Seasonal Changes in the Long-Distance Linkage Disequilibrium in *Drosophila melanogaster*

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Abstract

Seasonal environmental changes have the potential to influence the genetic structure of species with a short generation time, such as *Drosophila*. We previously found the seasonal change in linkage disequilibrium (LD) between the chemoreceptor (*Cr*) genes in a local Japanese population (Kyoto [KY]). This could be caused by fluctuation in the population size or selection in temporally heterogeneous environments or both. Here, we analyzed the scale of LD between 51 X-linked polymorphisms (10 *Cr* and 41 non-*Cr* gene markers) in the 2 seasonal samples from the KY population and an autumn sample from 106 localities in and around Japan (Ja03au). Many of the non-*Cr* genes have receptor function but fewer functional connections to each other. The magnitude of LD in Ja03au did not significantly differ from that in the KY autumn sample. The lack of local differentiation was confirmed in an autumn sample from another local Japanese population. On the other hand, the magnitude of LD was significantly larger in spring than in autumn in the 2 independent KY samples. This suggests that reduction in the population size during winter increased the magnitude of LD in spring in the mainland population in Japan. Long-distance LD could be a useful measure for assessing seasonal fluctuation in effective population size.

Key words: *Drosophila*, linkage disequilibrium, population bottleneck, population structure, seasonal change

Seasonal environmental changes have the potential to influence the genetic structure of species with a short generation time, such as *Drosophila*. Seasonal changes in the frequency of chromosomal inversions have long been known in *Drosophila* species, and it was proposed that they were caused by varying selection (Dobzhansky 1948, 1970). Furthermore, the population size likely varies seasonally, which can also affect the genetic structure of the population. In spite of extensive surveys of the genetic structure of natural populations of several *Drosophila* species, relatively little is known about seasonal fluctuation of population size (Lumme and Lakovaara 1983). A better assessment of the impact of seasonality on the genetic structure can deepen our understanding of currently acting selection and the causes of within-species variation.

The impact of random genetic drift in natural populations has been inferred from changes in allele frequency over generations (e.g., Fisher and Ford 1947; Schaffer et al. 1977; Mueller et al. 1985). Such studies require multiple samples for a single estimation, and thus, a seasonal comparison of their magnitudes is difficult. A recent population bottleneck can be tested by distortion of allele
frequency distribution from the neutrality (Cornuet and Luikart 1996; Luikart et al. 1998). However, this test is unlikely able to monitor short-term changes such as seasonal fluctuation because it would take a long time to recover the frequency distribution.

In this context, nonrandom association of polymorphisms at different sites, that is, linkage disequilibrium (LD), may serve as an alternative measure. LD can arise from physical linkage, finite population size, admixture and structure of populations, or epistatic natural selection, whereas it gradually decays by recombination. Therefore, long-distance LD is smaller in magnitude than short-distance LD and less dependent on the past history, the initial state of polymorphisms (ancestral haplotype), and their respective ages. Generally, in Drosophila, little LD is observed between pairs of sites separated by >2 kb (e.g., Miyashita and Langley 1988), except when polymorphic inversions are involved (e.g., Mukai et al. 1971; Charlesworth B and Charlesworth D 1973; Langley et al. 1974). This low level of background LD makes interlocus LD more sensitive to short-term demographic changes, and we may be able to find signatures of seasonal change in the genetic structure in terms of long-distance LD.

We previously found a greater amount of LD between polymorphisms in the 98 Drosophila chemoreceptor (Cr) genes in a spring sample compared with an autumn sample and a significant excess of associations between one frequent and one less common allele only for replacement polymorphisms in the spring sample (Takano-Shimizu et al. 2004). It seems unlikely that these were simply caused by seasonal bottlenecks associated with overwinter mortality. Because the Cr genes could be functionally associated with one another, we inferred that epistatic selection on these genes, in combination with bottleneck, was responsible for the seasonal changes in scale of LD. This can be tested by studying genes that are functionally independent to each other. Under this hypothesis, seasonal changes in LD for such genes are expected to be smaller than those for functionally connected genes.

