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Ancestral polymorphisms in *Drosophila pseudoobscura* and *Drosophila miranda*

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Summary

Ancestral polymorphisms are defined as variants that arose by mutation prior to the speciation event that generated the species in which they segregate. Their presence may complicate the interpretation of molecular data and lead to incorrect phylogenetic inferences. They may also be used to identify regions of the genome that are under balancing selection. It is thus important to take into account the contribution of ancestral polymorphisms to variability within species and divergence between species. Here, we extend and improve a method for estimation of the proportion of ancestral polymorphisms within a species, and apply it to a dataset of 33 X-linked and 34 autosomal protein-coding genes for which sequence polymorphism data are available in both *Drosophila pseudoobscura* and *Drosophila miranda*, using *Drosophila affinis* as an outgroup. We show that a substantial proportion of both X-linked and autosomal synonymous variants in these two species are ancestral, and that a small number of additional genes with unusually high sequence diversity seem to have an excess of ancestral polymorphisms, suggestive of balancing selection.

1. Introduction

An ancestral polymorphism is defined as a polymorphism that originated as a result of mutation prior to the speciation event that generated the species in which it segregates. The presence of ancestral polymorphisms within a species, and their fixation subsequent to speciation, can contribute to divergence from a closely related species; this influences estimates of rates of sequence evolution, and may also lead to incorrect inferences concerning phylogenetic relationships (e.g. Gillespie & Langley, 1979; Clark, 1997; Maddison, 1997; Arbogast *et al.*, 2002; Hudson & Coyne, 2002; McVicker *et al.*, 2009; Cutter & Choi, 2010). In addition, estimates of the abundance of ancestral polymorphisms provide a test for balancing selection, since an excess frequency of ancestral polymorphisms within a gene or genetic region, relative to the level that would be expected under neutrality, is a signature of long-term balancing selection (Wiuf *et al.*, 2004; Asthana *et al.*, 2005). For the purpose of interpreting the phylogenetic relationships of closely related species, and analysing the causes of variability within species, it is thus important to take into account the contribution of ancestral polymorphisms to variability within species and divergence between species.

Here, we extend a method for estimation of the proportion of ancestral polymorphisms among all polymorphisms within species, based on a comparison of three species, which was first introduced by Ramos-Onsins *et al.* (2004) and subsequently elaborated by Charlesworth *et al.* (2005). We apply it to a dataset of nearly 70 protein-coding genes for which DNA sequence polymorphism data are available in both *Drosophila pseudoobscura* and its close relative *Drosophila miranda*, using their relative *Drosophila affinis* as an outgroup (Haddrill *et al.*, 2010), in an attempt to estimate the true level of ancestral polymorphism for these two species. We show that a substantial proportion of the synonymous variants in these two species are ancestral, and that a small number of genes with unusually high sequence diversity seem to show evidence of an excess of ancestral polymorphisms, suggestive of balancing selection. Our methods offer a substantial improvement on those reported in Charlesworth *et al.* (2005), by introducing novel procedures for the estimation of the parameters

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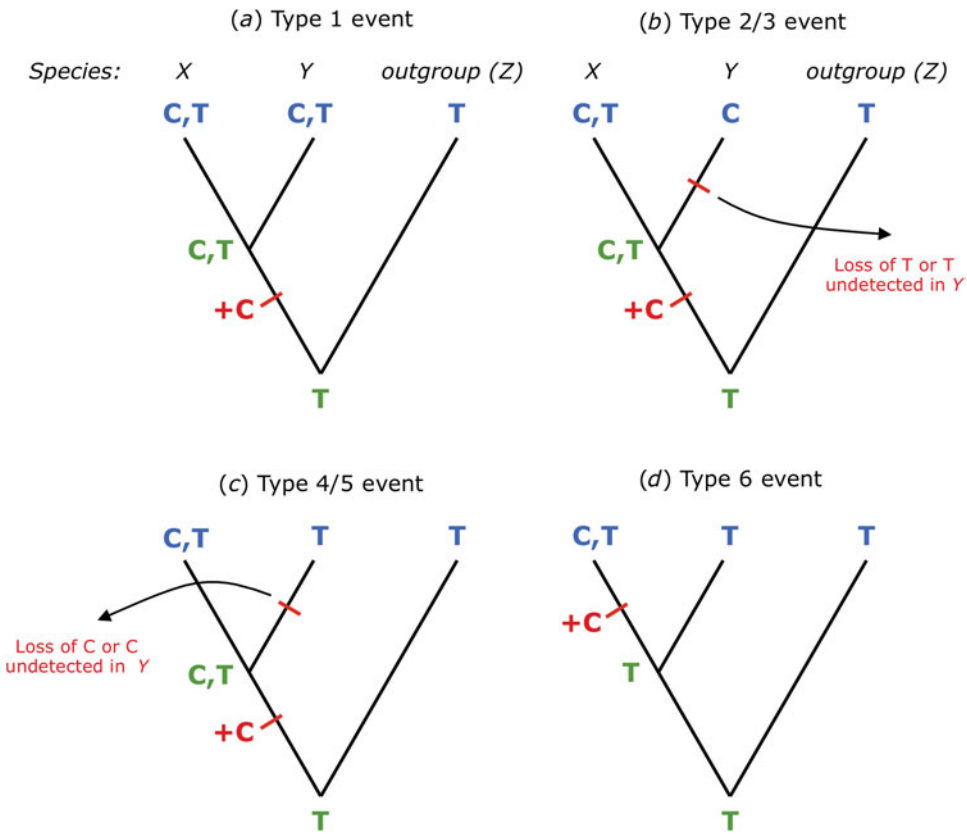

