DNA Sequence Variation at the *Period* Locus Reveals the History of Species and Speciation Events in the *Drosophila virilis* Group

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ABSTRACT

The virilis phylad of the Drosophila virilis group consists of five closely related taxa: D. virilis, D. lummei, D. novamexicana, D. americana americana and D. americana texana. DNA sequences from a 2.1-kb pair portion of the period locus were generated in four to eight individuals from each of the five taxa. We found evidence of recombination and high levels of variation within species. We found no evidence of recent natural selection. Surprisingly there was no evidence of divergence between D. a. americana and D. a. texana, and they collectively appear to have had a large historical effective population size. The ranges of these two taxa overlap in a large hybrid zone that has been delineated in the eastern U.S. on the basis of the geographic pattern of a chromosomal fusion. Also surprisingly, D. novamexicana appears to consist of two distinct groups each with low population size and no gene flow between them.

THE basic process of evolution that has led to the diversity of life is the formation of new species. Different genetical theories of speciation have been proposed (DOBZHANSKY 1937; MAYR 1942; CARSON 1968, 1975; WHITE 1978; TEMPLETON 1980), yet there is still much debate over the details of the process (BARTON and CHARLESWORTH 1984; TEMPLETON 1989; COYNE and ORR 1989; COYNE 1992). In general, speciation events cannot be observed directly because they occur on an evolutionary time scale. However, measurements of the pattern of genetic variation within and among closely related species can provide information suitable for exploring speciation processes and examining the role of population size, gene flow and population subdivision.

This research compares DNA sequence data from within and among the five closely related taxa in the virilis phylad of the Drosophila virilis species group (THROCKMORTON 1982). These five taxa are D. virilis, D. lummei, D. novamexicana, D. americana americana and D. americana texana. D. virilis is found in wild habitats in Japan and China, while in North America it is restricted to domestic habitats (PATTERSON and STONE 1952). In contrast, the other virilis phylad species are found exclusively in woodland settings (PATTERSON 1942a). D. lummei is found in northeastern Europe. The North American taxa, D. a. americana, D. a. texana, and D. novamexicana are closely related (PATTERSON and STONE 1952; THROCKMORTON 1982; SPICER 1992) and are collectively referred to as the americana complex (PATTERSON and STONE 1952). D. a. americana and D. a. texana reside in the eastern United States, and their

ranges overlap in a hybrid zone running through North Carolina, Tennessee and Arkansas (Figure 1). D. a. americana is found north of the hybrid zone, and D. a. texana is found to the south (PATTERSON 1942b; CARSON and BLIGHT 1952; PATTERSON and STONE 1952; THROCKMORTON 1982). The third North American species, D. novamexicana, is found in the drier habitat of lower river valleys of New Mexico and the surrounding states. D. novamexicana has a lighter mesothorax color than both D. a. americana and D. a. texana, which have a dark body color and are virtually indistinguishable morphologically from each other. The lighter mesothorax of D. novamexicana may be an adaptation for desiccation resistance (SPICER 1991a). It has been suggested that the change accompanied speciation and the ability to live in the drier habitat (SPICER 1991a).

The five virilis phylad taxa exhibit three different metaphase karyotypes. D. virilis possesses what is considered the primitive karyotype, with five pairs of rods and one pair of dots (HSU 1952; THROCKMORTON 1982). This pattern is shared by D. novamexicana and D. lummei. D. a. americana and D. a. texana both have a fusion of the second and third chromosomes, making a large Vshaped chromosome. D. a. americana has a unique fusion of the X and a fourth chromosome, also a large Vshaped chromosome, which causes both the Y and a free fourth chromosome (both rods) to be male limited (STURTEVANT and NOVITSKI 1941; HSU 1952; PAT-TERSON and STONE 1952; EVGEN'EV 1971; THROCKMOR-TON 1982; GUBENKO and EVGEN'EV 1984). It is this X-4 chromosomal fusion that distinguishes D. a. americana from D. a. texana, and the hybrid zone between these species has been described on the basis of measurements of the frequency of X-4 fusion karyotypes.

