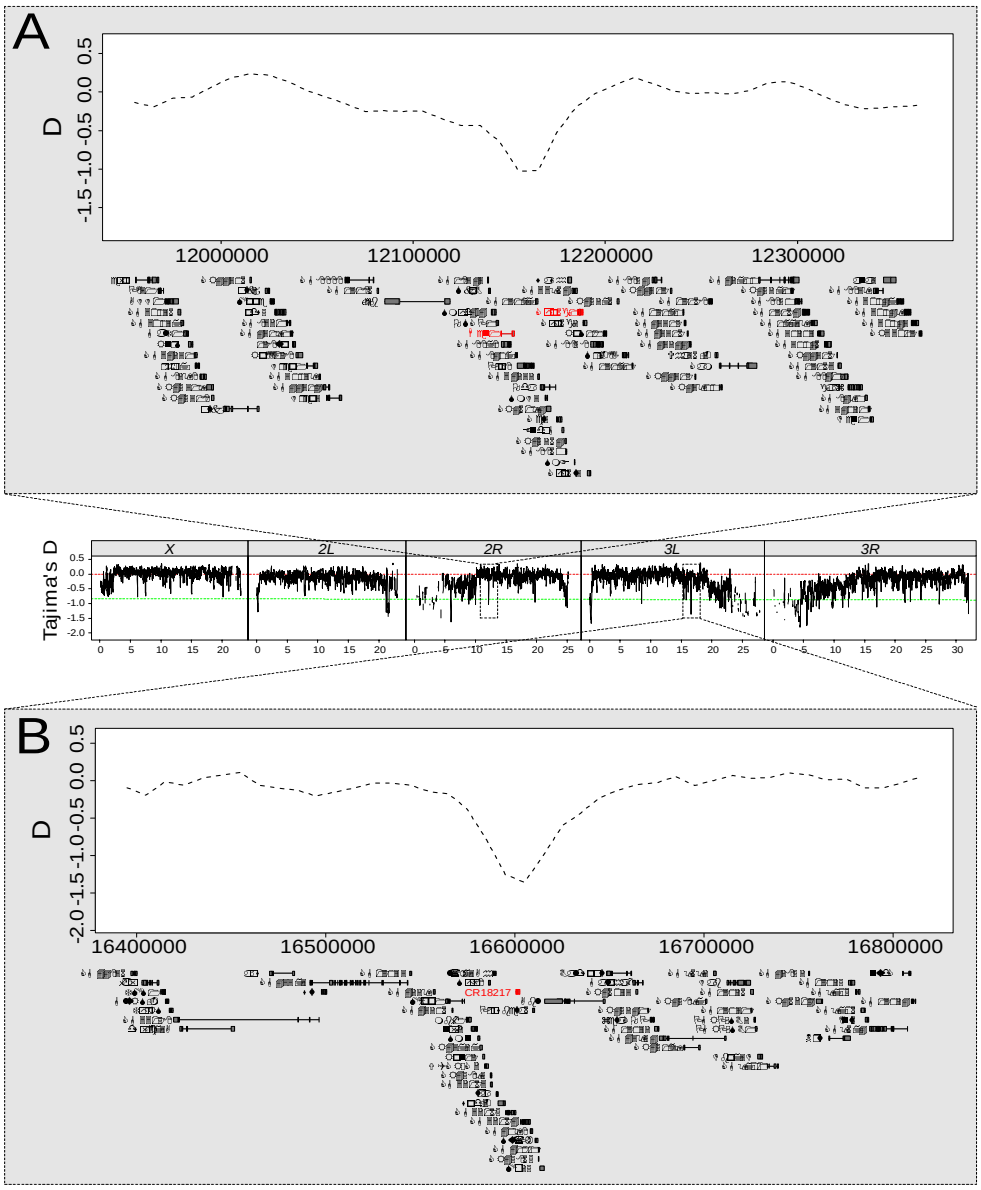

Figure 4 with 2 supplements. **Signals of selective sweeps.** The central panel shows the distribution of Tajima's *D* in 50 kb sliding windows with 40 kb overlap, with red and green dashed lines indicating Tajima's *D* = 0 and -1, respectively. The top panel shows a detail of a genomic region on chromosomal arm *2R* in the vicinity of *Cyp6g1* and *Hen1* (highlighted in red), genes reportedly involved in pesticide resistance. This strong sweep signal is characterized by an excess of low-frequency SNP variants and overall negative Tajima's *D* in all samples. Coloured solid lines depict Tajima's *D* for each sample (see SI Figure 4 for color codes); the black dashed line shows Tajima's *D* averaged across all samples. The bottom panel shows a region on *3L* previously identified as a potential target of selection, which shows a similar strong sweep signature. Notably, both regions show strongly reduced genetic variation (Figure 4 - figure supplement 1).

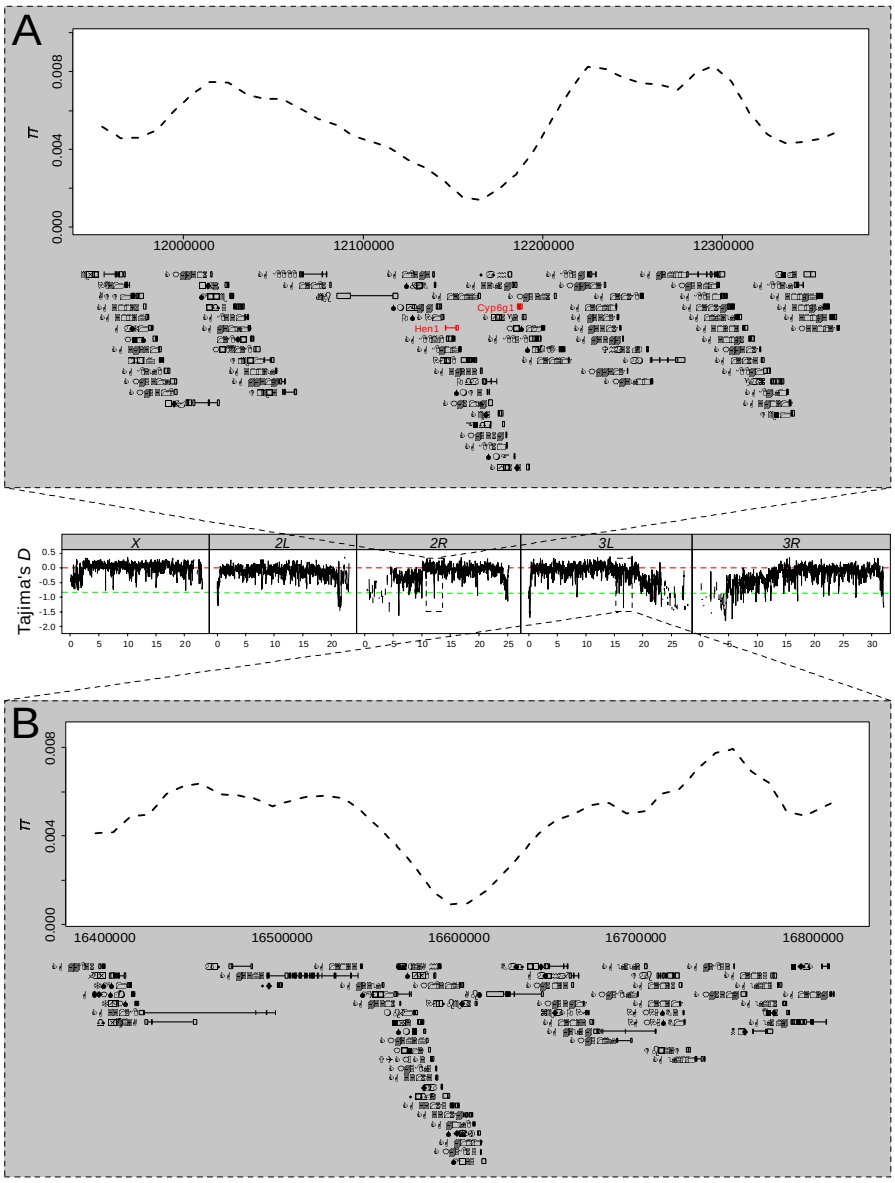

Figure 4 - figure supplement 1: Genetic variation in regions of putative selective sweeps. This figure is equivalent to Figure 4 in the main text but shows the distribution of genetic variation *(π)* in regions with depressed Tajima's *D* around the well-studied *Cyp6g1* locus (A) and around a previously known candidate region on *3L* (B). Similar to Tajima's *D*, *π* was calculated in 50 kb sliding windows with 40 kb overlap. See Table S6 for more examples. A legend for the color codes of the samples can be found in Figure 4 - figure supplement 2.

AT_14_Mau_1	UA_14_Ode_19	ES_14_Lle_35
AT_14_Mau_2	UA_14_Ode_20	FI_14_Aka_36
TR_14_Yes_3	UA_14_Ode_21	FI_14_Aka_37
TR_14_Yes_4	UA_14_Ode_22	FI_14_Ves_38
FR_14_Vil_5	UA_14_Kyi_23	DK_14_Kar_39
FR_14_Vil_7	UA_14_Kyi_24	DK_14_Kar_41
FR_14_Got_8	UA_14_Var_25	CH_14_Cha_42
UK_14_She_9	UA_14_Pyr_26	CH_14_Cha_43
UK_14_Sou_10	UA_14_Dro_27	AT_14_See_44
CY_14_Nic_11	UA_14_Cho_28	UA_14_Kha_45
UK_14_Mar_12	UA_14_Cho_29	UA_14_Kha_46
UK_14_Lut_13	SE_14_Lun_30	UA_14_Cho_47
DE_14_Bro_14	DE_14_Mun_31	UA_14_Cho_48
DE_14_Bro_15	DE_14_Mun_32	UA_14_Kyi_49
UA_14_Yal_16	PT_14_Rec_33	UA_14_Uma_50
UA_14_Yal_18	ES_14_Lle_34	RU_14_Vald_51

Figure 4 - figure supplement 2. Legend for color code in Figure 4, Figure 4 - figure supplement 1.

European populations are structured along an east-west gradient

We next investigated patterns of genetic differentiation due to demographic substructure. Overall, pairwise differentiation as measured by F_{ST} was relatively low, particularly for the autosomes (autosomal F_{ST} 0.013–0.059; *X*-chromosome F_{ST}: 0.043–0.076; Mann-Whitney-U test; $p < 0.001$; Table S1). The slightly elevated F_{ST} for the *X* chromosome is expected given its smaller effective population size (Hutter *et al.* 2007). One population, from Sheffield (UK), was unusually differentiated from the others (Table S1) and was excluded from analyses of neutral genetic differentiation. Despite overall low levels of among-population differentiation, European populations showed evidence of geographic substructure. To analyse this pattern in detail, we focused on SNPs most likely to reflect neutral population structure, those at 4-fold degenerate sites, in regions outside those showing signatures of selective sweeps, in regions of high recombination (r > 3cM/Mb; Comeron et al. 2011) and at least 1 Mb away from the breakpoints of common inversions.