Fig. 1. Interpretation of polymorphism patterns for a three-species model, using parsimony. Observed states are shown in blue, inferred states in green and inferred evolutionary events in red. Type 1 (*a*), type 2/3 (*b*) and type 4/5 (*c*) all represent ancestral polymorphisms, whereas type 6 (*d*) represents *de novo* polymorphisms that have arisen in one lineage of the tree. However, since type 4/5 is indistinguishable from type 6, they do not contribute to the observed fraction of ancestral polymorphisms. Adapted from Fig. 1 of Charlesworth *et al*. (2005).

of interest. We also incorporate the estimation of confidence intervals on these parameters, in order to assess error in our estimates. In addition, we analyse a much larger dataset than Charlesworth *et al*. (2005) (67 genes compared with three genes), which enables us to compare levels of ancestral polymorphism at X-linked and autosomal loci.

2. Materials and methods

(i) *Theoretical background*

The method uses an outgroup species and parsimony to infer the ancestral state of a given polymorphic site in two species for which DNA sequence polymorphism data are available (Ramos-Onsins *et al*., 2004; Charlesworth *et al*., 2005). The states of a given nucleotide site at the internal nodes of the phylogeny of that site are inferred from the observed state of the nucleotide in the outgroup species, from which a single DNA sequence is assumed to have been obtained. Thus, with three species denoted by *X*, *Y* and *Z*, where *X* and *Y* are close relatives for which polymorphism data are available and *Z* is the outgroup species, the state of a given nucleotide site in the outgroup is assumed to be the ancestral state for polymorphic sites in *X* and *Y* (see Fig. 1). In such a three-species comparison, the observed pattern of polymorphism at a nucleotide site across the three species can be assigned a 'type' that is consistent with the most parsimonious interpretation of the pattern (Charlesworth *et al*., 2005).

Figure 1 displays an example of a C, T polymorphism observed at a given polymorphic site in a focal species (*X*) in a group of three species; the following arguments are equally true for polymorphisms observed in species *Y*, interchanging *X* and *Y*. Slightly modifying the terminology of Charlesworth *et al*. (2005), we can define four distinct types of event that generate polymorphisms in species *X*: type 1, type 2/3, type 4/5 and type 6. Figure 1(*a*) shows a 'type 1' event: a CT polymorphism is observed in both species, while a T is present in the outgroup sequence. The most parsimonious interpretation is that the ancestral state for all three species was T, and that a T→C mutation occurred in the lineage leading to both species *X* and *Y*. In Fig. 1(*b*), a CT polymorphism is observed only in *X*, while *Y* is apparently fixed for C and the outgroup is T. Here, the most parsimonious explanation is that a T→C mutation that

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occurred in the common ancestor to X and Y gave rise to a CT polymorphism in both species, but in species Y either the C allele has gone to fixation (a 'type 2' event) or by chance the T allele was not found in the sample (a 'type 3' event). Although there is a clear distinction between a type 2 and a type 3 event, they are observationally identical, and both represent an ancestral polymorphism; they are thus pooled to constitute a 'type 2/3' event.

