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Evolution

Nucleotide sequence analysis of *Adh* genes estimates the time of geographic isolation of the Bogota population of *Drosophila pseudoobscura*

(alcohol dehydrogenase/nucleotide site polymorphism/molecular evolution/reproductive isolation)

STEPHEN W. SCHAEFFER* AND ELLEN L. MILLER

Department of Biology and Institute of Molecular Evolutionary Genetics, Pennsylvania State University, University Park, PA 16802

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ABSTRACT **The population of *Drosophila pseudoobscura* at Bogota, Colombia, is geographically and partially reproductively isolated from populations in the main body of the species in North America. The degree of genetic differentiation and time of divergence between populations at Bogota and Apple Hill, CA, were estimated by comparison of 3388 nucleotides in the alcohol dehydrogenase region (*Adh* and *Adh–Dup* genes) of 18 strains. Of the 146 polymorphic nucleotide sites detected, 68 and 31 were unique to the Apple Hill and Bogota samples, respectively, and 53 were shared. On the basis of an observed net divergence per nucleotide site of 0.264% between the two samples, the Bogota and North American populations were estimated to have been separated for at least 155,000 years. This divergence time suggests that *D. pseudoobscura* extended its range from North America to South America in a period of Pleistocene glaciation, when habitat suitable for the species presumably existed in lowland Central America.**

The time and mode of origin of the geographically disjunct population of the fruit fly *Drosophila pseudoobscura* at Bogota, Colombia (Fig. 1), has been a subject of speculation and controversy since its discovery in 1960 (1). Although Prakash (2) demonstrated that flies from Bogota are reproductively isolated from those in North America (F_1 males from crosses between Bogota females and North American males are sterile), he nonetheless suggested that the Bogota population is of very recent origin because it was genetically similar to North American populations at 19 enzyme loci assayed electrophoretically (3). Additionally, the chromosomal inversions in the Bogota population had earlier been shown to be a subset of those present in North American populations (1).

In contrast, Ayala and Dobzhansky (4) concluded that the Bogota population is old when an electrophoretic analysis of allelic variation at 25 additional enzyme loci revealed a level of differentiation from North American populations that is typical of many pairs of species. This led Ayala and Dobzhansky (4) to name the flies at the Bogota population a new subspecies, *Drosophila pseudoobscura bogotana*. Subsequently, sequential gel electrophoresis demonstrated the occurrence of unique alleles at the xanthine dehydrogenase and alcohol dehydrogenase loci in *D. p. bogotana* (5, 6). These studies strongly suggested that *D. pseudoobscura* had not recently colonized the Bogota region, but the data did not yield an estimate of the age of the population.

We here report the results of a nucleotide sequence analysis of the alcohol dehydrogenase region (*Adh* and *Adh–Dup* genes) (Fig. 2) of 18 strains of *D. pseudoobscura* from Bogota and a population at Apple Hill, CA.† The degree of nucleotide sequence divergence between the Bogota and California populations indicates a divergence time of at least 155,000 years.

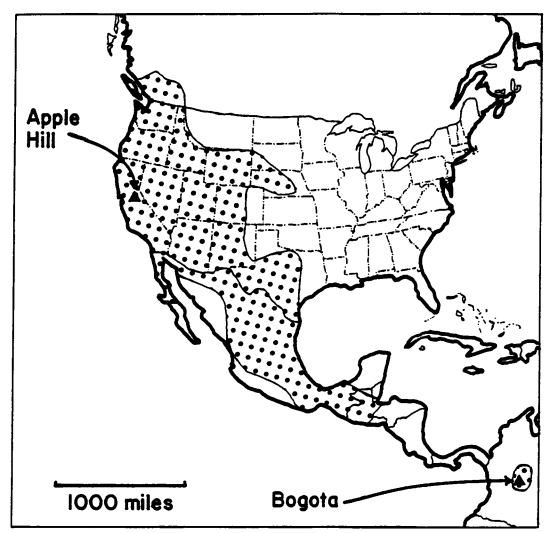

FIG. 1. Geographic distribution and sampling localities of *D. pseudoobscura* in the Americas.