313 The final filtered data set consisted of 8,727 SNPs. Within Europe, we found a weak but
314 significant pattern of isolation by distance (IBD). That is, pairwise F_{ST}, though low overall,
315 increased significantly with geographic distance (Mantel test; $p < 0.001$; r=0.65, max. F_{ST} ~
316 0.05; Figure 5A and Figure 5A – figure supplement 1A).
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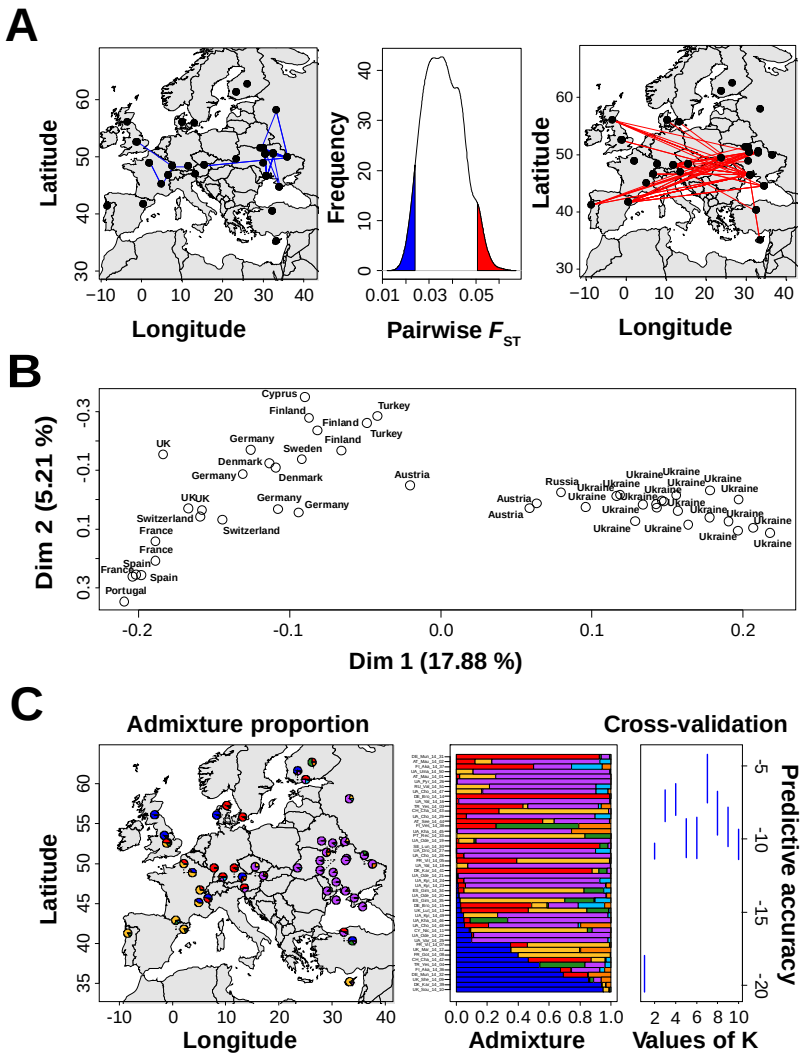

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319 **Figure 5** with 1 supplement**. Genetic differentiation among European populations**. (A) Average F_{ST} among
320 populations at putatively neutral sites. The centre plot shows the distribution of F_{ST} values for all 1,128 pairwise
321 population comparisons, with the F_{ST} values for each comparison obtained from the mean across all 8,727 SNPs used in
322 the analysis. Plots on the left and the right show population pairs in the lower (blue) and upper (red) 5% tails of the F_{ST}
323 distribution. (B) PCA analysis of allele frequencies at the same SNPs reveals population substructuring in Europe.
324 Hierarchical model fitting using the first four PCs showed that the populations fell into three clusters (indicated by colour),
325 with cluster assignment of each population subsequently estimated by *k*-means clustering. (C) Admixture proportions for
326 each population inferred by model-based clustering with *ConStruct* are highlighted as pie charts (left plot) or Structure
327 plots (centre). The optimal number of 7 spatial layers (K) was inferred by cross-validation (right plot).

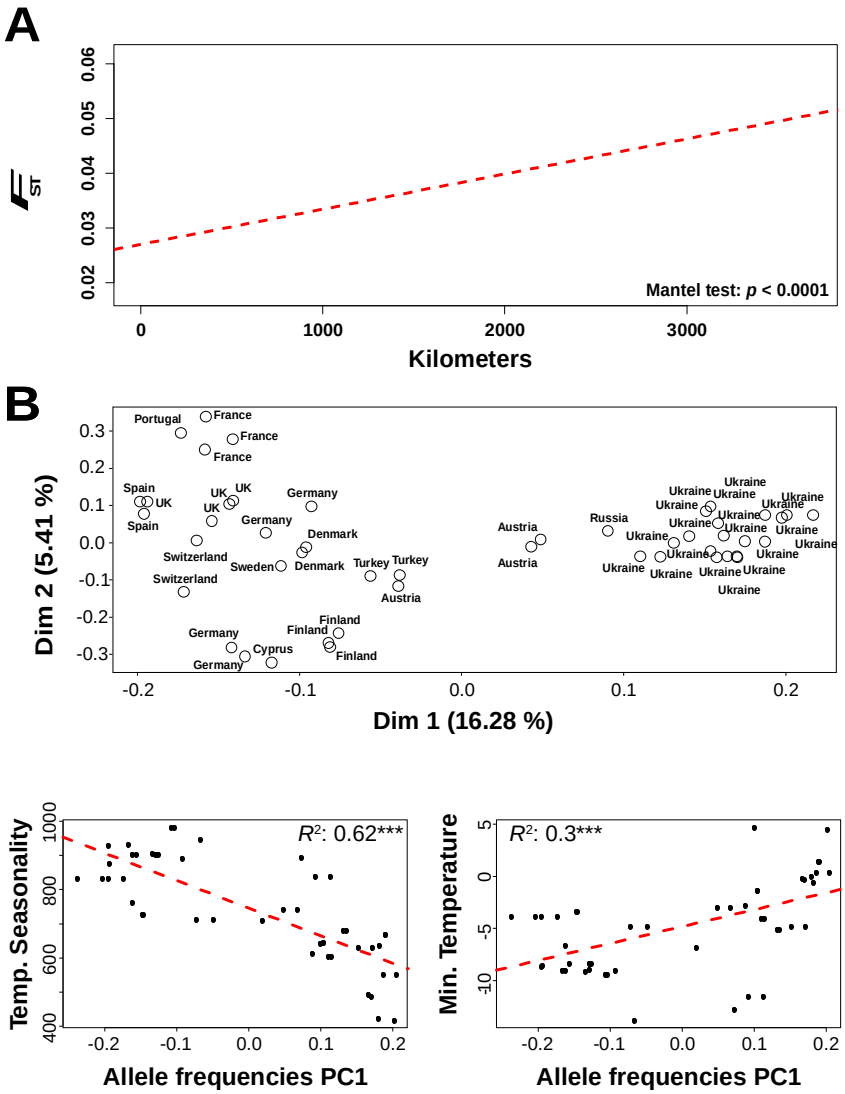


Figure 5 - figure supplement 1: Genetic differentiation among European populations. (A) Average F_{ST} for 8,727 putatively neutral SNPs is significantly negatively correlated with geographic distance (red dashed line shows the linear regression) (B) PCA-based inference of population structure similar to Figure 5B in the main text, but based on 20,008 SNPs located in short introns (<60bp). (C) We tested the top 5 PC for significant associations with 8 climatic variables obtained from the WorldClim database; the two significant regressions, between PC1 and Temperature seasonality (WorldClim Biovar 4; left) and between PC1 and minimum temperature of the coldest month (WorldClim Biovar 6; right) are shown.

We investigated population substructure using principal components analysis (PCA) on allele frequencies from the same set of SNPs at 4-fold degenerate sites. The first three PC axes explained >25% of the total variance (PC1: 17.88%, PC2: 5.2%, PC3: 4.7%, eigenvalues = 410, 101, and 92, respectively), with PC1 strongly correlated with longitude

and to a lesser extent with altitude (Table 2). This longitudinal stratification is expected under a simple model of IBD, as the continent extends further in longitude than latitude. As there was significant spatial autocorrelation between samples (as indicated by Moran's test on residuals from linear regressions with PC1), we repeated the analysis with an explicit spatial error model; the association between PC1 and longitude remained significant. Like PC1, PC2 is correlated with longitude and altitude. PC3, by contrast, is not associated with any variable examined (Table 2). No major PC axes were correlated with season, indicating that there were no shared seasonal differences across samples in our data. However, based on linear regressions comparing summer and fall values of PC1 (adjusted R^2: 0.98; p-value < 0.001), PC2 (R^2: 0.79; p-value < 0.001) and PC3 (R^2: 0.93; p-value < 0.001), we found very strong associations of genetic variation across seasons in the 10 locations that were sampled in summer and fall. This indicates a high degree of spatio-temporal stability in the levels of genetic variation.

Hierarchical model fitting based on the first three PC axes resulted in three distinct clusters (Figure 5B) separated along PC1, supporting the notion of strong longitudinal differentiation among European populations. Importantly, these results remain qualitatively unchanged when restricting the analysis to SNPs located in short introns (< 60 bp), which are also assumed to be relatively unaffected by selection (Figure 5 – figure supplement 1B; Haddrill *et al.* 2005; Singh *et al.* 2009; Parsch *et al.* 2010; Clemente & Vogl 2012; Lawrie *et al.* 2013).

Model-based spatial clustering showed qualitatively similar results, with populations separated mainly by longitude (Figure 5C; using ConStruct, with K=7 spatial layers chosen based on model selection procedure *via* cross-validation). We could also infer levels of

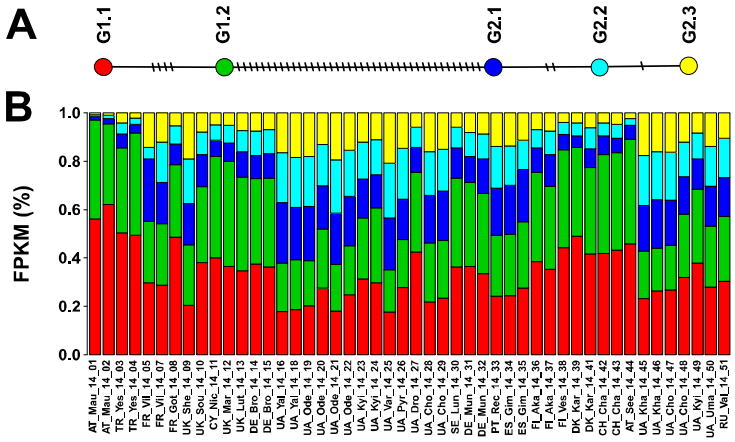
admixture among populations from this analysis; population samples from eastern and northwestern Europe showed low levels of admixture, while those from central Europe appeared locally well-mixed (Figure 5C).