In Fig. 1(*c*), there is a CT polymorphism in species X, but only T is found in species Y and the outgroup. One possibility is that a T→C mutation occurred in the ancestral population prior to the speciation of X and Y, but the C variant has been lost from species Y (a 'type 4' event) or is not present in the sample taken from Y (a 'type 5' event). Alternatively, a *de novo* polymorphism that arose only in species X could have produced this pattern (a 'type 6' event: Fig. 1(*d*)). Type 4, 5 and 6 events are observationally indistinguishable, but have distinct evolutionary causes: type 4/5 events are ancestral polymorphisms, but cannot be distinguished from a '*de novo*' polymorphism (type 6). Thus, it is this misclassification of type 4/5 polymorphisms as *de novo* (i.e. type 6) that constitutes the primary source of error in calculating the observed fraction of ancestral polymorphisms, which in its true sense is defined as the ratio of the sum of types 1 through to 5 to the total number of polymorphisms in a given species.

In order to estimate the fraction of ancestral polymorphisms among all polymorphisms in species X, we use the formulae of Charlesworth *et al.* (2005) for calculating the expected frequencies of types 1, 2 and 3 events among the total, on the assumption of selective neutrality. Let P_d be the probability that a polymorphic site, which was present in the common ancestor of species X and Y, is classed as type 1 or type 2/3 (i.e. as an observed ancestral polymorphism) in species X. Let the probability of detecting a type i polymorphism in species X be denoted by P_i. For $i=1$–3, expressions for these probabilities are given by eqns (5), (6) and (9), respectively, of Charlesworth *et al.* (2005), and can be summed to give P_d (eqn (11*a*) of Charlesworth *et al.* (2005):

$$\begin{aligned} P_d &= P_1+P_2+P_3 \\ &\approx \frac{1}{3}+\frac{(n-1)}{2(n+1)}\exp(-t) \\ &\quad+\frac{\{(n+1)(n+2)-6n\}}{6(n+1)(n+2)}\exp(-3t), \end{aligned} \tag{1}$$

where n is the sample size for species Y, and t is the time since the split of the two species in question, measured in units of $2N_e$ generations (here, N_e is the effective population size for the lineage leading to the species designated as species Y, i.e. the non-focal species).

In order to use this result, an estimate of t is required. Using the expressions for the P_i in Charlesworth *et al.* (2005), we can equate the following functions of the observed and theoretical frequencies of types 1, 2 and 3 polymorphisms:

$$\begin{aligned} &\frac{\frac{1}{2}\left(\frac{n+1}{n-1}\right)f_1+f_{[2+3]}}{f_1} \\ &=\frac{\frac{1}{2}\left(\frac{n+1}{n-1}\right)P_1+P_2+P_3}{P_1} \\ &=\frac{1}{n-1}\left\{1-\frac{n}{(n+2)}\exp(-2t)\right\} \\ &\quad+\frac{1}{3}\left\{\frac{n+1}{n-1}\right\}\left\{\exp(t)+\frac{\exp(-2t)}{2}\right\}, \end{aligned} \tag{2}$$

where f_1 is the observed fraction of type 1 polymorphisms and $f_{[2+3]}$ denotes the observed fraction of type 2/3 polymorphisms in species X. This provides a convenient exact formula for estimating t, which is more accurate than the approximate eqn (13) of Charlesworth *et al.* (2005).

Let the observed value of the expression on the left-hand side be denoted by r_{OBS}; this can be equated to the relatively simple theoretical formula on the right-hand side, in order to obtain an estimate of t. A simple Java program (*EstimateT*; available on request) utilizes the Newton–Raphson method for solving a non-linear equation of the form $f(x)=0$, by iterating $x_{i+1}=x_i-f(x_i)/f'(x_i)$, where $f(x)$ is a function of x and $f'(x)$ is its derivative. In the present case, replacing x with t, the function that yields the desired estimate of t can be written as

$$\begin{aligned} f(n,t)=&\left[\frac{1}{n-1}\left\{1-\frac{n}{(n+2)}\exp(-2t)\right\}\right. \\ &\left.+\frac{1}{3}\left\{\frac{n+1}{n-1}\right\}\left\{\exp(t)+\frac{\exp(-2t)}{2}\right\}\right]-r_{\mathrm{OBS}}, \end{aligned} \tag{3}$$

The partial derivative of f with respect to t is

$$\begin{aligned} f'(n,t)=&\left\{\frac{2n}{(n-1)(n+2)}\right\}\exp(-2t) \\ &+\left\{\frac{n+1}{3(n-1)}\right\}\{\exp(t)-\exp(-2t)\}. \end{aligned} \tag{4}$$

Iterations using these expressions quickly yield a stable estimate of t for given values of r_{OBS} and n. The method can be applied either to individual loci or a group of loci.