Here, we report LDs between 51 polymorphisms at 50 X-linked genes. These genes are distributed throughout the X chromosome and mostly loosely linked. Most of them have no obvious functional connection to one another. In addition, no inversion polymorphism is known to exist on the X chromosome. Thus, we could ignore these potentially confounding effects. The main objective of this study is to test whether the seasonality is unique to the Cr genes or a common feature of genes on the X chromosome. Other objectives are to examine the differences in LD between polymorphisms at replacement and silent sites and to evaluate the genetic structure of extant populations. For the latter purpose, we used a structured sample (i.e., a collection of 1 or 2 males from each of 106 localities in and around Japan) in addition to samples from local populations with different population densities. Population structure is expected to increase LD, and then its effect may be detected by comparing the amount of LD between such samples. The present study revealed that the seasonal change of the interlocus LD was common to the X-linked genes and that the scale of LD was little affected by sampling strategy. Long-distance LD could be a useful index for detecting short-term changes in effective population size.

Materials and Methods

Fly Samples

Ja03au Sample

By sweeping over sites attractive for Drosophila (harvested grapes, refuse at fruits market, or fermenting fruit waste in orchards) or with banana bait traps, male flies were collected at 106 localities in and around Japan (Figure 1), 1 in Hokkaido, 70 in the mainland, 4 in Shikoku, 24 in Kyushu, 3 in Okinawa and isolated islands, and 4 in Korea. All sites were at least several kilometers apart. Collections were carried out in 2003 between September and December. A sample of 198 male flies (n = 198 X chromosomes) was obtained, composed of 1 or 2 flies from each location.

KA03au Sample

The Katsunuma area is one of the centers of wine production in Japan. The town of Katsunuma (35.5°N) is rather small (34 km²), but more than 30 wineries and many grape orchards are densely distributed in the western parts of the town. KA03au sample comprised of 191 males collected on a single day by sweeping over harvested grapes.
in a winery in September 2003. We regarded the KA03 sample as a high-density population sample.

**Kyoto and Iriomote Samples**

Four Kyoto (KY) samples, KY01au (2001 autumn, n = 177), KY02sp (2002 spring, n = 187), KY02au (2002 autumn, n = 186), and KY03sp (2003 spring, n = 191), and 2 Iriomote (IR) samples, IR01au (2001 autumn, n = 192) and IR03sp (2003 spring, n = 191), were also used (Takano-Shimizu et al. 2004). At all locations, males were collected with banana bait traps. The climate and flora of IR (24.2°C), which is a subtropical island in the southernmost region of Japan, are considerably different from those of KY (35.0°C) and other localities in the mainland of Japan.

Field-caught males were crossed separately to an inbred attached X chromosome strain, TT-35 (C(1)RM, y w/y w). The F1 male progeny, which had an identical X chromosome to that of their father, was collected and used for typing.

**Markers and Data Sets**

In addition to the 10 markers in the 10 Cr genes (Takano-Shimizu et al. 2004), we newly identified 41 biallelic single nucleotide polymorphisms (SNPs) in 40 X chromosome genes including another Cr gene, Gr9α, (Supplementary Table 1). Many of these 40 genes have receptor functions but are thought to have fewer functional connections to one another. Of the 51 markers, 27 were replacement and 24 were silent polymorphisms. All are SNPs except Gr2α, which is a complex change of CAC/GGCC and categorized as a replacement polymorphism (Takano-Shimizu et al. 2004). The average distance between the 51 polymorphisms is 0.13 in terms of recombination frequency per generation.

In this study, we typed the above 51 markers for Ja03au and the 10 Cr gene markers for KA03au. In addition, the newly developed 41 markers were typed for KY01au and KY02sp. For the 10 Cr gene markers, we revisited the data of 6 samples in Takano-Shimizu et al. (2004): KY01au, KY02sp, KY02au, KY03sp, IR01au, and IR03sp. Consequently, we obtained the data of 3 samples (KY01au, KY02sp, and Ja03au) for the 51 markers and those of 8 samples (KY01au, KY02sp, KY02au, KY03sp, IR01au, IR03sp, KA03, and Ja03) for the 10 Cr gene markers.

**DNA Extraction, Polymerase Chain Reaction, and Marker Typing**

We extracted genomic DNA from F1 male progeny using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma, St Louis, MO). To amplify the variable sites, polymerase chain reaction (PCR) was carried out using the following protocol: 32 cycles of denaturing at 95 °C for 30 s, annealing at 60 °C for 30 s, and polymerizing at 72 °C for 1 min. Most genotyping was performed by allele-specific oligonucleotide hybridization (Saiki et al. 1986). The PCR primers and the allele-specific oligonucleotide probes are listed in the Supplementary Table 1. The polymorphisms in the Or2α, Gr5α, and Or7α were typed based on restriction fragment length polymorphisms according to Takano-Shimizu et al. (2004).