In addition to restricted gene flow between geographic areas, local adaptation may explain population substructuring, even at neutral sites, if closely related populations tend to respond to similar selective pressures. We thus probed whether this spatial substructuring is associated with any of nineteen climatic variables, obtained from the WorldClim database (Hijmans *et al.* 2005). These climatic variables represent averages interpolated averages across more than 50 years of observation at the geographic coordinates corresponding to our sampling locations. Only two variables are significant after Bonferroni correction (adjusted $\alpha = 0.0026$): between PC1 and 'temperature seasonality' (BioVar 4; Hijmans et al. 2005; $R^2 = 0.62$, $P<0.001$; Figure 5 – figure supplement 1C) and between PC1 and 'minimum temperature of the coldest month' ($R^2 = 0.3$, $P<0.001$; Figure 5 – figure supplement 1C). This suggests that the pronounced longitudinal differentiation along the European continent could at least partly be driven by the transition from oceanic to continental climate, leading to gradual changes in temperature seasonality and the severity of winter conditions which might impact demography, especially local survival. To the best of our knowledge, such strongly pronounced longitudinal structure and differentiation on a continent-wide scale has not yet been reported for *D. melanogaster.*

Mitochondrial haplotypes also exhibit longitudinal population structure

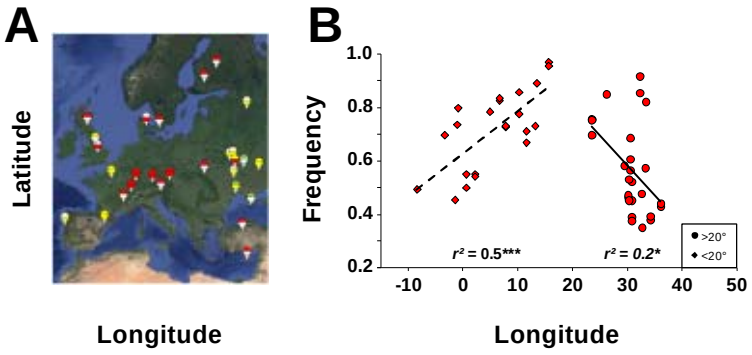
Our finding that European populations show strong longitudinal structure is also supported by an analysis of mitochondrial haplotypes. We identified two main mitochondrial haplotypes in Europe, separated by 41 mutations (G1.2 and G2.1; Figure 6A), with highly

391 variable frequencies among populations (Figure 6B). Qualitatively, three types of
392 European populations can be distinguished based on these haplotypes: (1) central
393 European populations with a high frequency (> 60%) of the G1 haplotypes, (2) Eastern
394 European populations in summer, with a low frequency (< 40%) of G1 haplotypes, and (3)
395 Iberian and Eastern European populations in fall, with a combined frequency of G1
396 haplotypes between 40-60% (Figure 6 - figure supplement 1A). These results are
397 consistent with analyses of mitochondrial haplotypes from a North American population
398 (Cooper *et al.* 2015) as well as from worldwide samples (Wolff *et al.* 2016), which revealed
399 a high level of haplotype diversity.



400

401 **Figure 6** with 1 supplement**. Mitochondrial haplotypes.** (A) TCS network showing the relationship of 5 common
402 mitochondrial haplotypes; (B) estimated frequency of each mitochondrial haplotype in 48 European samples.



403

404 **Figure 6 - figure supplement 1. Mitochondrial haplotypes.** (A) Graphical summary of the combined frequency of G1

haplotypes in Europe. Summer and Fall are represented at the top and bottom of the circles, respectively. White – no information; green, yellow and red represent a combined frequency of G1 haplotypes lower than 40%, in between 40% and 60% and higher than 60%, respectively. (B) Correlations between the combined frequency of G1 haplotypes and longitude (red diamonds for western populations below 20° and red circles for eastern populations above 20°).

Mitochondrial haplotypes also showed shifts in the relative frequencies of the two haplotype classes between summer and fall, but only in 2 of 9 possible comparisons. While there was no correlation between latitude and the frequency of G1 haplotypes, we found a weak but significant negative correlation between G1 haplotypes and longitude (r^2 = 0.10; $p < 0.05$), consistent with the longitudinal east-west population structure observed for SNPs at 4-fold degenerate sites. In a subsequent analysis, we divided the dataset at 20º longitude into an eastern and a western subset because in northern Europe 20º longitude corresponds to the division of two major climatic zones, temperate and cold (Peel *et al.* 2007). This split revealed a clear correlation between longitude and the combined frequency of G1 haplotypes, explaining as much as 50% of the variation in the western group (Figure 6 - figure supplement 1B). Similarly, in eastern populations, longitude and the combined frequency of G1 haplotypes were correlated, explaining approximately 20% of the variance (Figure 6 - figure supplement 1B). Thus, these data on mitochondrial haplotypes clearly confirm the pronounced east-west structure and differentiation among European populations of *D. melanogaster*.

The frequency of polymorphic TEs varies with longitude and altitude

To examine the population genetics of structural variants, we first focused on transposable elements (TEs). The repetitive content of the 48 samples ranged from 16% to 21% with respect to nuclear genome size (Figure 7). The vast majority of detected repeats were TEs, mostly represented by long terminal repeats (LTR) and long interspersed nuclear elements (LINE), as well as a few DNA elements (Class II). LTRs best explained total TE

content (LINE+LTR+DNA) (Pearson's $r = 0.87$, $p < 0.01$, *vs.* DNA $r = 0.58$, $p = 0.0117$, and LINE $r = 0.36$, $p < 0.01$ and Figure 7- figure supplement 1A).

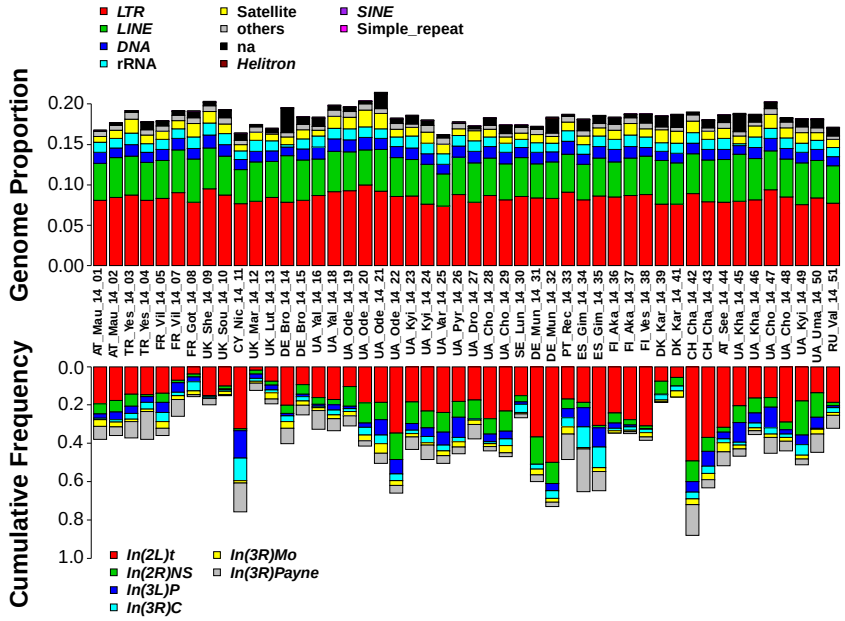


Figure 7 with 2 supplements. **Geographic patterns in structural variants**. The upper panel shows stacked bar plots with the relative abundances of TEs in all 48 population samples**.** The proportion of each repeat class was estimated from sampled reads with dnaPipeTE (2 samples per run, 0.1X coverage per sample). The lower panel shows stacked bar plots depicting absolute frequencies of six cosmopolitan inversions in all 48 population samples.

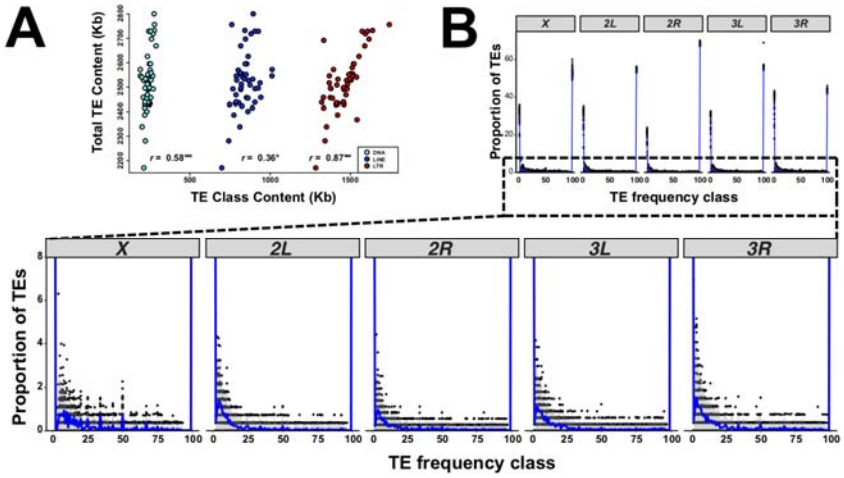


Figure 7- figure supplement 1. **Transposable Elements genome content and frequency distributions.** (A) Pearson's correlations between each main TE class (LTR, LINE and DNA) and the total TE content of each pool (LTR+LINE+DNA) in kb. (B) The site frequency spectrum of TE frequencies per chromosome arm. Each dot represents the proportion of TEs in each bin per sample and a smoother geometric line had been added to highlight the trend. Lower panel is a zoom in of the above panel.

We next estimated population frequencies of 1,630 TE insertions annotated in the *D. melanogaster* reference genome *v.6.04* using *T-lex2* (Table S7, Fiston-Lavier *et al.* 2015). On average, 56% of the TEs annotated in the reference genome were fixed in all samples. The majority of the remaining polymorphic TEs segregated at low frequency in all samples (Figure 7 - figure supplement 1A), potentially due to the effect of purifying selection (González *et al.* 2008; Petrov *et al.* 2011; Kofler *et al.* 2012; Cridland *et al.* 2013; Blumenstiel *et al.* 2014). However, we also observed 142 TE insertions present at intermediate (>10% and <95%) frequencies, which might be consistent with transposition-selection balance (Figure 7 - figure supplement 1B; Charlesworth *et al.* 1994).