We report DNA sequence data from the period (per)

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FIGURE 1.—Generalized range of *D. a. americana*, *D. a. texana*, and *D. novamexicana* including *D. a. americana* and *D. a. texana* hybrid zone. Compiled from data of PATTERSON and STONE (1952) and information on collection locations from The National Drosophila Species Resource Center.

locus. Mutations at the per locus have been found to affect circadian rhythms as well as courtship songs (KRYRIACOU and HALL 1984). In D. melanogaster, per is found on the X chromosome. An X chromosome location for *per* is also expected in species of the *virilis* phylad because of the high degree of conservation of chromosomal elements between D. melanogaster and D. virilis (STURTEVANT and NOVITSKI 1941; ALEXANDER 1976). This conservation of linkage groups between D. melanogaster and D. virilis has also been confirmed for many individual loci (TONZETICH et al. 1990; WHITING et al. 1989; NEUFELD et al. 1991; NURMINSKY et al. 1996). We generated data from a 2.1-kb region from four lines of D. virilis and D. lummei, seven lines of D. a. americana, eight lines of D. a. texana and six lines of D. novamexicana (Figure 2). The per locus was chosen for this analysis for a variety of reasons. KLIMAN and HEY (1993) studied variation in a 1.9-kb region of the per locus from six individuals of each of the four species of the D. melanogaster group and found it was a good choice for their study of speciation (KLIMAN and HEY 1993; HEY and KLIMAN 1993). Also, per evolves quickly, so that even when examining closely related species, there is ample variation for a variety of analyses (COLOT et al. 1988; KLIMAN and HEY 1993). Lastly, the expected X chromosome location of the per gene simplifies the procedure for generating single copy genomic DNA. DNA prepared from a single male contains sequences from X-



FIGURE 2.—Diagram of the *period* locus and the region sequenced. The specific region sequenced is marked with the lower black line that corresponds to bases 2870–4864 of COLOT *et al.* (1988). a marks the spot of the Thr-Gly repeat; b marks the three locations where sequence was not used in the analysis, see text for details.

TABLE 1

List of lines sequenced

Species name	Line no.	Location
D. virilis	1051.0	Pasadena, CA
	1051.8	Truckee, CA
	1051.9	Sendai, Japan
	1051.48	Texmelucan, Mexico
D. lummei	1011.1	Moscow, Russia
	1011.2	Overhalix, Sweden
	1011.4	Kukkola, Finland
	1011.8	Sakata, Japan
D. A. americana	0951.0°	Anderson, IN
	0951.1°	Poplar, MT
	0951.3°	Millersburg, PA
	0951.4°	Keelers Bay, VE
	0951.5°	Jackson, MI
	0951.6°	Chadson, NE
	0951.9°	Myrtle Beach, SC
D. A. texana	1041.0°	St. Francisville, LA
	1041.22°	New Orleans, LA
	1041.23 ^c	Morrilton, AR
	1041.25	So. Richmond, VA
	1041.26	Tallahassee, FL
	1041.27°	Goldenhead Branch, FL
	1041.29	Jamestown, SC
	1041.31	Hollandale, MS
D. novamexicana	1031.0^{a}	Grand Junction, CO
	1031.4^{b}	Moab, UT
	1031.7^{a}	Patagonia, AZ
	1031.8^{a}	San Antonio, NM
	1031.11^{b}	Gila, NM
	1031.12^{b}	Antlers, CO

All lines are from the National Drosophila Species Resource center.

^a A member of group Nova-A.

^b A member of group Nova-B.

^c These lines were checked for the appropriate metaphase chromosome compliment; see text for details.

linked genes in hemizygous, rather than diploid, proportion.

MATERIALS AND METHODS

The flies: All strains were obtained from the National Drosophila Species Resource Center (NDSRC) (Table 1). In this paper, strains are referred to by species name and the NDSRC extension number, for example, "virilis.0," corresponds to NDSRC #1051.0 (Table 1). Confirmation of chromosomal karyotype in some strains was done using mitotic squash protocol #1 in ASHBURNER (1989). In addition, JEANNE HNILICKA and B. CHARLESWORTH (personal communication) found the following lines obtained from the stock center had the expected chromosomal patterns (THROCKMORTON 1982): americana.0, americana.1, americana.3, americana.4, americana.6, americana.9, and texana.22 (Table 1).