Following Charlesworth *et al.* (2005), the observed frequency of type 1/2/3 polymorphisms among all polymorphisms can then be divided by P_d, in order to correct for the misclassification of type 4/5 ancestral polymorphisms as type 6, yielding the estimated

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fraction of ancestral polymorphisms as r_T. With independence among sites, this procedure is equivalent to a maximum likelihood estimate (see Supplementary material), assuming independence (linkage equilibrium) among nucleotide sites. While the assumption of linkage equilibrium is not completely accurate, polymorphism data show that linkage disequilibrium in these species falls off rapidly with distance between nucleotide sites (Schaeffer & Miller, 1993; Bachtrog & Andolfatto, 2006), so that it is unlikely that it will pose a major problem in the case of these data. A possible effect of non-independence was tested for using the distribution across loci of the numbers of type 2/3 polymorphisms for the X chromosome and autosome of *D. pseudoobscura*, the only cases in which there is more than a handful of loci with more than one putatively ancestral polymorphism (see Supplementary Table S1). The mean numbers of type 2/3 polymorphisms per locus were 0·79 and 1·06 for the X chromosome and autosome, respectively; chi-squared tests for agreement with the Poisson distribution gave values of 6·29 and 3·27, respectively (3 DF for each, $P > 0{\cdot}05$). Thus, there is no evidence for a non-random distribution across loci.

The variances and standard errors of t, P_d and r_T can be calculated using the delta method (Bulmer, 1980, p. 83), again assuming independence among sites so that the numbers of type 1, type 2/3 and type 4/5 and 6 polymorphisms are multinomially distributed (see Supplementary material). Alternatively, approximate confidence intervals for the estimates can be derived by bootstrapping. The dataset for the focal species is resampled (with replacement) k times, where k is equal to the number of polymorphic sites within the species, by randomly drawing a site from the array of sites from 1 to k, and storing it in a new array. This procedure is repeated 10 000 times; for each replicate, the f_1, $f_{[2+3]}$, $f_{de\ novo}$ (defined as the observed fraction of type 4/5 and 6 polymorphisms), t, P_d, r_{OBS} and r_T statistics are recalculated to create their sampling distributions; approximate 95% confidence intervals are then derived by extracting the 2·5 and 97·5 percentile values from these distributions.

(ii) *Nature of the data*

Our study species are *D. pseudoobscura* and *D. miranda*, with *D. affinis* as the outgroup species, as described by Charlesworth *et al.* (2005). *D. pseudoobscura* and *D. miranda* are very closely related, with a mean synonymous site divergence (K_S) of about 4% (Bartolomé & Charlesworth, 2006; Haddrill *et al.*, 2010). Introgression between the two species is thought to be absent in the wild, and laboratory hybrids are completely infertile (Dobzhansky & Tan, 1936), so that we should be safe to assume that the pattern of polymorphism observed here is not due to ongoing introgression between these species. This is important as ongoing hybridization would leave a pattern similar to that of ancestral polymorphism. *D. affinis* is another North American species that is relatively distantly related to our ingroup species, with a mean K_S of about 25% for the X chromosome and 28% for the autosome (A) (Haddrill *et al.*, 2010). The relatively large distance to the outgroup species poses some problems for the parsimony models used here, which are considered in section 2 (iii) below.

To estimate the incidence of ancestral polymorphism for *D. pseudoobscura* and *D. miranda*, the 67 loci that did not depart significantly from neutrality on the basis of a multilocus Hudson–Kreitman–Aguadé (HKA) test (Hudson *et al.*, 1987; Haddrill *et al.*, 2010) were screened for the presence of type 1, type 2/3 and type 4/5 and 6 synonymous polymorphisms in each species, using *D. affinis* as an outgroup. Gene sequence alignments for 34 autosomal (Muller element B or chromosome 4 in *D. pseudoobscura*) and 33 X-linked (Muller element A) loci that are orthologous in all three species were obtained (for details concerning Muller's elements, see Ashburner *et al.*, 2005). Each alignment consisted of 12–16 sequences from both *D. pseudoobscura* and *D. miranda*, and one sequence from the outgroup species *D. affinis*. A polymorphism dataset was constructed for each alignment using the relevant functions in the software package DnaSPv5 (Librado & Rozas, 2009).