In each of the 48 samples, TE frequency and recombination rate were negatively correlated on a genome-wide level (Spearman rank sum test; $p < 0.01$), as previously reported (Bartolomé *et al.* 2002; Petrov *et al.* 2011; Kofler *et al.* 2012). This pattern still held when only polymorphic TEs (population frequency <95%) were analysed, although it was not statistically significant for some chromosomes and populations (Table S8). In either case, the correlation was more negative when using broad-scale (Fiston-Lavier *et al.* 2010), rather than fine-scale (Comeron et al 2012), recombination rate estimates, indicating that broad-scale recombination patterns may best capture long-term population recombination patterns (Materials and methods, Tables S8).

We further tested whether the distribution of TE frequencies among samples could be explained by geographical or temporal variables. We focused on the 141 TE insertions that showed frequency variability among samples (interquartile range, (IQR) > 10; see Materials and Methods) and were located in regions of non-zero recombination according to both fine-scale (Comeron et al. 2012), and broad-scale (Fiston-Lavier et al. (2010)

estimations. Of these, 57 TEs showed significant associations with geographical or temporal variables after multiple testing correction (Table S9). We found significant correlations of 13 TEs with longitude, 13 with altitude, five with latitude, and three with season (Table S9). In addition, the frequencies of the other 23 insertions were significantly correlated with more than one of the above-mentioned variables. These TEs were scattered along the five main chromosome arms, with the majority located inside genes (42 out of 57; Table S9).

Two TE families were enriched in the 57 TE dataset: the LTR *297* family with 11 copies, and the DNA *pogo* family with five copies (χ^2-values after Yate's correction < 0.05; Table S10). Interestingly, 14 of these 57 TEs coincide with previously identified adaptive candidate TEs, suggesting that our dataset might be enriched for adaptive insertions, several of which seem to exhibit spatial frequency clines (Table S9; Rech *et al*. 2019).

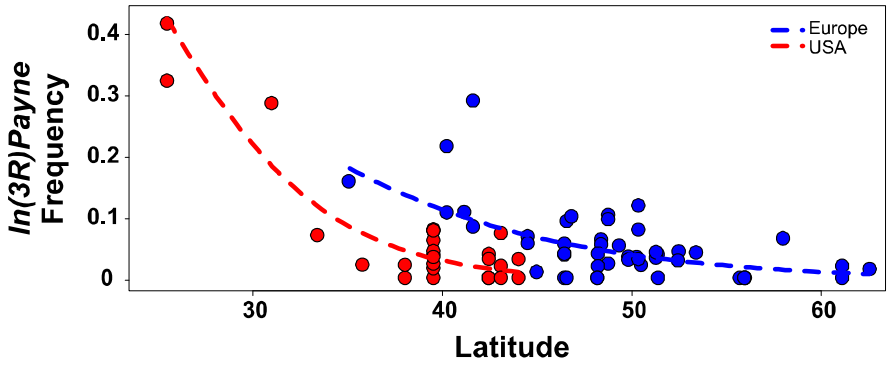
Inversions exhibit latitudinal and longitudinal clines in Europe

Another class of structural variants, chromosomal inversions, show spatial patterns in North American and Australian populations, potentially due to selection (reviewed in Kapun & Flatt 2019). In contrast to North America and Australia, inversion clines in Europe are poorly characterized (Lemeunier & Aulard 1992). Here, we examined the presence and frequency of six cosmopolitan inversions (*In(2L)t, In(2R)NS, In(3L)P, In(3R)C, In(3R)Mo, In(3R)Payne*) in our European samples, using a panel of inversion-specific marker SNPs (Kapun *et al.* 2014). All samples were polymorphic for one or more inversions (Figure 7). However, only *In(2L)t* segregated at substantial frequencies in most populations (average frequency = 20.2%); all other inversions were either absent or rare (average frequencies: *In(2R)NS* = 6.2%, *In(3L)P* = 4%, *In(3R)C* = 3.1%, *In(3R)Mo* =2.2%,

495 *In(3R)Payne* = 5.7%).

496

497 Despite their overall low frequencies, several inversions exhibited pronounced clinality
498 (Table 3). In particular, we observed significant latitudinal clines for *In(3L)P, In(3R)C* and
499 *In(3R)Payne*. Although they differed in overall frequencies, *In(3L)P* and *In(3R)Payne*
500 showed latitudinal clines in Europe qualitatively similar to clines previously observed along
501 the North American and Australian east coasts (Figure 7 - figure supplement 2 and Table
502 S11, Kapun *et al.* 2016a), which, at least in the case of *In(3R)Payne*, are maintained by
503 spatially varying selection (Kapun *et al*. 2016a,b; Durmaz *et al.* 2018; Anderson *et al.*
504 2005; Umina *et al.* 2005; Kennington *et al.* 2006; Rako *et al.* 2006).



505

506 **Figure 7 - figure supplement 2. Clinal variation of the inversion *In(3R)Payne* across continents.** Parallel frequency
507 clines of *In(3R)Payne* along the latitudinal axis at the North American east coast (red) and in Europe (blue) (see also
508 Table S11).

509

510 We also detected – for the first time – longitudinal clines for *In(2L)t* and *In(2R)NS*, with
511 both polymorphisms decreasing in frequency from east to west, a result consistent with the
512 strong longitudinal population differentiation in Europe. *In(2L)t* also increased in frequency
513 with altitude (Table 3). Except for *In(3R)C,* we did not find significant residual spatio-
514 temporal autocorrelation among samples for any inversion tested (Moran's $I \approx 0$, $p > 0.05$

for all tests; Table 3), suggesting that our analysis was not confounded by spatial autocorrelation for most of the inversions. Further studies are necessary to determine the extent to which these clines of inversion frequencies in Europe are shaped by selection.

European *Drosophila* microbiomes contain Entomophthora, trypanosomatids and unknown DNA viruses

We examined the bacterial, fungal, protist, and viral microbiota associated with *D. melanogaster* using the Pool-Seq data. The microbiota can affect life history traits, immunity, hormonal physiology, and metabolic homeostasis of their fly hosts (e.g., Trinder *et al.* 2017; Martino *et al.* 2017).

We characterised the taxonomic origin of the non-*Drosophila* reads in our dataset using MGRAST, which identifies and counts short protein motifs ('features') within reads (Meyer *et al.* 2008). We examined 262 million reads in total and of these most were assigned to *Wolbachia* (mean 53.7%; Figure 8), a well-known endosymbiont of *Drosophila* (Werren *et al.* 2008). The abundance of *Wolbachia* protein features relative to other microbial protein features (relative abundance) varied strongly between samples, ranging from 8.8% in a sample from the UK to almost 100% in samples from Spain, Portugal, Turkey and Russia (Table S12). Similarly, *Wolbachia* loads varied 100-fold between samples, as estimated from the ratio of *Wolbachia* protein features to *Drosophila* protein features (Table S12).

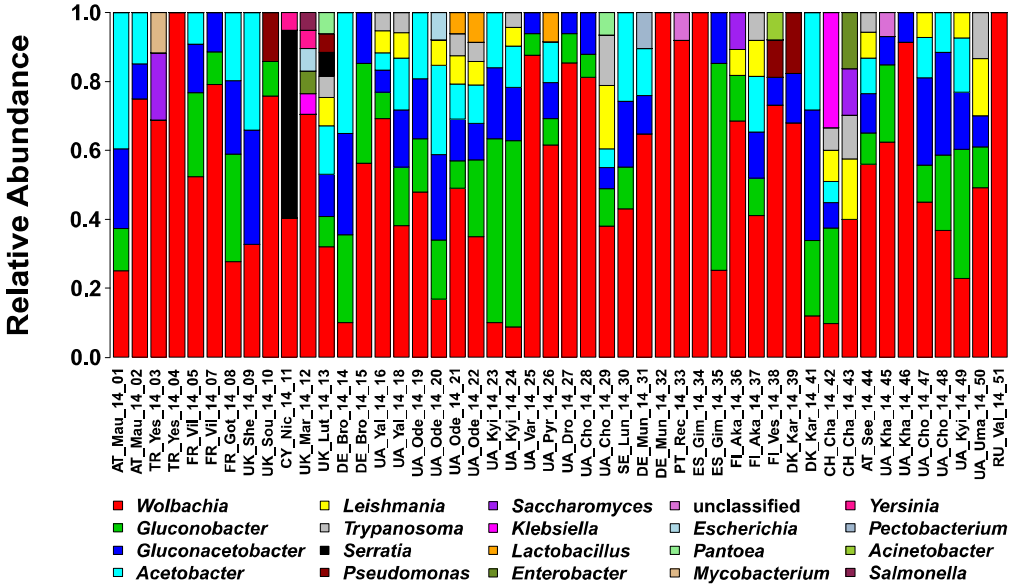


Figure 8: Microbiome. Relative abundance of *Drosophila*-associated microbes as assessed by MGRAST classified shotgun sequences. Microbes had to reach at least 3% relative abundance in one of the samples to be represented

Acetic acid bacteria of the genera *Gluconobacter*, *Gluconacetobacter*, and *Acetobacter* were the second largest group, with an average relative abundance of 34.4% among microbial protein features. Furthermore, we found evidence for the presence of several genera of Enterobacteria (*Serratia, Yersinia, Klebsiella, Pantoea, Escherichia, Enterobacter, Salmonella*, and *Pectobacterium*). *Serratia* occurs only at low frequencies or is absent from most of our samples, but reaches a very high relative abundance among microbial protein features in the Nicosia (Cyprus) summer collection (54.5%). This high relative abundance was accompanied by an 80x increase in *Serratia* bacterial load.