MATERIALS AND METHODS

Strains. Ten strains of *D. pseudoobscura* were collected at Apple Hill, CA (AH), in November, 1982 (8): AH43, AH54, AH69, AH100, AH122, AH133, AH135, AH144, AH162, and AH165 (Fig. 1). The eight strains of *D. p. bogotana* (BOG) came from two collections. Seven strains were collected in the 1960s: BOG 3389.1, BOG 3389.2, BOG 3389.3, BOG 3389.4, BOG 3389.5, BOG 3389.6, and BOG 3389.9 (3). One strain was collected in 1977: BOG ER (collected by H. F. Hoenigsberg and obtained from W. W. Anderson, University of Georgia, Athens). Single strains of the two sibling species *Drosophila persimilis* (DPER) and *Drosophila miranda* (DMIR) were obtained from the National *Drosophila* Species Resource Center at Bowling Green State University. The sibling species of *D. pseudoobscura* were included in the study to aid in the rooting of the phenogram of alleles.

DNA Sequencing. The DNA of each *Adh* region in the 20 strains listed above was amplified and sequenced by either molecular cloning techniques outlined in Schaeffer and Aquadro (7) or by direct sequencing of products from the polymerase chain reaction (9–11). Fig. 2 shows the fragment of DNA that was sequenced. A total of 3.5 kilobases of DNA

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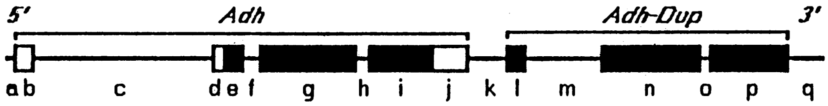
*To whom reprint requests should be addressed.

†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M60979–M60998).

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         a   b c                                                                                         d  ef           g
DPSE  TGACCACAAGGCACGTCCGCGTTTAACTGTGGCGGTGGGGTGTTATTTGAAGTATTCATTATGTTCCGTCTGATTTCGGAGGGATGATCCAAGGGTCGTACGTGCCTATCTAAAGGATGG
AH43  .....T...........A...C....A.............G.......A.......C..A.GT.......G.A.TAC...................A....A..G..A......C...
AH162 .....T...........A..........A...........G............G..C............G.....C.................A....A.....T.T........
AH135 .C...T.G.............................................................C............C.....A....AA..G.TAT........
AH133 .....T...........A......................G.........T.A.................C............T....AA....A.....T.T....C...
AH122 .....T.G..AA......A..............G......G..............G..............AC....................A....A...............
AH69  .....T...............C....A.............TG..........CATTAC.CAC...............C..................A....A...............
AH100 .....T...........A.......................GCTA..........G............T...G.A..AC....G.....T.......A....A..A..A.........
AH144 .....T...........A.......................G.........................A........C..................A.A..A...............
AH54  .....T...............C....A.............TG..........CATTAC.CAC...............C..................A....A...............
AH165 .....T.G..................................G.........................A.G.A...C..........T.......A....AA..G.TAT........
BOGER .....T...............C....A..............G........G.CATTAC..A.....A..........CT..................A....A....GTAT........
BOG.1 ....GT...........A....C..................G........G.CATTAC..A.....A..........CT..................A....A....GTAT........
BOG.2 ...T.T............................................GCATTAC..A.....A..........CT..................A....A....GTAT........
BOG.3 .....T...............C....A..............G........G.CATTAC..A.................CT..................A....A....GTAT........
BOG.4 .....T...............C....A..............G........G.CATTAC..A.................CT..................A....A....GTAT........
BOG.5 ....GT...........A....C..........T.....G........G.CATTAC..A.....A..........CT..................A....A......TAT........
BOG.6 ...T.T............................................GCATTAC..A.....A..........CT..................A....A.T..GTAT........
BOG.9 .....T.....A.T....A.....GGGG.CAAG.A..C.AA........................................G.....C..................A....A...............
DPERS .....T.....A.T....A................T...TC...GC............G..C...............G...AC..................AGAA.A......T.T........
DMIRA C.G..TT.TA.AC.AGGAAATG..GGGG.CAAG.A.......G...AATCC..T...T.C..A...TA.T..T..AT.AC.TCG.AGCAG...TCCT.CTG.AA.....T.TGGACTAAA
            10'       20'       30'       40'       50'       60'       70'       80'       90'      100'      110'      120'