For all analyses, only polymorphisms at synonymous sites were used, as these are likely to be closer to neutrality than non-synonymous changes. Any alignment gaps were also excluded from the analysis. Some additional autosomal and X-linked loci that were previously identified as potentially being under selection in either *D. pseudoobscura* or *D. miranda* on the basis of the HKA test (Haddrill *et al.*, 2010), and that were excluded from the main results presented here, are considered in the Discussion section.

A second Java program (*PolyFinder*; available on request) was written, which detects and classifies each type of polymorphism as a type 1, type 2/3 or type 4/5 and 6 for each species. When applied to the several hundred polymorphic sites in the samples from the two species, this program provides a simple and effective way of classifying polymorphisms under the parsimony assumption.

(iii) *Corrections for errors in the parsimony inferences*

A method of correcting errors in inferences from parsimony was described in the Appendix of Charlesworth *et al.* (2005), and was applied to the present dataset. This method requires estimates of the numbers of polymorphisms involving transitions and transversions, respectively; the relevant data are provided in Table S2 of the Supplementary material.

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Table 1. *Numbers of different types of polymorphisms found in* D. pseudoobscura *and* D. miranda*, before correcting for parsimony errors*

			D. pseudoobscura		*D. miranda*	
	k	Type 1 (shared)	Type 2/3 (ancestral)	Types 4/5 and 6 ('*de novo*')	Type 2/3 (ancestral)	Types 4/5 and 6 ('*de novo*')
A	34	4	36	258	5	61
X	33	6	26	198	10	42
Total	67	10	62	456	15	103

A and X refer to autosomal and X-linked loci, respectively.
k is the number of loci in each category.

We also require an estimate of the time since the divergence of *D. pseudoobscura* and *D. miranda* to initiate the parsimony-correction procedure, because it requires use of the estimates of P_2, P_3 and the *a priori* probability of an ancestral polymorphism, but we also need accurate values for the proportions of type 1 and type 2/3 polymorphisms to estimate t from eqns (3) and (4). The correction procedure was performed on a locus-by-locus basis, thereby taking into account the slight variation in sample size between genes.

For each of the branches leading to *D. pseudoobscura* and *D. miranda* from their common ancestor, we therefore calculated a parsimony-free initial estimate of the time since divergence, t_0, using the ratio of the synonymous divergence between the species in question (K_S) to the mean synonymous diversity (π_S) of the non-focal species; this is expressed on a time-scale of units of $2N_e$ generations, where N_e is the effective population size along the lineage in question, which we equated to the estimate of current effective size for the species (Hudson *et al*., 1987). These values were 2·72 and 2·24 for the X and A of *D. pseudoobscura*, and 10·3 and 9·43 for the X and A of *D. miranda*, respectively. The initial estimate for a given chromosome and focal species was then used to calculate the proportion of incorrect assignments, as described by Charlesworth *et al*. (2005), and the corrected values were then used to recalculate t via the Newton–Raphson method outlined in section 2(i). No further use was made of the divergence/diversity ratios in subsequent iterations. Iterations were carried out until estimates of both t and the corrected values of f_1 and $f_{[2+3]}$ converged to three decimal places.

3. Results

(i) *Frequencies of the different types of polymorphisms*

The observed counts of ancestral polymorphisms for the autosomal and X-linked genes in *D. pseudoobscura* and *D. miranda* are shown in Table 1 (for a locus-by-locus breakdown of all polymorphisms, see Table S1 of the Supplementary material). These counts represent the observed values prior to correction of errors in the parsimony assumptions used in their detection. The observed fraction of apparent ancestral polymorphisms is the sum of the type 1 and type 2/3 polymorphisms, divided by the total number of polymorphisms. These account for $(10+62)/528=0{\cdot}136$ and $(10+15)/128=0{\cdot}195$ of the total polymorphisms seen within *D. pseudoobscura* and *D. miranda*, respectively. As discussed previously, this observed fraction is likely to be biased in two ways, firstly, by the misclassification of 4/5 polymorphisms as *de novo* polymorphisms and secondly by errors in the parsimony methodology used to classify polymorphisms within this dataset. To deal with these problems, an estimate of divergence time between the two species is required, as described in section 2.

