Molecular Population Genetics of the Alcohol Dehydrogenase Locus in the Hawaiian Drosophilid *D. mimica*

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Sequence variation among 10 alleles of the alcohol dehydrogenase (*Adh*) gene of the Hawaiian drosophilid *D. mimica* was analyzed with reference to the evolutionary history of the Hawaiian subgroup as well as to levels and patterns of polymorphism of the *Adh* gene in continental drosophilid species. The *Adh* gene of *D. mimica* is less polymorphic than that of other drosophilid species, and no replacement substitutions were found. Statistical analyses of the *Adh* alleles suggested the action of balancing selection and revealed significant linkage disequilibrium among three of the variable sites. The effective population size was estimated to be only slightly smaller than that of continental species and, surprisingly, on the same order of magnitude as the actual size.

Introduction

Intraspecific variation in the nucleotide sequence of the alcohol dehydrogenase (*Adh*) gene in *Drosophila melanogaster* and in closely related species has been used to study relative rates of nucleotide substitution (Kreitman 1983), intragenic recombination and gene conversion (Stephens and Nei 1985), fixation of advantageous mutations (McDonald and Kreitman 1991), and the molecular basis of variation in enzyme activity (Laurie, Bridgham, and Choudhary 1991). The *Adh* locus is the most intensively studied gene in Drosophila population genetics and, while much is now known about the generation and maintenance of allelic variation at this locus in *D. melanogaster,* further insight regarding the evolutionary forces shaping patterns of polymorphism and divergence comes from analyses of the *Adh* sequences of distantly related drosophilid species. These investigations have identified a variety of evolutionary events at this locus that are unique to particular lineages (e.g., Yum, Starmer, and Sullivan 1991; Anderson, Carew, and Powell 1993; Long and Langley 1993).

Intraspecific comparisons of *Adh* alleles, until now only available for the *D. melanogaster* subgroup and *D. pseudoobscura,* should be particularly useful in understanding the differences in the evolutionary dynamics of this locus among the various species groups. Intraspecific DNA sequence studies of the *Adh* locus in *D. pseudoobscura* have been used to estimate population substructure and amounts of gene flow (Schaeffer and Miller 1992*a*), to measure linkage disequilibrium (Schaeffer and Miller 1993), and to show that, in contrast to the conclusions from studies of the *D. melanogaster* subgroup, adaptive evolution does not appear to have been a significant force (Schaeffer and Miller 1992*b*).

We have sequenced the *Adh* genes of nine individuals of *D. mimica,* one of several hundred species that are endemic to the Hawaiian Islands (Carson and Kaneshiro 1976). Extensive allozyme electrophoretic surveys of *D. mimica* and related species have been reported (Rockwood 1969; Rockwood et al. 1971; Steiner 1979). *D. mimica* is a member of the modified-mouthparts group, and is found almost exclusively in two nearly adjacent localities, Kipuka Ki (0.575 km^2) and Kipuka Puaulu (0.297 km^2), in Volcano National Park on the island of Hawaii (Richardson 1974).

Materials and Methods

Isofemale lines of *D. mimica,* cultured as described in Yoon (1985), were obtained from the Drosophila Species Resource Center at Bowling Green (table 1). DNA was extracted from individual flies by the method of Gloor and Engels (1991); primers for PCR and nucleotide sequencing (available from F.J.A. on request) were designed from the published *D. mimica Adh* sequence (Thomas and Hunt 1991); and a fragment of ~900 bp spanning the entire coding region was amplified. PCR products were purified with the QIAquick Spin PCR Purification Kit (Qiagen), and sequenced with an Applied Biosystems model 373A sequencer and the PRISM™ DyeDeoxy terminator kit. Nucleotides 49–873, numbered as in Thomas and Hunt (1991), were sequenced on both strands from all strains and were aligned by hand. The sequences have been deposited in GenBank, accession numbers L78920–L78928. The published sequence was also used in the analyses.