We also detected several eukaryotic microorganisms, although they were less abundant than the bacteria. The fraction of fungal protein features, for example, is larger than 3% in only three samples (from Finland, Austria and Turkey; Table S12). Among the eukaryotic microbiota, we found trypanosomatids in 16 samples. Trypanosomatids have been

551 previously reported to be associated with *Drosophila* (Wilfert *et al.* 2011; Chandler &
552 James 2013; Hamilton *et al.* 2015), and this appeared to have been confirmed in this first
553 systematic survey across a wide geographic range in *D. melanogaster*. We also found the
554 fungal pathogen *Entomophthora muscae* in 14 samples (Elya C et al. 2018).
555 Somewhat surprisingly, we found few yeast sequences. Yeasts are commonly found on
556 rotting fruit, the main food substrate of *D. melanogaster*, and have been found in
557 association with *Drosophila* before (Barata *et al.* 2012; Chandler *et al.* 2012). This result
558 suggests that, although yeasts can attract flies and play a role in food choice (Becher *et al.*
559 2012; Buser *et al.* 2014), they might not be highly prevalent in or on *D. melanogaster*
560 bodies but are rather actively digested and thus not part of the microbiome.
561
562 Our data also allowed us to identify DNA viruses. Only one DNA virus has been previously
563 described for *D. melanogaster* (Kallithea virus; Webster *et al.* 2015; Palmer *et al.* 2018)
564 and only two others more from other Drosophilid species (*Drosophila innubila* Nudivirus
565 [Unckless 2011], Invertebrate Iridovirus 31 in *D. obscura* and *D. immigrans* [Webster *et al.*
566 2016]).
567 Here, we found six different DNA viruses, five of which are new (Table S13).
568 Approximately two million reads came from *Kallithea* nudivirus (Webster *et al.* 2015),
569 allowing us to assemble the first complete *Kallithea* genome (>300-fold coverage in the
570 Ukrainian sample UA_Kha_14_46; Genbank accession KX130344). We also identified
571 around 1,000 reads from a novel nudivirus closely related to both *Kallithea* virus and to
572 *Drosophila innubila* nudivirus (Unckless 2011) in sample DK_Kar_14_41 from
573 Karensminde, Denmark (Table S13). As the reads from this virus in our data set were
574 insufficient to assemble the genome, we identified a publicly available dataset
575 (SRR3939042: 27 male *D. melanogaster* from Esparto, California; Machado *et al.* 2016)

576 with sufficient reads to complete the genome (provisionally named "*Esparto* Virus";
577 KY608910).
578 We further identified two novel Densoviruses (*Parvoviridae*). The first is a relative of *Culex*
579 *pipiens* densovirus, provisionally named "*Viltain* virus", found at 94-fold coverage in
580 sample FR_Vil_14_07 (Viltain; KX648535). The second is "*Linvill Road* virus", a relative of
581 *Dendrolimus punctatus* densovirus, represented by only 300 reads here, but with high
582 coverage in dataset SRR2396966 from a North American sample of *D. simulans*
583 (KX648536; Machado *et al.* 2016). In addition, we detected a novel member of the
584 *Bidnaviridae* family,"*Vesanto* virus", a bidensovirus related to *Bombyx mori* densovirus 3
585 with approximately 900-fold coverage in sample FI_Ves_14_38 (Vesanto; KX648533 and
586 KX648534). Finally, in one sample (UA_Yal_14_16) we detected a substantial number of
587 reads from an Entomopox-like virus, which we were unable to fully assemble (Table S13).
588 Using a detection threshold of >0.1% of the *Drosophila* genome copy number, the most
589 commonly detected viruses were *Kallithea* virus (30/48 of the pools) and *Vesanto* virus
590 (25/48), followed by *Linvill Road* virus (7/48) and *Viltain* virus (5/48), with *Esparto* virus
591 being the rarest (2/48).
592

593 Discussion

594 In recent years, large-scale population re-sequencing projects have produced major
595 insights into the biology of both model (Mackay *et al.* 2012; Langley *et al.* 2012; Auton et
596 al. 2015; Lack *et al.* 2015; Alonso-Blanco *et al.* 2016; Lack *et al.* 2016) and non-model
597 organisms (e.g., Hohenlohe *et al.* 2010; Wolf *et al.* 2010). In particular, such massive
598 datasets contribute greatly to our growing understanding of the processes that create and
599 maintain genetic variation in natural populations. However, the relevant spatio-temporal

scales for population genomic analyses remain largely unknown (e.g., Guirao-Rico and González 2019). Here we have applied – for the first time – a continent-wide sampling and sequencing strategy to European populations of *D. melanogaster* (Figure 1), allowing us to uncover previously unknown aspects of this species' population biology and evolutionary genetics. This is particularly important because the population genomics of this species in Europe has been poorly characterized to date.

We find that European *D. melanogaster* populations exhibit pronounced longitudinal differentiation. We observed this pattern for a genome-wide set of SNPs at 4-fold degenerate sites, which presumably evolve neutrally (Figure 5), as well as for mitochondrial haplotypes, inversions and TEs which might be subject to spatially varying selection (Figure 6 and 7). Longitudinal differentiation might be due to the transition from oceanic to continental climate along the longitudinal axis (Figure 5-Figure 5 supplement 1). While spatial differences in climatic conditions likely play a major role in driving this pattern, we note that it is remarkably similar to that observed for human populations (e.g., Cavalli-Sforza 1966; Xiao et al. 2004; Francalacci & Sanna 2008; Novembre *et al.* 2008). Indeed, east-west structure has been previously found in sub-Saharan Africa populations of *D. melanogaster*, with the split between eastern and western African populations having occurred ~70 kya ago (Michalakis & Veuille 1996; Aulard *et al.* 2002; Kapopoulou *et al.* 2018b), a period that – interestingly – coincides with a wave of human migration from eastern into western Africa (Nielsen *et al.* 2017). However, in contrast to the pronounced pattern observed in Europe, African east-west structure is relatively weak, explaining only ~2.7% of variation, and is due to an inversion whose frequency varies longitudinally. In contrast, our demographic analyses are based on SNPs located in >1 Mb distance from the breakpoints of the most common inversions. This makes it very unlikely that the strong

longitudinal pattern we have observed is driven by inversions.

Spatial patterns of differentiation were stronger for longitude than for latitude. In contrast, differentiation in North America has mainly been observed across latitude, for both neutral and adaptive polymorphisms (e.g., Machado *et al.* 2016; Kapun *et al.* 2016a; reviewed in Adrion *et al.* 2015). Although our present analysis showed that putatively neutral SNPs were primarily differentiated along longitude, latitudinal clines may still exist for adaptive polymorphisms. In fact, we detected latitudinal frequency clines for both inversions and TEs (Table 3 and Table S9). For the inversions *In(3L)P* and *In(3R)Payne*, the observed latitudinal clines were in qualitative agreement with parallel clines reported from North America and Australia, with the inversions decreasing in frequency as distance from the equator increases (Mettler *et al.* 1977; Knibb *et al.* 1981; Leumeunier & Aulard 1992; Fabian *et al.* 2012; Kapun *et al.* 2014; Rane *et al.* 2015; Kapun *et al.* 2016a). This pattern is widely thought to be a result of climate adaptation, with the inversions containing variants that make them better adapted to tropical or subtropical than to temperate, more seasonal climates (e.g., Kapun *et al*. 2016a). Several euchromatic TE insertions also showed geographic (or seasonal) patterns of variation (Table S9), indicating that they might play a role in local adaptation, particularly since many of them are located in regions where they might affect gene regulation. Further, 17 of them also show significant correlations with either geographical or temporal variables in North American populations (Lerat *et al.* 2019). Additionally, several inversions and TEs also exhibited longitudinal gradients.

We also examined signatures of selective sweeps in our data. Several of the identified regions have previously been reported as potential targets of positive selection (Figure 4,

650 Table S6B and SC). However, most of these sweeps were originally identified by analysing
651 a small number of populations (*e.g.* Kolaczkowski *et al.* 2011b; Daborn *et al.* 2002; Rogers
652 & Hartl 2012). Here, we identified 64 genes (including *wapl*, *CR18217*, and *mgl*) which
653 showed clear signatures of selection and which were widespread across Europe, thus
654 strengthening the case for their adaptive significance. In addition, we found several
655 regions with evidence of hard sweeps, some of them showing evidence of local climatic
656 adaptation (Table S6); these candidate regions represent a valuable resource for future
657 analyses of adaptation in European *Drosophila.*
658
659 Finally, our continent-wide analysis of the microbiota suggests that natural populations of
660 European *D. melanogaster* vary greatly in the composition and abundance of microbes
661 and viruses over space and time. Recent work suggests that at least parts of this variation
662 in microbiomes follows geographic patterns (Walters et al 2018, Wang et al 2019) and
663 contribute to phenotypic differences and local adaptation among populations, especially
664 given that there might be tight and presumably local co-evolutionary interactions between
665 fly hosts and their endosymbionts (e.g., Haselkorn *et al.* 2009; Richardson *et al.* 2012;
666 Staubach *et al.* 2013; Kriesner *et al.* 2016; Wang and Staubach 2018). Most notably, we
667 discovered five new DNA viruses of *D. melanogaster*. Despite this species being host to a
668 wide diversity of RNA viruses, we now have found that the DNA viruses of *D.*
669 *melanogaster* are also widespread, for instance with *Kallithea* virus detected in most
670 populations.
671
672 Our study demonstrates that sampling on a continent-wide scale and pooled sequencing
673 of a large number of natural populations can reveal fundamental and novel aspects of
674 population biology, even for a well-studied model species such as *D. melanogaster*. Our

675 extensive sampling was feasible only due to synergistic collaboration among many
676 research groups. Our efforts in Europe are paralleled in North America by the *Dros-RTEC*
677 consortium, with whom we are collaborating to compare population genomic data across
678 continents. Together, we have sampled both continents annually since 2014; we aim to
679 continue to sample and sequence European and North American *Drosophila* populations
680 with increasing spatio-temporal resolution in future years. With these efforts we hope to
681 provide a rich community resource for biologists interested in molecular population
682 genetics and adaptation genomics.
683

684 ## Materials and methods

685 The 2014 *DrosEU* dataset represents the most comprehensive spatio-temporal sampling
686 of European *D. melanogaster* populations to date (Table 1). It comprises 48 samples of *D.*
687 *melanogaster* collected from 32 geographical locations across Europe at different time
688 points in 2014 through a joint effort of 18 research groups. Collections were mostly
689 performed with baited traps using a standardized protocol (see Supplementary File 2).
690 From each collection, we pooled 33–40 wild-caught males. We used males as they are
691 more easily distinguishable morphologically from similar species than females. Despite our
692 precautions, we identified a low level of *D. simulans* contamination in our sequences; we
693 computationally filtered these sequences from the data prior to further analysis (see
694 below).
695

696 **DNA extraction, library preparation and sequencing**

697 We extracted DNA from each sample after homogenization with bead beating and
698 standard phenol/chloroform extraction. A detailed extraction protocol can be found in the
699 Supplementary File 2. In preparation for sequencing, 500 ng of DNA from each sample

was sheared with a *Covaris* instrument (Duty cycle 10, intensity 5, cycles/burst 200, time 30). Library preparation was performed using *NEBNext Ultra DNA Lib Prep-24* and *NebNext Multiplex Oligos* for Illumina-24 following the manufacturer's instructions. Each sample was sequenced as a pool (Pool-Seq; Schlötterer *et al.* 2014), as paired-end fragments on a *Illumina NextSeq 500* sequencer at the Genomics Core Facility of Pompeu Fabra University. Samples were multiplexed in 5 batches of 10 samples, except for one batch of 8 samples (Table S1). Each multiplexed batch was sequenced on 4 lanes at ~50x raw coverage per sample. The read length was 151 bp, with a median insert size of 348 bp (range 209-454 bp). The data are available from NCBI Bioproject PRJNA388788.