(ii) *Divergence times and corrections for parsimony error*

The estimates of the number of different types of polymorphisms after correction for parsimony errors, using the method outlined in section 2(iii), are shown in Table 2. The corrections reduce the number of observed ancestral polymorphisms (i.e. type 1/2/3) from 72 to ~33 (for *D. pseudoobscura*) and from 25 to ~15 (*D. miranda*). Thus, correcting for parsimony errors reduces the estimate of the proportion of type 1/2/3 polymorphisms by approximately half.

Estimates of the time since the divergence of *D. pseudoobscura* and *D. miranda*, after corrections for parsimony errors, were obtained as described in sections 2(i) and 2(iii), and are shown in Table 3. These divergence time estimates are in units of $2N_e$ generations, where N_e is the long-term effective population size of the lineage leading to the species chosen as Y in the comparisons, i.e. the partner to the focal species whose polymorphism data are being used (see section 2(i)). The estimated times for both the X chromosome and autosome are greater than 1, so that the use of the equations in section 2(i) is justified, since these require $t>0{\cdot}5$ (Charlesworth *et al*., 2005).

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Table 2. *Number of different types of polymorphisms found in* D. pseudoobscura *and* D. miranda*, after correcting for parsimony errors*

	D. pseudoobscura			*D. miranda*		
	Type 1 (shared)	Type 2/3 (ancestral)	Types 4/5 and 6 ('*de novo*')	Type 1 (shared)	Type 2/3 (ancestral)	Types 4/5 and 6 ('*de novo*')
A	1·1	14·4	282·5	1·7	1·6	66·7
X	4·6	13·0	212·4	4·8	7·1	46·1
Total	5·7	27·4	494·9	6·5	8·7	112·8

A and X refer to autosomal and X-linked loci, respectively.

The estimates of P_d, defined by eqn (1) of section 2(i), are all around 40%, indicating that there is a high probability of misclassification of an ancestral polymorphism. As described in section 2(i), this allows estimation of the fraction of ancestral polymorphisms by dividing the frequency of type 1/2/3 polymorphisms by the estimate of P_d. The results show that there is a significant frequency of ancestral polymorphisms on both the X chromosome and autosome in both species, which is higher for the X (especially in *D. miranda*), and higher in *D. miranda* than in *D. pseudoobscura*.

This method also offers a way of calculating the relative proportions of the indistinguishable type 4/5 (ancestral) and type 6 (true *de novo*) polymorphisms; the difference between the unadjusted and adjusted fraction of ancestral polymorphisms is attributable to the misclassified polymorphisms that were erroneously categorized as *de novo*. Since these are type 4/5 polymorphisms, this difference must be equal to the total proportion of type 4/5 polymorphisms in a given dataset. Thus, for the X chromosome in *D. pseudoobscura*, the (corrected) proportion of type 1/2/3 polymorphisms is 7·7% and the estimated proportion of ancestral polymorphisms is 19·5%, so that the estimate of the proportion of type 4/5 polymorphisms is 11·8%. It is interesting to note, therefore, that type 4/5 polymorphisms appear to contribute more to the overall fraction of ancestral polymorphisms than type 1 and type 2/3 combined. This reflects the fact that, as may be seen from Fig. 1, type 4/5 events involve the loss of the derived rather than ancestral variant from either the population or the sample; since derived variants are mostly present at low frequencies, their loss is more probable than the loss of the ancestral variant.

4. Discussion

The results presented here suggest that a significant fraction of polymorphisms in *D. pseudoobscura* and *D. miranda* arose in the ancestral population common to these species, prior to their complete separation by speciation. We estimate that the overall proportions of ancestral polymorphisms are 19·5±4·9% (standard error) and 14·9±3·8% for the *D. pseudoobscura* X chromosome and autosome, respectively; the corresponding values for *D. miranda* are 47·6±14·1% and 10·0±6·4%. Our result for the *D. pseudoobscura* autosome agrees well with a previous estimate of 18·6% (Charlesworth *et al.*, 2005), but is likely to have a substantially lower standard error due to the increased size of the present dataset.