Results and Discussion

Adh Sequences

The polymorphic nucleotide sites in the 824-bp sequenced region of *Adh* are shown in table 1. There were no insertions or deletions. Sites 132, 149, and 596 are located in the introns, the others are in the coding region, and all are silent (synonymous). Site 412 was heterozygous in two strains; in the calculations that follow one allele was chosen at random from each heterozygote.

Phylogenetic reconstructions of the *D. mimica Adh* alleles performed by the UPGMA, neighbor-joining (Kimura's two-parameter distance method), and maximum-parsimony (branch-and-bound method) algorithms with the MEGA program (Kumar, Tamura, and Nei 1993) yielded nearly identical topologies (data not shown).

Key words: alcohol dehydrogenase, *D. mimica,* Hawaiian Drosophila, effective population size, polymorphism, molecular evolution.

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Table 1
Collection Localities and Variable Sites in the *Adh* Sequences of *D. mimica*

Sequence[a]	Collection Locality[b]	Site[c]								
		1	1	2	2	4	5	6	8	8
		3	4	0	3	1	9	8	2	6
		2	9	8	8	2	6	0	1	9
Published	Thomas and Hunt (1991)	T	G	C	C	A	G	T	T	G
15292-2561.0	Kipuka Ki	A	T	T	T	T	·	·	A	·
15292-2561.1	Kipuka Puaulu	A	T	T	T	T	·	·	A	·
15292-2561.2	Papa	A	T	·	T	T	·	·	A	·
15292-2561.3	Kipuka Puaulu (Bird Park)	A	T	·	T	T	·	·	A	·
15292-2561.4	Kipuka Puaulu (Bird Park)	A	T	·	T	T	A	C	·	A
15292-2561.5	Kipuka Puaulu (Bird Park)	·	·	·	·	W	·	·	·	·
15292-2561.6	Kipuka Puaulu (Bird Park)	A	T	·	T	T	·	C	·	·
15292-2561.8	Kipuka Puaulu (Bird Park)	·	·	·	·	W	·	·	·	A
15292-2561.9	Kipuka Ki (Mohanaloa)	A	T	·	T	T	A	C	·	A

[a] The first sequence is the published sequence of Thomas and Hunt (1991); the rest are listed by catalog number from the Drosophila Species Resource Center.

[b] Collection localities are listed as in the catalogs.

[c] Nucleotide sites are numbered according to the published sequence. Nucleotides identical to those of the published sequence are indicated by dots (·). The letter W stands for a heterozygous position in which both the nucleotides A and T were found.

There was no tendency for the alleles from each collection site to cluster together, which is consistent with the results of an electrophoretic survey which also showed no significant differences between localities (Rockwood 1969). All 36 pairwise combinations of segregating sites were analyzed for linkage disequilibrium by Fisher's exact test of independence, with the significance level adjusted for multiple comparisons by the sequential Bonferroni method (Rice 1989). Sites 132, 149, and 238, which are in complete linkage disequilibrium, are significantly nonrandomly associated, each with $P = 0.001$. At least one recombination event is inferred from the "four gamete" test (Hudson and Kaplan 1985).

Tests of Selective Neutrality of Substitutions

Several statistical tests of the null hypothesis of selective neutrality of substitutions (Kimura 1983) may be applied to intraspecific DNA sequence data. Each of the three tests applied here is sensitive to a distinctive type of violation of the null model caused by the action of positive selection. However, because the relatively low level of polymorphism in the *D. mimica Adh* sequences greatly decreases the power of these tests, nonsignificant results must be viewed with caution.