**Data Analysis**

Average heterozygosity (H), minimum genetic distance (Dmin), and their standard errors were estimated as described in Nei and Roychoudhury (1974). We estimated fixation index (Wright 1951), Fst, without sampling correction using an expected H in Ja03au as the expected total H.

LD between polymorphisms was statistically tested by a 2-tailed Fisher’s Exact test. Because we performed 1275 tests in the analysis of the 51 X-linked polymorphisms, the critical value for Bonferroni multiple test correction was 5%/1275 = 0.0039%. Squared correlation coefficient (r2) was calculated as a measure of LD, excluding 7 closely located polymorphism pairs whose physical distances were less than 10 kb.

For the sign test (Lewontin 1995), alleles at all marker sites were assigned so that the LD sign was negative when there was an excess of chromosomes with one frequent and one less common allele. Polymorphisms were excluded when the frequency of the less common allele was 0.4 or greater. A singleton was also excluded from the sign test.

To statistically test a difference in the average r2 values between 2 samples, permutation tests were performed. Chromosomes from the 2 samples of sizes n1 and n2 were randomly divided into 2 samples of n1 and n2 chromosomes, and the average r2 value was calculated for each sample. By repeating this process 5000 times, we obtained the probability that the between-sample difference in r2 values was equal to, or larger than, the observed difference. The tests were 2 sided. We also used permutations to test differences in the increase in the average r2 value from winter to spring between the Cr and non-Cr genes. In this case, we calculated the average r2 values in KY01au and KY02sp samples for randomly selected 11 genes, and the distribution of their differences was used to establish significance thresholds.

**Recombination Frequency**

Recombination frequency between polymorphisms was calculated using the Kosambi formula (Kosambi 1944) and the standard genetic map of Drosophila melanogaster (Lindsley and Zimm 1992; Drysdale et al. 2005).

**Results**

**Fifty-One Polymorphisms in 3 Samples**

We typed 51 diallelic X chromosome polymorphisms for 3 sets of samples: Ja03au (198 chromosomes), KY01au (177 chromosomes), and KY02sp (187 chromosomes) (Supplementary Tables 2, 3, and 4). The average H was estimated to be 0.32 for all the 3 samples, meaning that there was no excess of homozygosity in the local KY samples compared...
with the combined fly sample (Ja03au). All 3 samples shared the same rare allele in 48 of 51 polymorphisms; the exceptions were CG6986, mys, and CG32704. The estimates of \( D_m \) were small (0.002 ± 0.001 between KY01au and KY02sp, 0.001 ± 0.001 between KY01au and Ja03au, and 0.003 ± 0.001 between KY02sp and Ja03au).

The results of LD analysis are given in Figure 2 and Table 1. Of the 1275 polymorphism pairs in Ja03au, KY01au, and KY02sp, 16, 21, and 53 pairs showed significant LDs at the 1% significance level and 5, 0, and 1 pairs did so after the Bonferroni correction, respectively (Figure 2). Excluding 7 closely located polymorphism pairs whose physical distances were less than 10 kb, the average \( r^2 \) values were 0.0062 in Ja03au, 0.0073 in KY01au, and 0.0095 in KY02sp (Table 1). Although the difference in average \( r^2 \) values between Ja03au and KY01au was not significant (\( P > 0.2 \)), the difference between KY01au and KY02sp was highly significant (\( P = 0/5000 \)). The differences between KY01au and KY02sp were all statistically significant even after dichotomizing the 51 markers into \( C_r \) and non-\( C_r \) genes and into replacement and silent sites. This finding indicates that the amount of LD increased from autumn to spring at the chromosome-wide level in the KY population. The increase in the amount of LD in the spring was most pronounced in the \( C_r \) genes (0.0074 in KY01au to 0.0135 in KY02sp in terms of average \( r^2 \); see Table 1), although the observed difference in the \( C_r \) genes was not significantly larger than that in the non-\( C_r \) genes (\( P = 0.06 \) by a permutation test).

The direction of LD was tested by the sign test (Lewontin 1995), and no significant deviation from the expectations was obtained regardless of the class of polymorphisms (replacement, silent, \( C_r \), non-\( C_r \), and all polymorphisms) and samples (Table 2).