Table 3. *Estimates of* t, P_d *and* r_T *after correcting for parsimony errors*

	D. pseudoobscura		*D. miranda*	
	A	X	A	X
t	3·59 (1·51)	2·17 (0·72)	1·35 (1·15)	1·66 (0·62)
	2·22/4·04	1·37/3·32	−0·11/2·48	0·84/2·78
P_d	34·7 (2·09)	39·0 (4·12)	46·6 (15·9)	42·9 (6·11)
	34·2/38·8	35·1/46·3	37·5/112·0	36·4/56·3
r_T	14·9 (3·81)	19·5 (4·94)	9·97 (6·38)	47·6 (14·1)
	8·46/22·5	11·2/29·4	1·26/26·7	23·3/74·5

Estimates are presented with standard errors in parentheses and 95% confidence intervals below (based on 10 000 replicate populations of the corrected data; see section 2(ii)). Time t is in units of $2N_e$ generations. P_d is the probability of classifying an ancestral polymorphism as such, r_T is the true fraction of ancestral polymorphisms; both are expressed as percentages.
A and X refer to autosomal and X-linked loci, respectively.

The main novelty of the approach used here is that it enables an adjustment to be made for the ancestral polymorphisms that are undetected in the sample (the type 4/5 polymorphisms; see section 2(i)), as well as using parsimony to detect polymorphisms that are ancestral but not shared between the two species in question (type 2/3 polymorphisms, shared polymorphisms being classed as type 1). Before this adjustment, but after correcting for parsimony error, the estimated fractions of ancestral polymorphisms were approximately 6% and 12% for *D. pseudoobscura* and *D. miranda*, respectively (Table 2). The adjustment therefore increases the estimated fraction of ancestral polymorphisms by a factor of more than two.

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Table 4. *Uncorrected numbers of different types of polymorphisms found in* D. pseudoobscura *and* D. miranda *for three autosomal and five X-linked genes with high nucleotide diversity in at least one of the species*

			D. pseudoobscura		*D. miranda*	
Locus	π_s (%) pse/mir	Type 1 (shared)	Type 2/3 (ancestral)	Types 4/5 and 6 ('*de novo*')	Type 2/3 (ancestral)	Types 4/5 and 6 ('*de novo*')
A						
GA13976	3·8/3·3	0	0	15	0	8
GA21851	0·9/2·7	2	2	1	3	3
Total		2	2	17	3	11
% of type		33·0	7·7	6·2	37·0	15·3
X						
GA12872	4·4/0·6	2	0	17	0	1
GA14306	1·1/3·1	0	0	6	2	6
GA15909	5·7/1·9	4	6	11	0	5
GA17538	1·6/3·7	0	2	7	5	6
GA21767	7·6/3·7	3	6	17	0	3
Total		9	14	58	7	21
% of type		60·0	35·0	23·0	41·0	33·3

π_s (%) pse/mir is the mean pairwise nucleotide diversity for *D. pseudoobscura* and *D. miranda*, respectively (from Table 2 of Haddrill *et al*., 2010).
A and X refer to autosomal and X-linked loci, respectively.
% of type is the percentage of all polymorphisms of that type contributed by the specific genes listed.

The parsimony correction used here is, however, somewhat crude (for details, see Charlesworth *et al*., 2005), and so the numerical results should be treated with caution. Our method of adjusting for misclassified ancestral polymorphisms would be more trustworthy when used with an outgroup species with a much lower divergence than that between *D. affinis* and the two focal species, so that no parsimony correction is required, but no such species is currently available.

It is clear, however, that it is not sufficient to infer the level of ancestral polymorphisms on the basis of the number of shared polymorphisms alone, as has been done in most previous studies. This underestimates the total number of ancestral polymorphisms by a factor of about two, which may bias estimates of sequence divergence (Gillespie & Langley, 1979; Patterson *et al*., 2006; McVicker *et al*., 2009; Cutter & Choi, 2010). In addition, use of the fraction of unequivocally identified shared polymorphisms alone may underestimate the role of balancing selection, which has previously been reported to be limited in a human–chimpanzee study of shared polymorphisms (Asthana *et al*., 2005).