The McDonald-Kreitman method (McDonald and Kreitman 1991) tests the neutral expectation of a correspondence between interspecific divergence and intraspecific polymorphism. The number of fixed substitutions in the *D. mimica* lineage was calculated by analyzing all available Hawaiian drosophilid *Adh* sequences with the MacClade 3.03 program (Maddison and Maddison 1992), using the phylogeny of Russo, Takezai and Nei (1995, fig. 2). The phylogenetic position of the Hawaiian drosophilid *D. adiastola* remains uncertain (e.g., Kambysellis et al. 1995 and references therein), and this drosophilid was therefore excluded from the analysis. The *Adh* sequence of *D. mettleri,* in the *D. repleta* group, was used as an outgroup to the Hawaiian sequences. The number of substitutions that lie on the branch basal to the radiation of the *D. mimica* sequences, on the node which separates *D. mimica* from the picture-winged group, was counted. The ratio of fixed to polymorphic replacement events in the *D. mimica Adh* sequences was 6:0, and the ratio of fixed to polymorphic synonymous events was 7:6. These ratios are not significantly different (Fisher's exact test of independence: $P = 0.109$). The analysis was repeated by extending the *D. mimica* branch to the node which separates the (*D. mimica,* picture-winged) clade from *D. nigra,* in the fungus-feeder group (see Russo, Takezaki, and Nei 1995, fig. 2). The respective ratios then become 7:0 and 14:6, which are also not significantly different from each other (Fisher's exact test of independence: $P = 0.155$). Therefore, the null hypothesis of selective neutrality of the amino acid replacements is not rejected by this analysis.

The Tajima test of selective neutrality examines the relationship between two estimates of the population parameter θ: an estimate calculated from the average number of pairwise nucleotide differences, and another based on the total number of segregating sites, both of which should have the same value under selective neutrality (Tajima 1989). The two estimates for the *D. mimica Adh* sequences are not significantly different ($k = 3.933$, $S/a_1 = 3.181$; $D = 1.03$; $P > 0.1$) and, therefore, again the null hypothesis is not rejected. (Sampling different sets of alleles from the heterozygous strains does not alter this conclusion; $P > 0.1$ in every case.)

A third test of selective neutrality, the Fu and Li test (Fu and Li 1993), examines the expected relative numbers of substitutions that have occurred on the internal and external branches of a genealogy. Among the nine evolutionary events ($\eta = 9$) that have occurred in the *D. mimica Adh* sequences, there are no external substitutions ($\eta_e = 0$). On the basis of the Fu and Li test, the null hypothesis of selective neutrality is rejected ($D = 1.59$; $P < 0.025$), in a direction consistent with bal-

Table 2
Population Parameters of Drosophilid Species

Species[a]	k[b]	r[c]	θ_s[d]	$\text{var}_r(\theta_s)$	$\text{var}_l(\theta_s)$	θ_U[e]	$\text{var}(\theta_U)$
D. melanogaster	14	12	4.64	1.54	5.21	6.10	5.76
D. simulans	11	6	4.82	2.11	8.63	7.85	14.18
D. yakuba.....	17	12	5.63	1.86	7.28	n.a.[f]	n.a.
D. pseudoobscura ...	33	99	6.39	1.24	3.73	16.49	8.86
D. mimica.....	6	10	2.12	0.75	1.61	3.19	2.32

[a] *D. melanogaster, D. simulans,* and *D. yakuba* data from (McDonald and Kreitman 1991), *D. pseudoobscura* data from (Schaeffer and Miller 1993).

[b] The number of segregating synonymous sites at the *Adh* locus.

[c] The number of *Adh* sequences.

[d] θ_s is the estimate of θ calculated from the number of segregating sites, $\text{var}_r(\theta_s)$ is the variance under free recombination of sites, $\text{var}_l(\theta_s)$ is the variance under complete linkage.

[e] θ_U and $\text{var}(\theta_U)$ are the estimates calculated by the UPBLUE method.

[f] Because many of the *D. yakuba* sequences contain several heterozygous sites, unambiguous distance measures between pairs of alleles cannot be obtained; consequently, the UPBLUE procedure could not be used to estimate θ for these sequences.

ancing selection. (If only alleles which contain T at site 412 are sampled from the heterozygous strains, then η_e becomes 1, and $D = 1.092$; $P > 0.05$. All other combinations yield significant results.)