### Ten \( C_r \) Polymorphisms for 8 Samples

To compare the KY fly samples with other local population samples, we typed 10 of the above 11 \( C_r \) polymorphisms on 191 X chromosomes of the Katsunuma sample (KA03au).
Table 2. Numbers of positive- and negative-phase LDs

<table>
<thead>
<tr>
<th>Sample</th>
<th>Polymorphisms</th>
<th>Positive or zero</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ja03au</td>
<td>All</td>
<td>332/329</td>
<td>371/374</td>
</tr>
<tr>
<td></td>
<td>Cr</td>
<td>12/14</td>
<td>16/14</td>
</tr>
<tr>
<td></td>
<td>Non-Cr</td>
<td>217/203</td>
<td>218/232</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>100/108</td>
<td>131/123</td>
</tr>
<tr>
<td></td>
<td>θ</td>
<td>56/58</td>
<td>64/62</td>
</tr>
<tr>
<td>KY01au</td>
<td>All</td>
<td>394/382</td>
<td>428/440</td>
</tr>
<tr>
<td></td>
<td>Cr</td>
<td>17/14</td>
<td>12/15</td>
</tr>
<tr>
<td></td>
<td>Non-Cr</td>
<td>251/244</td>
<td>277/284</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>150/152</td>
<td>175/173</td>
</tr>
<tr>
<td></td>
<td>θ</td>
<td>46/48</td>
<td>59/57</td>
</tr>
<tr>
<td>KY02sp</td>
<td>All</td>
<td>374/379</td>
<td>450/445</td>
</tr>
<tr>
<td></td>
<td>Cr</td>
<td>23/22</td>
<td>22/23</td>
</tr>
<tr>
<td></td>
<td>Non-Cr</td>
<td>213/209</td>
<td>254/258</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>118/132</td>
<td>161/147</td>
</tr>
<tr>
<td></td>
<td>θ</td>
<td>57/61</td>
<td>79/75</td>
</tr>
</tbody>
</table>

* Between Cr and Cr polymorphisms.
* Between non-Cr and non-Cr polymorphisms.
* Between replacement polymorphisms.
* Between silent polymorphisms.

(Supplementary Table 5). These 10 polymorphisms were previously typed for 2 other KY samples, KY02au and KY03sp, and 2 IR samples, IR01au and IR03sp (Takano-Shimizu et al. 2004). Table 3 gives the estimates of average H and Dm for the 8 samples including Ja03au, KY01au, and KY02sp. As in the samples harboring 51 polymorphisms, the 4 KY samples did not show a reduction in H compared with Ja03au. The genetic distances between the populations were concordant with their geographical locations; the distance between Katsunuma and IR was the largest. The Fst estimate was 0.08 based on the average H in the three 2003 local samples (KY03sp, KA03au, and IR03sp) and that in Ja03au, implying little differentiation among the localities.

Figure 3 illustrates the average r^2 values between the 10 Cr polymorphisms in the 8 samples. Despite possible differences in demographic characteristics, the values in Ja03au, KA03au, IR01au, and IR03sp were very similar to those in the 2 KY autumn samples. In contrast, the amounts of LD in the 2 KY spring samples were consistently larger than those in the other samples. The permutation probability that between-sample differences in average r^2 value are equal to or larger than the observed difference was significant in both the KY01au–KY02sp (P = 0.03) and KY02au–KY03sp comparisons (P = 0.001). These findings implied that the amounts of LD between the Cr polymorphisms are significantly larger in spring than in autumn in the 2 consecutive years. In addition, the similar amounts of r^2 values in the KYau and KAau samples suggested that population density did not greatly affect the amount of LD at the present resolution level.