DNA preparation and sequencing: DNA preparations were made from single male flies (protocol 48 in ASHBURNER 1989). A 2.1-kb region of the *per* gene was PCR amplified using 20-mer oligonucleotide primers starting at positions 2803 ("+" primer 5' base) and 4911 ("-" primer 5' base) of COLOT *et al.* (1988; GenBank accession X13877). PCR and DNA sequencing methods were identical to those of KLIMAN and

HEY (1993). Sequences have been submitted to GenBank, acquisition numbers (L81296-L81324).

Alignments: Sequence alignment was first done by eye and then with the Genetics Computer Group program PILEUP (DEVEREUX and HAEBERLI 1991). Three small areas within introns revealed large amounts of insertion-deletion (indel) variation and were not included in the study because of alignment uncertainty. One area that was removed (corresponding to position 4197–4227 of COLOT *et al.* 1988) contained variations on a CT repeat, ranging from four CT pairs in all *D. virilis* lines to 21 pairs in a *D. novamexicana* line. The other regions that were not included correspond to positions 4436– 4456 and 4585–4635 of COLOT *et al.* (1988).

Estimating Nm and Mantel test: Nm, the product of effective population size and migration rate, was estimated using the Fst estimate of HUDSON et al. (1992). To test whether the divergence between subspecies is greater than expected by chance, the nonparametric Mantel test (MANTEL 1967) was used to compare the similarity between two matrixes. The first matrix contained, for a set of DNA sequences from two subspecies, the number of differences between all pairs of sequences. The second matrix represented the hypothesis that sequences were more similar within subspecies and was made up of zeros and ones. A zero was placed in the matrix at those positions that corresponded to positions in the first matrix that contained the pairwise difference between sequences drawn from the same subspecies. A one corresponded to a difference between sequences from different subspecies. The test of association between the two matrices is straightforward: a coefficient of association, z, is calculated as the sum of all pairwise matrix cell projects (i.e., the product of matrix 1 cell *i*,*j* with matrix 2 cell *i*,*j* summed over all *i* and j; an empirical distribution of this statistic is determined by a repeated process of 1000 random permutation of rows and columns of one matrix and recalculation of z for each permutation, and the probability of getting an equal or more extreme value than the observed value of z is assessed by comparing the observed value with the random distribution. The Mantel test was carried out using the NTSYS (ROHLF 1985) computer program package.

Measuring variation: The average number of pairwise nucleotide differences, π , is calculated from

$$\pi = \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \frac{k_{ij}}{\binom{n}{2}},\tag{1}$$

where *n* is the number of sequences sampled and k_{ij} is the number of differences between sequences *i* and *j*. A second measure of sequence variation, θ , is a simple function of the number of polymorphic sites, *S*, and the sample size (WATT-ERSON 1975)

$$\theta = S / \sum_{i=1}^{n-1} \frac{1}{i}.$$
 (2)

Both π and θ have expected values of 4Nu, where N is the effective population size and u is the neutral mutation for the locus per generation. For a sex-linked gene, in the case where the effective population size is similar for males and females, the expected value for π and θ is 3Nu.

RESULTS

Grouping the lines: The three taxa in the americana complex, *D. a. americana*, *D. a. texana*, and *D. novamexicana*, have been distinguished on the basis of morphological and chromosomal comparisons. Typically, one individual has been chosen to represent each taxa in later studies of the *D. virilis* group (*e.g.*, REINBOLD and

COLLIER 1990). However, these *a priori* hypotheses of species and subspecies status, based on limited genetic data, are not supported by our genealogical study. We have two examples where comparative sequence data do not support the prior species designations. First, *D. a. americana* and *D. a. texana* appear to be indistinguishable on the basis of the *per* data. Second, our *D. novamexicana* sequences seem to have come from two groups that have not recently exchanged genes.

Three analyses of the per data failed to reveal a pattern of divergence between the D. a. americana and the D. a. texana samples. First, these two groups had 33 shared polymorphisms, which are base pair positions where both D. a. americana and D. a. texana were segregating the same two bases. We also found no fixed differences (positions where all of the D. a. americana lines had one base and all of the D. a. texana had a different base) between these two groups. Second, the Fst estimate of Nm was 55.166 (HUDSON et al. 1991). Typically an Nm value greater than or equal to one leads to considerable homogeneity among populations (WRIGHT 1940). Third, a comparison of pairwise differences within and between D. a. americana and D. a. texana was no different from random contrasts, as determined by a Mantel test (the observed divergence was not different than zero; P =0.176; see MATERIALS AND METHODS). For the rest of the analyses we have treated D. a. americana and D. a. texana as one group under the name, D. americana.