Mapping pipeline and variant calling

Prior to mapping, we trimmed and filtered raw FASTQ reads to remove low-quality bases (minimum base PHRED quality = 18; minimum sequence length = 75 bp) and sequencing adaptors using *cutadapt* (v. 1.8.3; Martin 2011). We retained only pairs for which both reads fulfilled our quality criteria after trimming. FastQC analyses of trimmed and quality filtered reads showed overall high base-qualities (median range 29-35), with ~1.36% of bases lost after trimming. We used *bwa mem* (v. 0.7.15; Li 2013) with default parameters to map the trimmed reads. To avoid paralogous mapping, we mapped to a compound reference, consisting of the genomes of *D. melanogaster* (v.6.12) and common commensals and pathogens, including *Saccharomyces cerevisiae* (GCF_000146045.2), *Wolbachia pipientis* (NC_002978.6), *Pseudomonas entomophila* (NC_008027.1), *Commensalibacter intestine* (NZ_AGFR00000000.1), *Acetobacter pomorum* (NZ_AEUP00000000.1), *Gluconobacter morbifer* (NZ_AGQV00000000.1), *Providencia burhodogranariea* (NZ_AKKL00000000.1), *Providencia alcalifaciens* (NZ_AKKM01000049.1), *Providencia rettgeri* (NZ_AJSB00000000.1), *Enterococcus*

faecalis (NC_004668.1), *Lactobacillus brevis* (NC_008497.1), and *Lactobacillus plantarum* (NC_004567.2). We used Picard (v.1.109; http://picard.sourceforge.net) to remove duplicate reads and reads with a mapping quality below 20. In addition, we re-aligned sequences flanking indels with GATK (v3.4-46; McKenna *et al.* 2010).

After mapping, we filtered reads due to *D. simulans* contamination, using the method of Bastide *et al*. (2013). To do this, we used fixed differences between *D. simulans* and *D. melanogaster* to identify reads from *D. simulans*. For the nine samples that had a contamination level > 1% (range 1.2 - 8.7%; Table S1), we used custom software to remove reads that mapped preferentially to the *D. simulans* genome (Hu *et al.* 2013) using competitive mapping to references from both species. After applying our decontamination pipeline, contamination levels dropped below 0.4 % for all nine samples.

We used *Qualimap* (v. 2.2., Okonechnikov *et al.* 2016) to evaluate average mapping qualities per population and chromosome, which ranged from 58.3 to 58.8 (Table S1). Sequencing depth ranged from 34x to 115x for autosomes and from 17x to 59x for *X*-chromosomes (Table S1). We then combined individual *bam* files from all samples into a single *mpileup* file using *samtools* (v. 1.3; Li & Durbin 2009). Due to the large number of samples, we implemented quality control criteria for all libraries jointly to call SNPs. To call SNPs, we developed custom software (*PoolSNP*; see Supplementary File 2; available at doi: https://doi.org/10.5061/dryad.rj1gn54) using stringent heuristic parameters: (1) minimum coverage 10x for each sample, (2) maximum coverage < 95th coverage percentile for a given chromosome and sample (to avoid paralogous regions duplicated in the sample but not in the reference), (3) for each allele, a minimum read count > 20x and a minimum read frequency > 0.001, across all samples pooled. These parameters were

optimized based on simulated Pool-Seq data to maximize true positives and minimize false positives (Supplementary File 2). We also excluded SNPs (1) for which more than 20% of all samples did not fulfil the above-mentioned coverage thresholds, (2) which were located within 5 bp of an indel with a minimum count larger than 10x in all samples pooled, and (3) which were located within known TEs based on the *D. melanogaster* TE library v.6.10. We annotated our final set of SNPs with *SNPeff* (v.4.2; Cingolani *et al.* 2012) using the Ensembl genome annotation version BDGP6.82.

Additional samples

We obtained genome sequences from African flies from the *Drosophila* Genome Nexus (DGN; http://www.johnpool.net/genomes.html; see Table S5 for SRA accession numbers). We used data from 14 individuals from Rwanda and 40 from Siavonga (Zambia). We mapped these data as described above and built consensus sequences for each haploid sample by only considering alleles with > 0.9 allele frequencies. We converted consensus sequences to *VCF* and used *VCFtools* (Danecek et al. 2011) for downstream analyses.

Genetic variation in Europe

We characterized patterns of genetic variation among the 48 samples for the five major chromosomal arms (*X*, *2L*, *2R*, *3L*, *3R*) by estimating π, Watterson's θ and Tajima's *D* (Watterson 1975; Nei 1987; Tajima 1989), using corrections for Pool-Seq data (Kofler *et al.* 2011). To perform these analyses for our set of SNPs, we re-implemented the methods of Kofler *et al*. (2011) in Python (PoolGen; doi: https://doi.org/10.5061/dryad.rj1gn54). To calculate unbiased window-wise estimates of parameters, we used an output file of our SNP calling pipeline (*PoolSNP*; doi: https://doi.org/10.5061/dryad.rj1gn54), which indicates for any given site in the reference, if it passed the filtering parameters used for SNP

775 calling. These data allow for the calculation of the effective window-size, which is the
776 difference between the total window-size and the number of sites that did not pass the
777 quality criteria. Using effective windows-sizes as the denominator for the calculation of
778 window-wise averages yields unbiased average estimates. In contrast, dividing the
779 summed statistics in a given window by the total window-size, which is common practice in
780 most software tools, results in an underestimation of averaged parameters. Before
781 calculating the estimators, we subsampled the data to an even coverage of 40x for
782 autosomes and 20x for the *X*-chromosome, as Watterson's θ and Tajima's *D* are sensitive
783 to coverage variation (Korneliussen *et al.* 2013). We calculated chromosome-wide
784 averages of π, θ and Tajima's *D* for autosomes and *X* chromosomes using *R* (R
785 Development Core Team 2009). We tested for correlations between these estimators and
786 latitude, longitude, altitude, and season using a linear regression model: $y_i = Lat + Lon +$
787 $Alt + Season + \varepsilon_i$, where y_i represents π, θ or *D*. We used *Lat*, *Lon* and *Alt* as continuous
788 predictors (Table 1) and *Season* as a categorical factor with two levels, corresponding to
789 collection dates before and after 1st September ('summer' and 'fall'), respectively, following
790 Bergland *et al.* (2014) and Kapun *et al.* (2016a). To test for residual spatio-temporal
791 autocorrelation among the samples (Kühn & Dormann 2012), we calculated Moran's *I*
792 (Moran 1950) with the *R* package *spdep* (v.06-15., Bivand & Piras 2015) for the residuals
793 of the above models. For this analysis, we considered samples within 10° latitude /
794 longitude to be neighbours, based on the pairwise geographical distances between
795 collection locations. Whenever these tests revealed significant autocorrelations indicating
796 non-independence, we repeated the above regressions using a spatial weights matrix
797 based on nearest neighbours as described above to test for remaining spatial patterning in
798 residuals as implemented in *spdep*. We also fitted models with run ID as a random factor
799 using the *R* package *lme4* (v.1.1-14; see Supplementary File 2) to test for confounding

effects of variation in error rates among sequencing runs. As these models did not fit significantly better than simpler models, we excluded it from final analysis (see Supplementary File 2 and Table S3).

To investigate genome-wide patterns of variation, we averaged π, θ, and *D* in 200 kb non-overlapping windows for each sample and chromosomal arm separately and plotted the distributions in *R.* In addition, to investigate fine-scale deviations from neutral expectations, we also calculated Tajima's *D* in 50 kb sliding windows with a step size of 10 kb. We normalized diversity statistics using log-transformation and tested for correlations between π and recombination rate for 100 kb non-overlapping windows in *R* and plotted these data using the *ggplot2* (v.2.2.1., Wickham 2016). We used both fine-scale (Comeron *et al.* 2012) and broad-scale (Fiston-Lavier *et al.* 2010) estimates of recombination rate, after converting their coordinates to reference genome v 6.

To identify regions under selection, we used *Pool-hmm* to calculate the SFS (Site Frequency Spectrum) for each sample in the *pileup* format file with the following parameters *–prefix* (to assign a name to each sample), *-n* (number of chromosomes), *--only-spectrum* (for the SFS calculation), *--theta* 0.005 (default), and *-r* 100 (subsampling of 1/100 SNPs). We then split the *pileups* by chromosome and ran *Pool-hmm* with the following parameters: *--prefix*, *-n*, *-k* (per site transition probability between hidden states), *-s* (frequency spectrum file from previous step) and *-e sanger* (*Phred quality* = 33). For the 18 samples for which Tajima's *D* was very low, *Pool-hmm* identified the majority of the genome to be under selection; we thus removed those samples from our analysis. We used three different *k parameters* depending on the sample: $k=1e^{-10}$, $k=1e^{-30}$, and $k=1e^{-40}$ (Table S6A). For windows with significantly low Tajima's D in euchromatic regions, we

identified genes using *bedtools intersect* (v2.27.1) and the *D. melanogaster* v6.12 annotation file from Flybase (Thurmond et al 2019). For genes significant in all populations, we checked whether average Tajima's *D* was among the lowest 10% per chromosome. We tested for enrichment of involvement in particular biological processes using *DAVID* with default parameters (Huang et al 2009).

Genetic differentiation and population structure in European populations

To estimate genome-wide pairwise genetic differences, we used custom software to estimate SNP-wise F_{ST} using the approach of Weir and Cockerham (1984) for all pairwise combinations of samples. For each sample, we averaged pairwise F_{ST} between that sample and the other 47 samples and ranked the 48 population samples by overall differentiation.

We inferred demographic patterns by focusing on putatively neutrally evolving SNPs. For this, we used either 4-fold degenerate sites (defined using the genome sequences and the annotation features of the *D. melanogaster* reference genome version 6.12) or short introns (<60 bp; Haddrill *et al.* 2005; Singh *et al.* 2009; Parsch *et al.* 2010; Clemente & Vogl 2012; Lawrie *et al.* 2013). We also restricted our analyses to SNPs that were at least 1 Mb distant from major chromosomal inversions (see below) and those located in genomic regions with high recombination rates (r > 3cM/Mb; Comeron et al. 2012) to minimize the effects of linkage, which may confound analyses of neutral evolution. As the Sheffield (UK) population showed unusually high differentiation from other populations, we repeated the following analyses without the Sheffield sample. To assess isolation by distance (IBD), we averaged pairwise F_{ST} values across all neutral markers. We calculated geographic distance using the haversine formula (Green & Smart 1985), which takes the

spherical curvature of the planet into account. We tested for correlations between linearized genetic differentiation (Slatkin's distance: $F_{ST}/([1-F_{ST}])$ and $\log_{10}$-scaled geographic distance (Slatkin 1985) using Mantel tests implemented in *ade4* (v.1.7-8., Dray & Dufour 2007) with 1,000,000 iterations. In addition, we plotted the 5% smallest and largest F_{ST} values from all 1,128 pairwise comparisons among the 48 population samples onto a map to visualize geographic patterns of genetic differentiation.