       h                    i        j k  l  m                  n                o        p                   q
DPSE  ATAACACTCAATTTGAACCCCTAGGTCTCTCTCACGCCCCCCATCCGTGCATACCTCATATGTGGCTGGTAACGAAGATGGCCTCGGCTTCCCGAGTTCCACTGCTCCGGCTAAT
AH43  ..........................TC..................................C...G....T.A..C...A.....C.G.................A.......G......C
AH162 ........................A.TCG.................................C...GC...TT..T..G.A............T.....G...AA..................
AH135 .......CA...AAATT.......A.TC..........A............C...C...G....T...T.G..A.GC.............C.G..........T...C.........
AH133 .......CA..........T....A.............................T.C...C...G....T.A.TC....A....G.............G..CAA..AT....C.G.....T.
AH122 ..........................TC.........T..................C...G....T....T....A....G....CG......G........T..............
AH69  TAGTTT............T.........TC.............G..........C...C..........T..AT.G..T.......A..C.......T....AAC.........TG.......
AH100 TAGTTT....................CTC...........................C...TC...CC.......T.G..A.GC.............C.G.......................T.
AH144 TACTTT....................CTC...............................TC..C...C.T...T.G..A....................G...AA.........C.......T.
AH54  TAGTTT............T.........TC.............G..........C...C..........T..AT.G..T.......A..C.......T....AAC.........TG.......
AH165 .......CA...AAATT.......A.TC.G..........T.....................C...G...AT...T....A.....C...C.....C.....AAC.AT......G.......
BOGER TAGTTT....................TC....................................TCT.C..T......T....A.......A..C.......G....................
BOG.1 TACTTT....................CTC...................................TCT....T......T....A...................G..CAA...T.............
BOG.2 TACTTT....................TC.......................................C.....T......T....A..............A...G..CAA...T.............
BOG.3 TACTTT....................TC.......................A.....TCT.C..T..T....CG..A.G....A..C.......G...AA.G.........G...GG..
BOG.4 TACTTT....................TC.......................A.....TCT.C..T..T....CG..A.G....A..C.......G...AA..........G...GG..
BOG.5 .......CA...AAATT..........CG....................................TCT.C..T......T....A.......A..C.......G....................
BOG.6 TACTTT....................TC.......................................C.....T......T....A...................G..CAA...T.............
BOG.9 TACTTT..................A..C......T...................................C..C.....T....T.G..A......C.GT.........G.......A......C.G.......
DPERS .......CA...AAATT....CG..CTC.........T...T..G............TC...G.....T...T....AG..C...T..........G.ACA.......G....G.....T.
DMIRA TAGTTTT..TGC..C...T.TCGT..TC..GGGT.A.T....G.GGTA..T.CTTC.G.G....T.A.T...AAT.....A.....C.C..GT..........TG..GATT....
          130'      140'      150'      160'      170'      180'      190'      200'      210'      220'      230'
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FIG. 2. Variable nucleotide sites in the *Adh* region of *D. pseudoobscura* and its close relatives. The fine structure of the two genes *Adh* and *Adh–Dup* is shown at the top of the figure. The functional domains of the *Adh* region are abbreviated as follows: a, 5′ flank; b, adult leader; c, adult intron; d, larval leader; e, exon 1; f, intron 1; g, exon 2; h, intron 2; i, exon 3; j, 3′ leader; k, intergene; 1, exon 1; m, intron 1; n, exon 2; o, intron 2; p, exon 3; and q, 3′ flank. The names of the sequences are abbreviated as follows: AH, Apple Hill; BOG, Bogota; DMIRA, *D. miranda*; DPERS, *D. persimilis*; and DPSE, *D. pseudoobscura* (7). The Bogota names BOG.1–BOG.6 and BOG.9 are a further abbreviation of the Bogota 3389.1–Bogota 3389.6 and Bogota 3389.9 lines, respectively.

sequence was generated for each strain. The full nucleotide sequences are available in the EMBO/GenBank data bases.