A second question about how to group individuals arose in D. novamexicana. When taken as a group of six sequences, there was little to distinguish them from D. americana. There was just one fixed difference between the D. novamexicana and the D. americana sequences (a synonymous change at position 468). The D. novamexicana samples, when grouped together, where highly variable; however this was misleading. The D. novamexicana sequences include two divergent sets, each of three very similar sequences. Nearly all of the variation within the D. novamexicana sample occurs as differences between these two groups, which we have named "Nova-A" and "Nova-B". There were 23 fixed differences between Nova-A and Nova-B, and no shared polymorphisms. The Fst estimate of Nm between the two groups was 0.01, revealing little evidence of intermixing at per. For the remainder of the analysis, D. novamexicana was treated both as one group, as well as treating Nova-A and Nova-B separately.

Both Nova-A and Nova-B revealed little within-group polymorphism (Table 3, Figure 3). In Nova-A, lines nova.7 and nova.8 were identical, sharing a unique 3bp insertion in the coding sequence. The third member of Nova-A, nova.0, was also different from the other two, in having a unique intron base change. In group Nova-B, each of the three lines, nova.4, nova.11, and nova.12, differed by one or two changes. This is quite different from *D. americana* where after lines texana.26 and texana.27, which were identical, the next most closely related lines differed by 14 changes. Base

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FIGURE 3.—Variable sites in *period*. The first rows indicate the base position of variable sites within the sequenced region. In the comment row, s, synonymous substitution; r, amino acid replacement substitution; I, intron change; d, deletion change. There are three noncoding intron regions that correspond to base positions 1015–1088, 1311–1432, and 1574–2088. The virilis.0 (1051.0) sequence is used as a reference. Nucleotides identical to the reference are indicated by a dash. At amino acid replacement sites, the nucleotide is followed in parentheses by the one letter code for the resulting amino acid (M, Met; L, Leu; P, Pro; A, Ala; S, Ser; I, Ile; D, Asp; E, Glu; Q, Gln; T, Thr; N, Asn; V, Val; G, Gly; H, His; K, Lys; W, Trp; R, Arg). Length variation is indicated by * in sequences shortened relative to others.

Eleven of the 23 fixed differences between the two *D. novamexicana* groups are polymorphic within *D. americana*. In other words, at 11 base positions where *D. americana* was found to segregate two bases, it was also found that the three Nova-A sequences all possessed one of the bases found in *D. americana* while the three Nova-B sequences all possessed the other base that was found in *D. americana*. Of the 12 remaining fixed differ-

ences between Nova-A and Nova-B, five base changes are unique to Nova-A (*i.e.*, Nova-A sequences are different from Nova-B and *D. americana*) and seven are unique to Nova-B. These 12 base changes may have arisen since each group has become isolated from the species that was ancestral to *D. americana* and *D. novamexicana*. It is also possible that these changes are polymorphisms in *D. americana* that did not appear in our