We tested for population substructure using two different approaches. First, we performed principal component analysis (PCA) based on unscaled allele frequencies of the neutral marker SNPs, as suggested by Menozzi *et al*. (1978) and Novembre and Stephens (2008), using *LEA* (v. 1.2.0., Frichot *et al.* 2013). We focused on the first three principal components (PCs) and used *mclust* (v. 5.2., Fraley & Raftery 2012) to estimate the number of clusters *via* maximum likelihood and assigned population samples to clusters *via k*-means. In addition, we examined the first three PCs for correlations with latitude, longitude, altitude, and season using general linear models and tested for spatial autocorrelation as above. A Bonferroni-corrected α threshold ($\alpha' = 0.05/3 = 0.017$) was used to correct for multiple testing.

In a second, complementary approach, we inferred population delineation using model-based clustering as implemented in *ConStruct* (v.1.0.2; Bradburd *et al.* 2018). In contrast to most clustering-based methods, *ConStruct* incorporates continuous isolation by distance to avoid inflating estimates of the number of clusters and allows estimating admixture among populations. We ran spatial models with three MCMC chains per run and 10,000 iterations and compared the goodness of fit for models incorporating 1 to 10 spatial layers by cross-validation.

875

876 **Mitochondrial DNA**

877 To obtain consensus mitochondrial sequences for each of the 48 European populations,
878 we aligned reads from individual FASTQ files and replaced minor variants with the major
879 variant using *Coral* (Salmela & Schröder 2011). This method prevents ambiguities from
880 interfering with the assembly process. We assembled a genome for each population from
881 the modified FASTQ files using *SPAdes* with standard parameters and *k*-mers of size 21,
882 33, 55, and 77 (Bankevich *et al.* 2012). Mitochondrial contigs were retrieved by *blastn*,
883 using the *D. melanogaster* NC 024511 sequence as a query and each genome assembly
884 as the database. To avoid nuclear mitochondrial DNA segments (numts), we ensured that
885 only contigs with a higher than average coverage of the genome were retrieved. When
886 multiple contigs were available for the same region, the one with the highest coverage was
887 selected. Possible contamination with *D. simulans* was assessed by looking for two or
888 more consecutive sites that show the same variant as *D. simulans* and looking for
889 alternative contigs for that region with similar coverage. As an additional quality control
890 measure, we also examined the presence of pairs of sites showing four gametic types
891 using *DNAsp* 6 (Rozas *et al.* 2017) – given that there is no recombination in mitochondrial
892 DNA no such sites are expected. The very few sites presenting such features were
893 rechecked by looking for alternative contigs for that region and were corrected if needed.
894 The uncorrected raw reads for each population were mapped on top of the different
895 consensus haplotypes using *Express* as implemented in *Trinity* (Grabherr *et al.* 2011). If
896 most reads for a given population mapped to the consensus sequence derived for that
897 population the consensus sequence was retained, otherwise it was discarded as a
898 possible chimera between different mitochondrial haplotypes. The repetitive mitochondrial
899 hypervariable region is difficult to assemble and was therefore not used; the mitochondrial

region was thus analysed as in Cooper *et al*. (2015). Mitochondrial genealogy was estimated using statistical parsimony (TCS network; Clement *et al.* 2000), as implemented in *PopArt* (http://popart.otago.ac.nz), and the surviving mitochondrial haplotypes. Frequencies of the different mitochondrial haplotypes were estimated from FPKM values using the surviving mitochondrial haplotypes and expressed as implemented in *Trinity* (Grabherr *et al.* 2011).

Transposable elements

To quantify transposable element (TE) abundance in each sample, we assembled and quantified repeats from unassembled sequenced reads using *dnaPipeTE* (v.1.2., Goubert *et al.* 2015). Only the left read of each pair were used. As the vast majority of high-quality trimmed reads were longer than 135 bp, we discarded reads shorter than this before sampling. Reads matching mtDNA were filtered out by mapping to the *D. melanogaster* reference mitochondrial genome (NC_024511.2. 1) with *bowtie2* (v. 2.1.0., Langmead & Salzberg 2012). Prokaryotic sequences, including reads from symbiotic bacteria such as *Wolbachia*, were filtered out from the reads using the implementation of *blastx vs.* the non-redundant protein database (nr) using *DIAMOND* (v. 0.8.7, Buchfink *et al.* 2015). To quantify TE content, we subsampled a proportion of the raw reads (after filtering) corresponding to a genome coverage of 0.1X (assuming a genome size of 175 MB), and then assembled these reads with *Trinity* (Grabherr *et al.* 2011). Due to the low coverage of the genome obtained with the subsampled reads, only repetitive DNA present in multiple copies should be fully assembled (Goubert *et al.* 2015). To assess the constancy of the estimates, we repeated this process with three iterations per sample, as recommended by the program guidelines.

We further estimated frequencies of TEs present in the reference genome with *T-lex2* (v. 2.2.2., Fiston-Lavier *et al.* 2015), using all annotated TEs (5,416 TEs) in version 6.04 of the *D. melanogaster* genome from flybase.org (Gramates *et al.* 2017). For 108 of these TEs, we used the corrected coordinates as described in Fiston-Lavier *et al*. (2015), based on the identification of target site duplications at the site of the insertion. We excluded TEs nested or flanked by other TEs (<100 bp on each side of the TE), and TEs, which are part of segmental duplications, since *T-lex2* does not provide accurate frequency estimates in complex regions (Fiston-Lavier *et al.* 2015). We additionally excluded the INE-1 TE family, as this TE family is ancient, with 2,234 insertions in the reference genome, which appear to be mostly fixed (Kapitonov & Jurka 2003). After applying these filters, we were able to estimate frequencies of 1,630 TE insertions from 113 families from the three main orders, LTR, non-LTR, and DNA across all *DrosEU* samples. Because the mapper used by *T-lex2* to detect the presence of insertions (presence module) only accepts reads ≤127 bp, we trimmed reads longer than 100 bp into two equally sized fragments using *Trimmomatic* (v. 0.35; Bolger *et al.* 2014) with the CROP and HEADCROP parameters.

To avoid inaccurate TE frequency estimates due to very low numbers of reads, we only considered frequency estimates based on at least 3 reads. Despite the stringency of *T-lex2* to select only high-quality reads, we additionally discarded frequency estimates supported by more than 90 reads, i.e. 3 times the average coverage of the sample with the lowest coverage (CH_Cha_14_43, Table S1), in order to avoid non-uniquely mapping reads. This filtering allows to estimate TE frequencies for ~96% (92.9% to 97.8%) of the TEs in each population. For 85% of the TEs, we were able to estimate their frequencies in more than 44 out of 48 *DrosEU* samples.

We tested for correlations between TE insertion frequencies and recombination rates using Spearman's rank correlations as implemented in *R*. For SNPs, we used

recombination rates from Comeron *et al*. (2012) and from Fiston-Lavier *et al.* (2010) in non-overlapping 100 kb windows and assigned to each TE insertion the recombination rate of the corresponding window.

To test for spatio-temporal variation of TE insertions, we excluded TEs with an interquartile range (IQR) < 10. We tested the population frequencies of the remaining 141 insertions for correlations with latitude, longitude, altitude, and season using generalized linear models (ANCOVA) following the method used for SNPs but with a binomial error structure in *R*. We further tested if significant correlations with either of the predictor variables deviated from expectations under neutral evolution. To this end, we repeated the ANCOVA analyses on 8,727 presumably neutrally evolving 4-fold degenerate sites that we described previously in the demographic analyses. Based on *F*-ratios obtained from the ANCOVA models for each neutral SNP and predictor, we built empirical density functions and calculated empirical *p*-values for each TE by integrating over the area of the curve that is delineated by the *F*-value specific for the given TE and the maximum *F*-ratio in the neutral dataset.

We also tested for residual spatio-temporal autocorrelations in TE insertion frequencies, with Moran's *I* test (Moran 1950; Kühn & Dormann 2012). We used Bonferroni corrections to account for multiple testing (α'= 0.05/141 = 0.00035) and only considered Bonferroni-corrected p-values < 0.001 to be significant. To test TE family enrichment among the significant TEs we performed a χ^2 test and applied Yate's correction to account for the low number of some of the cells.

Inversion polymorphisms

Since Pool-Seq data precludes a direct assessment of the presence and frequencies of chromosomal inversions, we indirectly estimated inversion frequencies using a panel of

approximately 400 inversion-specific marker SNPs (Kapun *et al.* 2014) for six cosmopolitan inversions (*In(2L)t, In(2R)NS, In(3L)P, In(3R)C, In(3R)Mo, In(3R)Payne*). We averaged allele frequencies of these markers in each sample separately. To test for clinal variation in the frequencies of inversions, we tested for correlations with latitude, longitude, altitude and season using generalized linear models with a binomial error structure in *R* to account for the biallelic nature of karyotype frequencies. In addition, we Bonferroni-corrected the *α* threshold (*α*'= 0.05/7 = 0.007) to account for multiple testing, accounted for residual spatio-temporal autocorrelations and tested if *F*-ratios of the ANCOVAs deviated from neutral expectations as explained above.

Microbiome

Raw sequences were trimmed, and quality filtered as described for the genomic data analysis. The remaining high-quality sequences were mapped against the *D. melanogaster* genome (v.6.04) including mitochondria using *bbmap* (v. 35; Bushnell 2016) with standard settings. The unmapped sequences were submitted to the online classification tool, *MGRAST* (Meyer *et al.* 2008) for annotation. Taxonomy information was downloaded and analysed in *R* (v. 3.2.3; R Development Core Team 2009) using the *matR* (v. 0.9; Braithwaite & Keegan) and RJSONIO (v. 1.3; Lang) packages. Metazoan sequence features were removed. For microbial load comparisons, the number of protein features identified by *MGRAST* for each taxon and sample was divided by the number of sequences that mapped to *D. melanogaster* chromosomes *X*, *Y, 2L*, *2R*, *3L*, *3R* and *4*.