Population Parameters

Because of their recent origin, extensive radiation, and restricted geographic ranges (Carson and Kaneshiro 1976), it has often been assumed that the effective population sizes, N_e, of the Hawaiian Drosophila must be very small (*e.g.,* DeSalle and Templeton 1988; Ohta 1993). But this assumption has not been tested and is, furthermore, incongruent with evidence from allozyme studies that many of these species, including *D. mimica,* have heterozygosities that exceed those of species with much larger geographic ranges, including *D. melanogaster* and *D. pseudoobscura* (Rockwood et al. 1971; Templeton 1980). Because N_e can have a profound effect on the patterns of genetic polymorphism and divergence (Nei and Graur 1984; Kimura 1983), we have endeavored to estimate N_e from the *Adh* sequences.

N_e was calculated by estimating the parameter $\theta = 4N_e\mu_s$, where μ_s is the synonymous mutation rate per gene per generation. An unbiased estimator, θ_s, is provided by $\theta_s = k/a_{r-1}$ (Ewens 1979, Eq. 9.73), where k is the number of segregating sites and $a_{r-1} = (1/1) + (1/2) + \cdots + (1/(r-1))$, where r is the number of sequences. This calculation yields an estimate of $\theta_s = 2.12$. On the assumption of free recombination among all segregating sites, the variance of this estimate is $\theta_s/a_{r-1} = 0.75$; and under complete linkage, the variance is $\theta_s/a_{r-1} + \theta^2((1/1) + (1/4) + \cdots + (1/(r-1)^2))/(a_{r-1})^2 = 1.61$ (Ewens 1979, eq. 9.74). The actual variance of θ_s lies between these values; while some linkage disequilibrium has been shown, the entire gene is not under complete linkage. Another estimate, θ_u, is provided by the UPBLUE method of Fu (1993). In addition to the number of segregating sites, this algorithm incorporates phylogenetic information (in the form of distance measures) from the alleles, and it typically yields a relatively smaller variance than calculations based on the number of segregating sites alone. θ_u was calculated as 3.19, with a variance of 2.32.

By calibrating the times of speciation events to the times of formation of the islands on which the species are endemic, Rowan and Hunt (1991) estimated the synonymous substitution rate in the Hawaiian Drosophila to be 1.5×10^{-8} substitutions per nucleotide per year. Given 161 synonymous sites in the sequenced region of the *Adh* gene (calculated by the method of Li, Wu, and Luo [1985], which was also the method used by Rowan and Hunt [1991]) and five generations per year (Nei and Graur 1984), $\mu_s = 4.83 \times 10^{-7}$ substitutions per gene per generation. Therefore, N_e was estimated as 1.1×10^6 from θ_s, and as 1.7×10^6 from θ_u.

The population parameters of several drosophilid species are presented in table 2. These estimates were obtained as above from the sequences of the *Adh* locus of *D. melanogaster, D. simulans, D. yakuba* (McDonald and Kreitman 1991), and *D. pseudoobscura* (Schaeffer and Miller 1993). Only the synonymous sites from the region homologous to the sequenced segment of the *D. mimica Adh* alleles were used.

Tests of constancy of the molecular clock have shown that the third codon positions of most drosophilid *Adh* sequences evolve at a constant rate (Takezaki, Rzhetsky, and Nei 1995). However, *D. pseudoobscura* is among the exceptions, evolving more slowly than the others at the 1% significance level. Thus, while the θ's for the *D. melanogaster, D. simulans, D. yakuba,* and *D. mimica* sequences may be compared directly to assess their relative N_e's, the estimate of N_e for *D. pseudoobscura* will be even higher than expected from the relative values of θ. We also note that balancing selection increases genetic variation within a population (Li 1978; Nei 1980; Maruyama and Nei 1981) and may therefore have inflated the estimates of θ in *D. mimica* as well as those in *D. melanogaster* (Hudson, Kreitman, and Aguadé 1987).