Base	11	1111	111	11	1 1	111	1	1	1111111	111111	111111	L1	1111	11111	1111:	11111	1111	.11111		TTT
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•	01	1557	900	12	2 2	688	8	9	9122223	234888	801225	56	7888	89999	9001	12226	6778	89990	0112	2222
	93	5174	028	73	4 7	812	6	1	2912394	807036	946588	32	0358	90124	9021	32688	9396	94782	25490)123
comment	sr	rssr	ssr	sr	r r	srr	r	r	siiidii	iissss	SSSSS	sr	siii	iiiii	iiii:	iiiii	iiii	iiiii	iide	ppp
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Lummei-4	GT(S)	- A - A (Q	T(N)	TC (D)	-(-)G(A)C-C	(S) - (-) - (-)	- A *CT		C-T-C-	(-)		A1-	CIC-		A P	TAGG.		
Lummei-8	GT(S)	- A - A (Q) T (N)) TC (D)	- (-)G(A)CGC	(A) - (-) A (Q)	- A *CT	CC	C-T-C	(-)	11	T -	CTC-0	2	A P	TAGG-	- C - * *	****
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Amer-1	G-(-)	- A - A (Q) C - T (N)) TC (D)	- (-)G(A) G-C	(S)A(E) - (-)	AA *CT	- ACC	C-T-C	(-)	TT	· T -	CTC-(2	- A - A	TAGG-	C	
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Amer-4	G-(-)	-A-A(0) - TT (N)) TC (D)	- (-)G(A) G-C	(S)A(E) - (-)	- AT - *CT	CC	C-T-C	A-(-)	A - TT	T-	CT (cc-	- A - A	TAGG	· C	
Amer-5	G-(-)	-A-A(0) C - T (N)) TC (D)	- (-)G(A)G-C	(S) A (E) - (-)	- A *AT	- ACC	C-T-C	(-)	TT	T -	CTC - (C C -	- A - A	AAGG	C	
Amer-6	G-(-)	- A - A (Õ	C-T (N	TC (D)	- (-)G(A)G-C	(S)A(E) - (-)	-A*CT	- AC - T -	C-T-C	(-)	TT	T -	CTC-	c	- A - A	TAGG	C	
Amor-9	G- (-)	- 2 - 2 (0	C-T(N	TC (D)	- (-)G(A)G-C	(S)A(E) - (-)	- A *CT	- ACC	C-T-C	(-)	- ATT	T-	CTC-	c	- A - A	TAGG	c	C
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Novamex-4	G-(-)) - A - A (Q) C - T (N) TC (D)	A(T)G(A) G - C	(S)A(E	(-)	- A *CT	- ACC	C-T-C	(-)	TI	r TC	CTC -	C C -	- A - A	ATAGG	CG-	
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Novamex-7	G-(-)) - A - A (Q) C - T (N) TC (D))-(-)G((A) G-C	(S)A(E	:) - (-)	-A-T*CT	- ACC	C - T - C	A-(-)	TI	r - T -	CTC -	cc-	- A - Z	TAGG	- C	
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Novamex-0	G- (-)	-A-A(0) C - T (N) TC (D)) - (-) G ((A) G-C	(S)A(E) - (-)	- A - T*CT	- ACC	C - T - C	A-(-)	TT	ст-	CTC -	c c -	- A - A	TAGG	- C	
Base position	11111	1111111 7777777	111111	111111	1111111 7777777	11111	111111 788888	11111	1111111 8888888	111111 888888	111111	11111 88889	11111	11111	1111	11122 99900	2222	222222	22222	22222
Base position	1111: 7777 2222:	1111111 7777777 2233333	111111 777777 333334	11111: 77777 44444	1111111 7777777 4444555	11111 777777 67777	111111 788888 845555	11111 88888 55555	1111111 8888888 6666666	111111 888888 667777	11111 88888 77789	11111 88889 99990	11111 99999 01124	11111 999999 155566	1111 9999 7888	11122 99900 89900	2222	222222	22222	22222 00000 33333
Base position	11111 7777 22222 4567	1111111 7777777 2233333 8901234	111111 777777 333334 567890	11111 77777 44444 12345	111111 777777 4444555 5789017	11111 777777 67777 740123	111111 788888 845555 591234	11111 88888 55555 56789	1111111 8888888 6666666 0123456	111111 888888 667777 790245	11111 888888 77789	11111 88889 99990 23588	L1111 99999 01124 35732	11111 999999 155566 201369	1111 99999 7888 3357	11122 99900 89900 94507	2222 00000 2222 234	222222 00000 222222 56789	22222 0000 33333 01234	22222 00000 33333 45678