Nucleotide Sequence Alignment. The 20-nucleotide sequences were aligned by eye by minimizing the number of mismatches and sequence length variants. Sequence length variation was ignored in all subsequent analyses because the mechanisms that generate insertions and deletions are poorly understood at this time and will be presented elsewhere.

Analysis of Segregating Site Data. Each segregating nucleotide position in the sample of 18 sequences from Apple Hill and Bogota was classified as either unique to its respective population or shared between the two populations. Six nucleotide sites had three segregating nucleotides in one or both populations. If the multiple segregating nucleotides were observed in only one of the populations, these sites were counted as two unique segregating sites. If the multiple segregating sites were observed in both populations, these sites were counted as two shared segregating sites. If only a pair of nucleotides were shared between populations and the other nucleotide was unique to one population, this situation was scored as one shared segregating site and one unique segregating site in its respective population.

Bootstrap analysis was used to determine if the observed number of shared segregating sites in *Adh* from Apple Hill and Bogota differs from the expected number assuming these samples were obtained from a single statistical population. For each bootstrap replicate, we generated a new set of alleles for Apple Hill ($n = 10$) and Bogota ($n = 8$) by drawing nucleotides with replacement within polymorphic sites that assumes independence of segregating sites. The set of alleles from a replicate was used to calculate the numbers of unique and shared segregating sites for the Apple Hill and Bogota populations. Observed values for each type of segregating site were compared to their respective bootstrap distributions over the 1000 replicates to determine statistical significance.

The net nucleotide divergence between the Apple Hill and Bogota population was calculated by the method of Nei (12). If similar alleles are present in both populations then net divergence between Apple Hill and Bogota should be zero. Bootstrap analysis was used to determine if the observed net

divergence between the two populations is significantly greater than zero. For each bootstrap replicate, we sampled the polymorphic sites with replacement, for a total of 146 sites. The set of alleles within each replicate was used to calculate heterozygosity within each population and the difference between populations. A net divergence value of zero was compared to the bootstrap distribution of net divergence to determine statistical significance of our estimate.

Genetic Similarity of Alleles. Genetic distances between all pairwise comparisons of the 20 *Adh* alleles sequenced in this study and the previously published sequence (7) were estimated with the method of Nei (12). Genetic distance measures will reflect the true phylogenetic history of a set of alleles provided that intragenic recombination is relatively rare. Schaeffer *et al.* (8) showed that intragenic recombination was an important force generating diversity in the *Adh* region of *D. pseudoobscura*. Thus, a gene genealogy will not necessarily reflect the true phylogenetic history of alleles in our sample. We used the neighbor-joining method (13) to cluster the 21 alleles into similar groups reflecting the current similarity of these alleles.

RESULTS

An examination of 3388 nucleotide sites, excluding insertions and deletions, in the sample of 18 *Adh* alleles from Apple Hill and Bogota revealed that 146 sites were segregating for two nucleotides in either or both of the populations. An additional six sites had three nucleotides segregating, for a total of 152 polymorphic sites (Fig. 2). Bogota and Apple Hill shared 53 (35%) of the segregating sites, 68 (45%) of the polymorphic sites were unique to Apple Hill, and 31 (20%) were recorded only in the Bogota sample. Bootstrap analysis shows that the Apple Hill and Bogota populations have each accumulated an excess of unique segregating sites ($P < 0.001$) and the two populations share fewer segregating sites than expected if they were recent derivatives of a single population ($P < 0.001$) (Table 1). We obtained similar results in a bootstrap analysis that assumed nonindependence of segregating sites (i.e., alleles were sampled with replacement) except that the number of unique polymorphisms in Bogota was not statistically different from bootstrap values ($P > 0.158$) due to a larger variance of the sampling distribution.