The estimated divergence time for the autosome is greater than that in the case of the X chromosome in *D. pseudoobscura*, and the values with regard to *D. miranda* are both smaller than for *D. pseudoobscura*. These differences are in the opposite direction from what is expected based on the estimates of N_e for these chromosomes and species, given current levels of synonymous nucleotide site diversities (Haddrill *et al*., 2010, 2011). These indicate that both species have N_e values for the X chromosome that do not differ significantly from three-quarters of that for the autosomes, as expected for species with little non-random variance in male mating success due to sexual selection (Charlesworth, 2009). In addition, *D. miranda* has about one-quarter the silent site diversity of *D. pseudoobscura*, so that its scaled divergence time should be four times that of *D. pseudoobscura*; the X chromosome in both species has about three-quarters of the diversity of the autosomes, so that its scaled divergence time should be four-thirds of that of the autosomes. While the X–A difference in *D. pseudoobscura* is not significant, the t values for *D. miranda* are highly significantly different from four times the *D. pseudoobscura* values, and are very different from the estimates based on the ratio of divergence to diversity given in section 2(iii). These discrepancies almost certainly reflect the fact that *D. pseudoobscura* has undergone a recent population expansion, whereas the unusually low diversity of *D. miranda* suggests that it may have experienced a past contraction of population size (Haddrill *et al*., 2010, 2011). Thus, the long-term N_e along the *D. pseudoobscura* lineage is probably much smaller, and the value for *D. miranda* much larger, than indicated by contemporary diversity estimates. While the t estimates in Table 3 are fairly noisy, it seems likely that they are less biased than the estimates from divergence/diversity ratios. Our estimates of the fraction of ancestral polymorphisms should not, however, be affected by shifts in population size along the

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D. pseudoobscura and *D. miranda* lineages, if we regard the effective sizes in the relevant equations as representing the harmonic mean effective sizes along the relevant lineage (Charlesworth & Charlesworth, 2010, pp. 225–226).

In *D. miranda*, there is a large difference between the estimated proportion of ancestral polymorphisms on the X chromosome and the autosome, in the direction opposite to that expected from the difference in effective population sizes, and hence t, for these two chromosomes. From the expression given on p. 154 of Charlesworth *et al.* (2005), the expected ratio of X to A ancestral polymorphism levels is approximately equal to exp $(t_A - t_X)$, where the subscripts denote the divergence time for the chromosome in question. If the estimated t values for *D. miranda* from Table 3 are substituted into this expression, the ratio of expected ancestral polymorphism levels is thus exp $(-0{\cdot}31) = 0{\cdot}73$. More conservatively, if we use the lower confidence limit for t_X (equal to 0·84) and estimate t_A as three-quarters of this, we obtain a ratio of 0·81. By multiplying the observed proportion of autosomal ancestral polymorphisms by this ratio, we can adjust this proportion to a scale on which it can be compared with the fraction for the X chromosome.

Even if the more conservative estimate is used for this purpose, the confidence interval for the adjusted A value does not overlap with that for X (the upper limit for A is 21·6 compared with a lower limit for X of 23·2). This suggests that there may be a real difference between X and A, compared to what is expected on the model used to generate the predictions. A possible explanation for this is that a relatively recent reduction in effective population in the *D. miranda* lineage has had a greater effect on the X (with its lower effective population size) in causing loss of the most recent derived polymorphisms, leading to a deficiency of type 6 events (see Fig. 1).

Finally, we examine how ancestral polymorphism levels may relate to the expectation of an elevated synonymous site diversity associated with long-term balancing selection (for a review of the theory of this effect, see Charlesworth & Charlesworth, 2010, pp. 393–398). Haddrill *et al.* (2010) found seven additional loci that appeared to show significantly elevated synonymous diversity in one or other of the two species, on the basis of a multilocus HKA test (Hudson *et al.*, 1987); this implies that their elevated diversity cannot simply be a consequence of a randomly generated, long coalescence time and that a non-neutral process is likely to be needed to explain the properties of these genes. Table 4 shows the uncorrected estimates of the numbers of ancestral synonymous polymorphisms for these loci. Comparison with Table 1 suggests that there may indeed be an excess of type 1/2/3 polymorphisms when compared with loci that show no deviations from neutral expectations by the HKA test. This possibility can be tested using a 2×2 contingency table for 'ancestral' vs. '*de novo*' polymorphisms for the two categories of loci. For the X chromosome, the 2×2 χ^2 is 8·15 ($P < 0{\cdot}01$), and for the autosome (with much less data) it is 1·85 (non-significant). While this test is somewhat crude, it suggests that the possibility of balancing selection acting on at least some of these genes is worth further investigation.

In summary, we have extended and substantially improved a method for estimation of the proportion of ancestral polymorphisms within species by introducing novel procedures for estimation of the scaled divergence time between species, correcting for parsimony and generating confidence intervals on the parameters of interest. We have applied this improved method to a dataset of polymorphisms in almost 70 protein-coding genes, distributed across the X chromosome and an autosome in *D. pseudoobscura* and *D. miranda*, and find that a substantial proportion of synonymous variants in these two species are ancestral.

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5. Supplementary material

The online data are available at http://journals.cambridge.org/GRH

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