Table 3. Average H and Dm with standard error for 10 Cr polymorphisms in 8 samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>H</th>
<th>Dm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KY01au</td>
<td>KY02sp</td>
</tr>
<tr>
<td>Ja03au</td>
<td>0.414 ± 0.031</td>
<td>0.000 ± 0.001</td>
</tr>
<tr>
<td>KY01au</td>
<td>0.414 ± 0.031</td>
<td>0.007 ± 0.004</td>
</tr>
<tr>
<td>KY02sp</td>
<td>0.414 ± 0.026</td>
<td>0.001 ± 0.001</td>
</tr>
<tr>
<td>KY03sp</td>
<td>0.402 ± 0.032</td>
<td>0.000 ± 0.001</td>
</tr>
<tr>
<td>KA03au</td>
<td>0.396 ± 0.021</td>
<td>0.015 ± 0.004</td>
</tr>
<tr>
<td>IR01au</td>
<td>0.385 ± 0.026</td>
<td>0.042 ± 0.021</td>
</tr>
<tr>
<td>IR03sp</td>
<td>0.384 ± 0.022</td>
<td>0.001 ± 0.001</td>
</tr>
</tbody>
</table>
treated as deterministic process, whereas mutation and selection are neglected. At generation 0, the allele frequencies are assumed to be 0.5 at both loci, and they are in linkage equilibrium. The $r^2$ value was calculated from 200 gametes randomly sampled in each run and time point, and the average values were obtained from 104 independent runs. Our simulation results (Figure 4) indicate the change in amount of LD and suggest that if the observed change in the magnitude of LD during winter was caused by bottlenecks of short duration, severe reduction in population size would be required. Therefore, long-distance LD could be useful for assessing the short-term fluctuation in population size.

Although the difference in the average $r^2$ values between KY03sp and KY02sp was not statistically significant ($P > 0.2$), the increase in the magnitude of LD was larger in the KY02au–KY03sp than in the KY01au–KY02sp (Figure 3). This could be caused by milder winter conditions in 2001–2002. Indeed, the winter of 2001–2002 was the record mildest winter in KY. The monthly mean air temperatures of January, March, and April in 2002 were the ninth, first, and fourth highest since 1881, respectively, whereas those in 2002–2003 were ordinary (the climatic statistics of Japan Meteorological Agency: http://www.jma.go.jp/jma/index.html).

We previously studied LD between 98 Cr genes in the entire Drosophila genome and found a large difference not only in the magnitude of LDs but also in their direction between KY01au and KY02sp (Takano-Shimizu et al. 2004). There is a significant shortage of coupling disequilibrium with associations of less common alleles but only for replacement polymorphism pairs in KY02sp. Such a bias in LD direction was not found in all the samples in this study (Table 2). Most of the genes studied here have no obvious functional connection to one another. Although further study is needed, the significant bias in the Cr genes might be due to their functional associations.

In contrast to the mainland population, a southern island population shows little seasonal difference in the magnitude of LD (Takano-Shimizu et al. 2004). This is not surprising, given the smaller seasonal climatic change in the southernmost region of Japan. The southern island populations are known to carry much larger additive genetic variance in viability than a northern mainland population of Japan, and diversifying selection is suggested to be the cause of the excessive variation (Tachida et al. 1983; Kusakabe and Mukai 1984; Takano et al. 1987). In light of the present findings, there may be other factors that contribute to the difference in genetic variance between the northern and southern populations, that is, heterogeneity in seasonal change. Reduction of population size can purge highly recessive deleterious mutations (Kirkpatrick and Jarne 2000; Grelmin 2003). However, the purging effect alone cannot explain the reduced variation in the mainland population because mildly deleterious mutations that are largely responsible for the excessive genetic variance in the southern populations are only mildly recessive (the degree of dominance $= 0.2–0.5$; Mukai and Nagano 1983; Tachida

Figure 3. Average $r^2$ values between 10 Cr polymorphisms.

Figure 4. Changes in average $r^2$ values when population size fluctuates through bottlenecks. Population size alternates cyclically between $N_1$ and $N_2$, with the duration time of 10 and 2 generations, respectively (the lower panel). Results for 3 different $N_2$ values (square: 200; cross: 500; and triangle: 1000) are illustrated; $N_1$ is assumed to be 5000 throughout. Essentially, the same results were obtained with $N_1 = 10 000$ (data not shown). The expected $r^2$ value may be obtained by $E[r^2] = \frac{1}{2N_e(c^2 + \frac{c}{n}) + \frac{1}{n}}$, where $c$ is the recombination rate, $N_e$ is the effective population size, and $n$ is the sample size (Weir and Hill 1980). In the present case, this becomes 0.0054 for $N_e = 5000$, 0.0072 for $N_e = 1000$, 0.0093 for $N_e = 500$, and 0.0158 for $N_e = 200$. 

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et al. 1983). It is also conceivable that selection is more severe during winter. Increased selection intensity, in combination with population bottleneck, during winter may contribute to the reduction of the genetic load.

Repeated bottlenecks frequently occur in natural populations. Long-distance LD could be a powerful measure for assessing their impact on the genetic structure.

Supplementary Material
Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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