The number of segregating sites may not always be the best parameter to examine variation in populations because polymorphism is not independent of sample size. Heterozygosity per nucleotide site, which is defined as the average number of nucleotide differences observed between two randomly chosen sequences, may be a better parameter to use because it is unbiased with respect to sample size. The observed heterozygosity in Apple Hill is greater than that in Bogota (Table 2). The difference in heterozygosity between Bogota and Apple Hill was greater than zero in each of the 1000 bootstrap samples of the segregating sites ($P < 0.001$). The net divergence per nucleotide site between Apple Hill and Bogota was 0.264% as estimated by the method of Nei (ref. 12 and Table 2). Bootstrap analysis of the segregating site data showed that the net divergence is greater than the expectation of zero, assuming no genetic differentiation between Apple Hill and Bogota ($P < 0.001$).

Table 1. Number of segregating sites in the *Adh* region of *D. pseudoobscura*

Type(s) of sites and population(s)	Observed		Expected	
	S	Proportion of all sites	S	Proportion of all sites
Unique				
Apple Hill	68	0.020	29.5	0.009
Bogota	31	0.009	20.5	0.006
Shared (both)	53	0.016	76.9	0.023
Total				
Apple Hill	121	0.036	106.4	0.032
Bogota	84	0.025	97.4	0.029

Total number of sites compared, $N = 3388$; S, number of segregating sites; proportion of segregating sites, S/N.

Table 2. Average pairwise number of differences and heterozygosity per nucleotide site in the *Adh* region of *D. pseudoobscura*

Comparison	k	H
Within population		
Apple Hill	39.60	0.0117
Bogota	28.32	0.0084
Between populations		
Apple Hill/Bogota	43.01	0.0127
Apple Hill/Bogota net	8.95	0.0026

Total number of sites compared, $N = 3388$; k, pairwise number of differences; heterozygosity per nucleotide site, $H = k/N$.

We can determine the time of divergence between the Bogota and Apple Hill populations if net nucleotide divergence is calibrated to a known rate of nucleotide substitution. Lemeunier *et al.* (14) proposed that *Drosophila simulans* diverged from both *D. mauritiana* and *Drosophila seychellia* approximately one million years ago. Caccone *et al.* (15) recorded a 1°C change in the mean melting temperature (ΔT_m) of nuclear DNA·DNA hybrids between *D. simulans* and *D. seychellia*. This corresponds to 1.7% nucleotide mismatch, based on DNA·DNA hybridization of known mitochondrial nucleotide sequences (16). The estimated divergence time between the Bogota and Apple Hill populations is between 155,000 and 534,000 years ago, depending on the calibration value used. The time based on the *Drosophila* calibration of 1.7% nucleotide substitutions per million years (15) is shorter than the age derived from the mammalian calibration value of 0.5% nucleotide substitutions per million years (17).

We constructed a phenogram to summarize the relationships among the 21 *Adh* alleles in the *obscura* group of *Drosophila* (Fig. 3). The tree was rooted at its midpoint. The *Adh* alleles from the Bogota population are found in two lineages that are distinct from the Apple Hill alleles. The

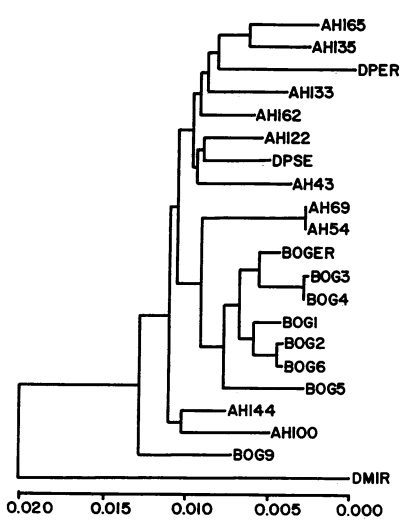

FIG. 3. Phenogram of nucleotide sequences in the alcohol dehydrogenase region for members of the *obscura* group of *Drosophila*. The neighbor-joining method of Saitou and Nei (13) was used to derive the phenogram. The tree was rooted at the midpoint. The names of the sequences are abbreviated as in Fig. 2.