Base position comment	11111 7777 22222 4567 dddd	1111111 7777777 2233333 8901234 1dddddd	111111 777777 333334 567890 dddddd	111111 77777 44444 12345 ddddd	111111 777777 4444555 5789017 dddddii	11111 77777 67777 740123 11111	111111 788888 845555 591234 iidddd	11111 88888 55555 56789 Iddddd	1111111 8888888 6666666 0123456 ddddddd	111111 888888 667777 790245 diiiii	11111 88888 77789 67911 diiii	11111 88889 99990 23588 iiiii	1111 99999 01124 35732	11111 999999 155566 201369 11111	1111 99999 7888 3357 iiii	11122 99900 89900 94507 iiiii	22222 00000 22222 2345	222222 00000 222222 567896 1dddd	22222 00000 33333 01234 1ddd	22222 00000 33333 45678 ddddd
Base position comment Virilis-0	11112 7777 22222 4567 ddddo TCCA	1111111 7777777 2233333 8901234 1dddddd AATGTGC	111111 777777 333334 567890 dddddd A*****	11111: 77777 44444 12345 ddddd	111111 777777 4444555 5789017 dddddii ****GAA	11111 777777 67777 740123 Liiiii	111111 788888 845555 591234 iidddd CG****	11111 88888 55555 56789 Idddd	1111111 8888888 6666666 0123456 ddddddd *******	111111 888888 667777 790245 diiiii *TGAAC	11111 888888 77789 67911 diiii ACAAC	11111 88889 99990 23588 iiiii TTAGG	11111 99999 01124 35732 11111 3GGTA	11111 999999 155566 201369 11111 ATATAT	1111 99999 7888 3357 iiii ATCG	11122 99900 89900 94507 iiiii ATTAC	2222 00000 2222 2349 1ddd	222222 00000 222223 567896 1ddddo 36GTA3	22222 00000 33333 01234 1ddda FAGC2	22222 00000 33333 45678 ddddd ATATC
Base position comment Virilis-0 Virilis-8	11112 7777 22222 4567 dddd TCCA	111111 777777 2233333 8901234 1dddddd AATGTGC	111111 777777 333334 567890 dddddd A*****	11111 77777 44444 12345 ddddd	1111111 777777 4444555 5789017 dddddii ****GAA	11111 777777 667777 740123 Liiiii AATGAG	111111 788888 845555 591234 iidddd CG****	11111 88888 55555 56789 ddddd	1111111 8888888 6666666 0123456 ddddddd *******	111111 888888 667777 790245 diiiii *TGAAC	11111 88888 77789 67911 diiii ACAAC	11111 88889 99990 23588 iiiii TTAGO	11111 99999 01124 35732 11111 3GGT7	11111 999999 155566 201369 11111 ATATAT	1111 99999 7888 3357 iiii ATCG	11122 99900 89900 94507 iiiii ATTAC	2222 00000 2222 2349 dddd CAATO	222222 00000 222222 567890 1dddd 3GGTA	22222 0000 33333 01234 1ddd FAGC2	22222 00000 33333 45678 ddddd ATATC S
Base position comment Virilis-0 Virilis-8 Virilis-9	11111 77777 22222 45677 ddddd TCCA	1111111 7777777 2233333 8901234 4dddddd AATGTGC	111111 777777 333334 567890 dddddd A****	11111 77777 44444 12345 dddddd	111111 777777 4444555 5789017 dddddii ****GAP	11111 77777 67777 40123 111111 AATGAG	111111 788888 845555 591234 iidddd CG**** - TGGA T	11111 88888 55555 56789 Iddddd	1111111 8888888 6666666 0123456 ddddddd *******	111111 888888 667777 790245 diiiii *TGAAC TC	11111 888888 77789 67911 diiii ACAAC	11111 88889 99990 23588 iiiiii TTAGO	11111 99999 01124 35732 11111 3GGTA	11111 999999 555566 201369 111113	1111 99999 7888 3357 iiii ATCG	11122 99900 89900 94507 iiiiii ATTAC	22222 00000 22222 2349 1dddc 2AATC	222222 00000 222222 567896 1dddd 3GGTA	22222 0000 33333 01234 1ddd FAGC2	22222 00000 33333 45678 ddddd ATATC G S