We also surveyed the datasets for the presence of novel DNA viruses by performing *de novo* assembly of the non-fly reads using *SPAdes* 3.9.0 (Bankevich *et al.* 2012) and using conceptual translations to query virus proteins from *Genbank* using *DIAMOND* '*blastp*'

(Buchfink *et al.* 2015). In three cases (*Kallithea* virus, *Vesanto* virus, *Viltain* virus), reads from a single sample pool were sufficient to assemble a (near) complete genome. In two other cases, fragmentary assemblies allowed us to identify additional publicly available datasets that contained sufficient reads to complete the genomes (*Linvill Road* virus, *Esparto* virus; completed using SRA datasets SRR2396966 and SRR3939042, respectively). Novel viruses were provisionally named based on the localities where they were first detected, and the corresponding novel genome sequences were submitted to Genbank (KX130344, KY608910, KY457233, KX648533-KX648536). To assess the relative amount of viral DNA, unmapped (non-fly) reads from each sample pool were mapped to repeat-masked *Drosophila* DNA virus genomes using *bowtie2*, and coverage normalized relative to virus genome length and the number of mapped *Drosophila* reads.

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Tables

Table 1. Sample information for all populations in the *DrosEU* dataset. Origin, collection date, season and sample size (number of chromosomes: *n*) of the 48 samples in the *DrosEU* 2014 data set. Additional information can be found in Table S1.

ID	Country	Location	Coll. Date	Number ID	Lat (°)	Lon (°)	Alt (m)	Season	n	Coll. name
AT_Mau_14_01	Austria	Mauternbach	2014-07-20	1	48.38	15.56	572	S	80	Andrea J. Betancourt
AT_Mau_14_02	Austria	Mauternbach	2014-10-19	2	48.38	15.56	572	F	80	Andrea J. Betancourt
TR_Yes_14_03	Turkey	Yesiloz	2014-08-31	3	40.23	32.26	680	S	80	Banu Sebnem Onder
TR_Yes_14_04	Turkey	Yesiloz	2014-10-23	4	40.23	32.26	680	F	80	Banu Sebnem Onder
FR_Vil_14_05	France	Viltain	2014-08-18	5	48.75	2.16	153	S	80	Catherine Montchamp-Moreau
FR_Vil_14_07	France	Viltain	2014-10-27	7	48.75	2.16	153	F	80	Catherine Montchamp-Moreau
FR_Got_14_08	France	Gotheron	2014-07-08	8	44.98	4.93	181	S	80	Cristina Vieira
UK_She_14_09	United Kingdom	Sheffield	2014-08-25	9	53.39	-1.52	100	S	80	Damiano Porcelli
UK_Sou_14_10	United Kingdom	South Queensferry	2014-07-14	10	55.97	-3.35	19	S	80	Darren Obbard
CY_Nic_14_11	Cyprus	Nicosia	2014-08-10	11	35.07	33.32	263	S	80	Eliza Argyridou
UK_Mar_14_12	United Kingdom	Market Harborough	2014-10-20	12	52.48	-0.92	80	F	80	Eran Tauber
UK_Lut_14_13	United Kingdom	Lutterworth	2014-10-20	13	52.43	-1.10	126	F	80	Eran Tauber

DE_Bro_14_14	Germany	Broggingen	2014-06-26	14	48.22	7.82	173	S	80	Fabian Staubach
DE_Bro_14_15	Germany	Broggingen	2014-10-15	15	48.22	7.82	173	F	80	Fabian Staubach
UA_Yal_14_16	Ukraine	Yalta	2014-06-20	16	44.50	34.17	72	S	80	Iryna Kozeretska
UA_Yal_14_18	Ukraine	Yalta	2014-08-27	18	44.50	34.17	72	S	80	Iryna Kozeretska
UA_Ode_14_19	Ukraine	Odesa	2014-07-03	19	46.44	30.77	54	S	80	Iryna Kozeretska
UA_Ode_14_20	Ukraine	Odesa	2014-07-22	20	46.44	30.77	54	S	80	Iryna Kozeretska
UA_Ode_14_21	Ukraine	Odesa	2014-08-29	21	46.44	30.77	54	S	80	Iryna Kozeretska
UA_Ode_14_22	Ukraine	Odesa	2014-10-10	22	46.44	30.77	54	F	80	Iryna Kozeretska
UA_Kyi_14_23	Ukraine	Kyiv	2014-08-09	23	50.34	30.49	179	S	80	Iryna Kozeretska
UA_Kyi_14_24	Ukraine	Kyiv	2014-09-08	24	50.34	30.49	179	F	80	Iryna Kozeretska
UA_Var_14_25	Ukraine	Varva	2014-08-18	25	50.48	32.71	125	S	80	Oleksandra Protsenko
UA_Pyr_14_26	Ukraine	Pyriatyn	2014-08-20	26	50.25	32.52	114	S	80	Oleksandra Protsenko
UA_Dro_14_27	Ukraine	Drogobych	2014-08-24	27	49.33	23.50	275	S	80	Iryna Kozeretska
UA_Cho_14_28	Ukraine	Chornobyl	2014-09-13	28	51.37	30.14	121	F	80	Iryna Kozeretska
UA_Cho_14_29	Ukraine	Chornobyl Yaniv	2014-09-13	29	51.39	30.07	121	F	80	Iryna Kozeretska
SE_Lun_14_30	Sweden	Lund	2014-07-31	30	55.69	13.20	51	S	80	Jessica Abbott
DE_Mun_14_31	Germany	Munich	2014-06-19	31	48.18	11.61	520	S	80	John Parsch
DE_Mun_14_32	Germany	Munich	2014-09-03	32	48.18	11.61	520	F	80	John Parsch
PT_Rec_14_33	Portugal	Recarei	2014-09-26	33	41.15	-8.41	175	F	80	Jorge Vieira

ES_Gim_14_34	Spain	Gimenells (Lleida)	2014-10-20	34	41.62	0.62	173	F	80	Lain Guio
ES_Gim_14_35	Spain	Gimenells (Lleida)	2014-08-13	35	41.62	0.62	173	S	80	Lain Guio
FI_Aka_14_36	Finland	Akaa	2014-07-25	36	61.10	23.52	88	S	80	Maaria Kankare
FI_Aka_14_37	Finland	Akaa	2014-08-27	37	61.10	23.52	88	S	80	Maaria Kankare
FI_Ves_14_38	Finland	Vesanto	2014-07-26	38	62.55	26.24	121	S	66	Maaria Kankare
DK_Kar_14_39	Denmark	Karensminde	2014-09-01	39	55.95	10.21	15	F	80	Mads Fristrup Schou
DK_Kar_14_41	Denmark	Karensminde	2014-11-25	41	55.95	10.21	15	F	80	Mads Fristrup Schou
CH_Cha_14_42	Switzerland	Chalet à Gobet	2014-07-24	42	46.57	6.70	872	S	80	Martin Kapun
CH_Cha_14_43	Switzerland	Chalet à Gobet	2014-10-05	43	46.57	6.70	872	F	80	Martin Kapun
AT_See_14_44	Austria	Seeboden	2014-08-17	44	46.81	13.51	591	S	80	Martin Kapun
UA_Kha_14_45	Ukraine	Kharkiv	2014-07-26	45	49.82	36.05	141	S	80	Svitlana Serga
UA_Kha_14_46	Ukraine	Kharkiv	2014-09-14	46	49.82	36.05	141	F	80	Svitlana Serga
UA_Cho_14_47	Ukraine	Chornobyl Applegarden	2014-09-13	47	51.27	30.22	121	F	80	Svitlana Serga
UA_Cho_14_48	Ukraine	Chornobyl Polisske	2014-09-13	48	51.28	29.39	121	F	70	Svitlana Serga
UA_Kyi_14_49	Ukraine	Kyiv	2014-10-11	49	50.34	30.49	179	F	80	Svitlana Serga
UA_Uma_14_50	Ukraine	Uman	2014-10-01	50	48.75	30.21	214	F	80	Svitlana Serga
RU_Val_14_51	Russia	Valday	2014-08-17	51	57.98	33.24	217	S	80	Elena Pasyukova

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Table 2. **Clinality of genetic variation and population structure.** Effects of geographic variables and/or seasonality on genome-wide average levels of diversity (π, θ and Tajima's D; top rows) and on the first three axes of a PCA based on allele frequencies at neutrally evolving sites (bottom rows). The values represent F-ratios from general linear models. Bold type indicates F-ratios that are significant after Bonferroni correction (adjusted α'=0.0055). Asterisks in parentheses indicate significance when accounting for spatial autocorrelation by spatial error models. These models were only calculated when Moran's I test, as shown in the last column, was significant. *p < 0.05; **p < 0.01; ***p < 0.001.

Factor	***Latitude***	***Longitude***	***Altitude***	***Season***	***Moran's I***
$\pi_{(X)}$	4.11*	1.62	**15.23*** **	1.65	0.86
$\pi_{(Aut)}$	0.91	2.54	**27.18*** **	0.16	-0.86
$\theta_{(X)}$	2.65	1.31	**15.54*** **	2.22	0.24
$\theta_{(Aut)}$	0.48	1.44	**13.66*** **	0.37	-1.13
$D_{(X)}$	0.02	0.38	5.93*	3.26	-2.08
$D_{(Aut)}$	0.09	0.76	5.33*	0.71	-1.45
PC1	0.06	**120.72*** (***)**	5.35*(*)	2.53	4.15***
PC2	3.5	**10.22** **	**15.21*** **	1.97	-1.96
PC3	0.14	0.11	0.01	1.29	0.22

Table 3. **Clinality and/or seasonality of chromosomal inversions.** The values represent *F*-ratios from generalized linear models with a binomial error structure to account for frequency data. Bold type indicates deviance values that were significant after Bonferroni correction (adjusted *α'*=0.0071). Stars in parentheses indicate significance when accounting for spatial autocorrelation by spatial error models. These models were only calculated when Moran's *I* test, as shown in the last column, was significant. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

Factor	***Latitude***	***Longitude***	***Altitude***	***Season***	***Moran's I***
In(2L)t	2.2	**10.09****	**43.94*****	0.89	-0.92
In(2R)NS	0.25	**14.43*****	2.88	2.43	1.25
In(3L)P	**21.78*****	2.82	0.62	3.6	-1.61
In(3R)C	**18.5***(***)**	0.75	1.42	0.04	2.79**
In(3R)Mo	0.3	0.09	0.35	0.03	-0.9
In(3R)Payne	**43.47*****	0.66	1.69	1.55	-0.89

Supplementary Files

Supplementary File 1. Supplementary Tables.

This file contains the 13 supplementary tables mention in the text.

Supplementary File 2. Additional methods.

This file contains the additional methods mention in the text.