BOG9 sequence is distantly related to all other sequences, while the other Bogota alleles form a single group that apparently shared a common ancestor with the AH69 and AH54 alleles from Apple Hill. The Apple Hill population has at least three other *Adh* lineages.

The *Adh* allele from the sibling species *D. miranda* (DMIR) is the most divergent sequence found in this study. A restriction endonuclease analysis of the *Adh* region (7) and the amylase region (18) found that the *D. persimilis* sequence is more closely related to some *D. pseudoobscura* sequences than some *D. pseudoobscura* alleles are related to one another. The phenogram of *Adh* alleles based on complete nucleotide sequences also shows the same relationship between the *D. persimilis* allele and alleles from *D. pseudoobscura* (Fig. 3).

DISCUSSION

Age and Biogeography of the Bogota Population. The number of nucleotide substitutions is an accurate predictor of the time of divergence between populations or species if random genetic drift and mutation are the predominant forces responsible for the observed nucleotide diversity of genes (19). The rate of selectively neutral substitutions at nucleotide sites is unaffected by natural selection acting on linked sites (20); however, the number of polymorphic nucleotides is profoundly affected by selection acting on linked sites. Directional selection reduces the number of segregating sites in a gene region, while balancing selection increases the number of segregating sites (21–24). We have found (unpublished results) that the *Adh* region has not been acted upon by directional or balancing selection in the recent history of these two populations. Hence, the net divergence between these populations in the *Adh* region should accurately reflect the age of the Bogota population.

All analyses of the *Adh* region indicate that the Bogota population of *D. pseudoobscura* is genetically distinct from the Apple Hill population. We confidently conclude *D. pseudoobscura* did not recently invade the Bogota region because the number of segregating sites that are shared between the Apple Hill and Bogota populations is less than would be expected if these two populations were recently derived from the same population (Table 1). In addition, the Bogota alleles form two distinct clusters (Fig. 3), which would not be expected if the Bogota population was geographically isolated in the recent past. If *D. pseudoobscura* had repeatedly invaded Bogota, then *Adh* alleles in Bogota would be scattered throughout the phenogram.

Prakash (2) suggested that the partial reproductive isolation observed between Bogota and the United States was unusual because it seemed that insufficient time had elapsed for the evolution of isolating mechanisms. Our estimate of the divergence time of at least 155,000 years shows that partial reproductive isolation of the Bogota and United States populations of *D. pseudoobscura* did not occur recently. Our results are consistent with Mayr's (25) view that reproductive isolation occurs through the slow accumulation of genetic differences between isolated populations. Orr (26) has recently expanded on the work of Dobzhansky (27) to show that there is a strong genetic component to the male sterility found in offspring of crosses between Bogota females and North American males. Male sterility is caused by maternal effects, a major effect due to the X chromosome, and a small but significant effect of the autosomal genes.

We can only speculate on the biogeographical factors relating to the origin of the Bogota population of *D. pseudoobscura*. One possible scenario is that the geographic distribution of *D. pseudoobscura* was more or less continuous across the Isthmus of Panama during periods of maximum glaciation in the Pleistocene when the climate in the present-day tropical regions was cool and dry. What little is known concerning the ecology of *D. pseudoobscura* in Central America suggests that lower altitude habitats are inhospitable. *D. pseudoobscura* extends its North American range as far south as Guatemala and prefers temperate to cold climates at elevations 5000 or more feet above sea level where the flies tend to occur in drier habitats on the edges of forests (28).