The methods used to estimate θ assume an infinite-site model at equilibrium, selective neutrality of substitutions, no recombination, and an absence of population subdivision—assumptions which are likely to be violated. The adaptive fixation of replacement substitutions in the *D. melanogaster* subgroup (McDonald and Kreitman

1991) may have affected the evolutionary dynamics of closely linked synonymous sites through genetic hitchhiking (Wiehe and Stephan 1993; Kaplan, Hudson, and Langley 1989). For the *D. mimica* alleles, a violation of the assumption of selective neutrality is inferred from the observation of linkage disequilibrium, as well as from the rejection of the null hypothesis by the Fu and Li test. For the *D. pseudoobscura* alleles, linkage disequilibrium (Schaeffer and Miller 1993), recombination (Schaeffer and Miller 1993), and population subdivision (Schaeffer and Miller 1992*a*) have been demonstrated. Therefore, estimates of θ must be viewed with caution.

Given the lower value of μ_s and much larger values of θ for *D. pseudoobscura*, N_e for this species appears to be significantly larger than for *D. mimica*. N_e apparently is also larger in species of the *D. melanogaster* subgroup than in *D. mimica*, but the significance is marginal at best, depending on the choice of estimates, and, in fact, the N_e's of these species could be of the same order of magnitude. Inasmuch as theoretical, simulation, and empirical analyses suggest that a 5- to 10-fold difference in N_e can significantly affect rates of fixation and amounts of polymorphism (Ohta 1973; Ohta and Tachida 1990; Gillespie 1994; Aquadro, Lado, and Noon 1988), the differences in θ for the species listed in table 2 may be evolutionarily significant.

The actual population size of *D. mimica* was estimated at three million from capture-recapture methods (W. W. M. Steiner and R. H. Richardson, personal communication cited in Nei and Graur [1984]). This value is, surprisingly, reasonably close to the estimates of N_e. Continental Drosophila, including *D. melanogaster* subgroup species and *D. pseudoobscura*, in contrast, must have actual population sizes many orders of magnitude greater than their effective population sizes (see Johnston and Heed [1976] and references therein).

The results of this study contribute to an understanding of the variation in the patterns of replacement substitution observed among the *Adh* sequences of various Drosophila subgroups. Dorit and Ayala (1995) observed that while some segments of the ADH protein exhibit similar patterns of constraint or divergence in all examined drosophilid subgroups, conspicuous differences also occur between subgroups. For example, certain segments of the ADH sequence, most notably amino acid positions 163–171, were unusually variable in the Hawaiian subgroup (including at least three substitutions, at sites 167, 170, and 171, unique to the *D. mimica* lineage), while the same segments were strongly conserved in the *D. melanogaster* and *D. repleta* subgroups. If the greater divergences in the Hawaiian sequences were due to relaxed selective constraints on these regions of the protein, as a result either of the unique features of the Hawaiian environment or of the induction of covariant change (Fitch and Markowitz 1970), then an increase in within-species replacement polymorphism would also be expected in this region in *D. mimica*. However, this was not observed.

The increased divergences among the Hawaiian Drosophila might be adaptive. This explanation requires that all of the examined Hawaiian lineages have independently undergone repeated cycles of adaptive fixation within regions of the ADH sequence which have remained invariant in other subgroups. Alternatively, the higher level of interspecific divergence may reflect the stochastic fixation of slightly deleterious substitutions during periods of relatively low N_e (Ohta 1973, 1976). Under this scenario, a reduced N_e (during bottlenecks or following founding events) renders weakly deleterious mutations effectively neutral and allows them to drift toward fixation. After N_e has increased to current levels, the power of natural selection to distinguish among slight variants is increased: newly arising deleterious mutations are no longer tolerated, while silent polymorphisms are allowed to accumulate. Either of these two scenarios could plausibly account for the heightened divergences of the Hawaiian subgroup *Adh* sequences, as well as the polymorphism data presented here. Further insight regarding the evolutionary forces that have shaped the contrasting divergence patterns will come from comparative sequence analyses of other loci among these species groups.

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