The formation of the Isthmus of Panama 3–4 million years ago (29) provided a possible land bridge for *D. pseudoobscura* to expand its geographic range southward from North America. It may have been able to move into Central American habitats as far as the mountains around Bogota, Colombia, during periods of glacial maxima in the Pleistocene when the climate of Central America was cool and dry rather than warm and humid. Populations of *D. pseudoobscura* became extinct in Central America when the climate became warm and humid during a long interglacial period, which led to isolation of the Bogota population from populations in Guatemala. Our estimate of 155,000 years as the time of divergence between the Apple Hill and Bogota populations is consistent with this explanation, because this date coincides with the beginning of a long interglacial period (30). It is entirely possible that the ancestral refugia of *D. pseudoobscura* during the Pleistocene was in South America rather than North America. Collections of *Drosophila* from high elevations in the Andes may uncover other populations of *D. pseudoobscura* that are necessary to evaluate this alternative hypothesis.

The data presented here do not preclude the possibility that *Adh* alleles in populations in Mexico and Guatemala are more similar to alleles in Bogota than to those in Apple Hill. However, this possibility is excluded by analysis of nucleotide sequences of the *Adh* region for 21 additional alleles collected throughout the North American distribution of *D. pseudoobscura* (unpublished data). The North American alleles show no strong pattern of geographic differentiation, which indicates that there is extensive gene flow between populations. Two alleles collected in British Colombia, Canada, and Tulancingo, Mexico, are identical in nucleotide sequence, with the exception of a single insertion; yet, the geographic distance between these two populations is greater than that between Guatemala and Bogota. Although *D. pseudoobscura* has the ability to migrate great distances, none of the North American alleles has been found in Bogota. Therefore, we conclude that the Apple Hill population is adequately representative of the North American populations.

Genetic Variation in Subspecies and Sibling Species. Heterozygosity in the Bogota population is reduced compared to the Apple Hill population. The phenogram of *Adh* alleles in Fig. 3 shows that *Adh* alleles in Bogota occur in two major lineages. One lineage is comprised of seven alleles that are very closely related, whereas the other lineage is made up of a single allele. Dobzhansky *et al.* (1) also observed reduced chromosomal variation in the Bogota population compared to populations in Guatemala. These combined data suggest that either the Bogota population was founded by a small number of individuals with few chromosomal types and/or that the Bogota population has maintained a smaller effective population size relative to other populations in the main geographic range of the species.

Powell (31) found that *D. pseudoobscura* and *D. persimilis* share mitochondrial DNA haplotypes. He suggested that hybridization between these species permitted introgression of mitochondrial DNA haplotypes but that selection against backcross females prevented nuclear genes from being transferred to either parental species. The relationship of the *D. persimilis Adh* allele to the *D. pseudoobscura* alleles is inconsistent with Powell's (31) explanation, because the

nucleotide sequence of *Adh* of *D. persimilis* is more similar to some *D. pseudoobscura* sequences than some *D. pseudoobscura* alleles are to one another.

D. persimilis is reproductively isolated from *D. pseudoobscura* (F_1 males are sterile in either of the reciprocal crosses of the parent species; F_1 females are fertile in both crosses). Thus, *D. persimilis* has a stronger barrier to gene flow in crosses with *D. pseudoobscura* than do flies from Bogota, yet the *D. persimilis* allele is more similar to *D. pseudoobscura* alleles than any of the Bogota alleles. These findings may indicate that either ancestral polymorphisms have been maintained in *D. pseudoobscura* and *D. persimilis* or that there is a small amount of nuclear gene introgression between *D. pseudoobscura* and *D. persimilis*. If repeated introgression has introduced nuclear genes into the two species, then *Adh* genes from *D. persimilis* should be spread throughout a phylogenetic tree of alleles of both species with some identical alleles occurring in both species. But, if little introgression has occurred in the history of these two species and ancestral polymorphisms have been maintained, the *Adh* alleles of *D. persimilis* should be clustered together in the same manner that the Bogota alleles are clustered in our phenogram. In either case, more *Adh* sequences from *D. persimilis* are needed to distinguish between these alternative explanations.

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