Base position comment Virilis-0 Virilis-8 Virilis48	11111 7777 22222 45677 ddddd TCCA	1111111 777777 2233333 8901234 ddddddd AATGTGC	111111 777777 333334 567890 dddddd A****	111111 77777 44444 12345 dadada	111111 777777 4444555 6789017 dddddii ****GAA	11111 77777 67777 740123 Liiiii AATGAG	111111 788888 845555 591234 iidddd CG**** TGGA T	11111 88888 55555 6789 ddddd	1111111 8888888 6666666 ddddddd ******* CTATAAA	111111 888888 667777 790245 diiiii *TGAAC TC	11111 888888 77789 67911 diiii ACAAC	11111 88889 99990 23588 iiiiii TTAGO	11111 99999 01124 35732 11111 3GGTA -A-A-	11111 999999 55566 201369 11111 ATATAT	1111 99999 7888 3357 iiii ATCG	11122 99900 89900 94507 iiiiii ATTAC	22222 00000 22222 2349 1dddd CAATO	222222 00000 222222 567890 1dddd 3GGTA	22222 00000 3333 01234 1dddd FAGC2	22222 00000 33333 45678 ddddd ATATC G G
Base position Virilis-0 Virilis-8 Virilis-9 Virilis48	11111 7777 22222 45677 ddddd TCCA G	1111111 7777777 2233333 8901234 ddddddd AATGTGC	111111 777777 33334 567890 dddddd A*****	11111: 77777 44444 12345 dadada	1111111 7777777 4444555 6789017 dddddii ****GAA	11111 77777 67777 40123 111111 AATGAG	111111 788888 845555 591234 iidddd CG**** - TGGA T TGGA T- TTGA	11111 88888 55555 6789 ddddd TCCTT	1111111 8888888 6666666 0123456 ddddddd ****** CTATAAA CTATAAA	111111 888888 667777 790245 diiiii *TGAAC TC TC	11111 888888 77789 67911 diiii ACAAC	11111 88889 99990 23588 iiiiii TTAGO	11111 99999 01124 85732 11111 GGGTA -A-A- -A-A-	11111 999999 55566 201369 11111 ATATATATATATATATATATATATATATATATA	1111 99999 7888 3357 .iiii ATCG	11122 99900 89900 94507 iiiii ATTAC	22222 00000 22222 2349 1dddc 2AATC	222222 00000 222223 567890 1dddd 3GGTA	22222 00000 33333 1234 1444 FAGC2	22222 00000 33333 45678 ddddd ATATC G G G S
Base position Virilis-0 Virilis-8 Virilis-9 Virilis48 Lummei-1 Lummei-2	11111 7777 2222 4567 ddddd TCCAJ	1111111 7777777 2233333 8901234 4dddddd AATGTGC	111111 777777 333334 567890 ddddddd A*****	11111: 77777 44444 12345 dddddd *****	111111 777777 4444555 6789017 dddddii ****GAA	11111 77777 67777 40123 Liiiii AATGAG	111111 788888 845555 5912344 iidddd CG**** - TGGA T- TGGA T- TTGA	11111 88888 55555 6789 ddddd ***** TCCTT TCCTT	1111111 8888888 6666666 0123456 ddddddd ****** CTATAAA CTATAAA CTATAAA	111111 888888 667777 790245 diiiii *TGAAC TC TC TC T-	11111 888888 77789 67911 ACAAC TC-6	11111 88889 99990 23588 iiiii TTAGC	11111 99999 01124 35732 11111 3GGTA -A-A- -A-A- -A-A-	11111 99999 55566 201369 11111 ATATAT ATATAT	1111 9999 7888 3357 .iiii ATCG	11122 99900 89900 94507 iiiii ATTAC	22222 00000 22222 2345 24ddd CAATO	222222 00000 22222 56789 1dddd 3GGTA	22222 00000 33333 1234 1ddda FAGC2 ((22222 00000 33333 45678 ddddd ATATC G GA GA
Base position Virilis-0 Virilis-9 Virilis-9 Virilis48 Lummei-1 Lummei-2	11111 7777 22222 45677 ddddd TCCAJ	1111111 7777777 2233333 8901234 1dddddd AATGTGC	111111 777777 333334 567890 ddddddd A*****	11111 77777 44444 12345 dddddd *****	111111 777777 444455 5789017 dddddii ****GAA *GC	11111 777777 67777 740123 111111 AATGAG A A A A A	111111 788888 845555 591234 iidddd CG**** - TGGA T- TGGA T- TTGA T- TTGA	11111 88888 55555 ddddd TCCTT TCCTT TCCTT TCCTT	1111111 8888888 6666666 0123456 ddddddd ****** CTATAAA CTATAAA CTATAAA CTATAAA	111111 888888 667777 790245 diiiii *TGAAC TC TC TC T- TC T-	11111 88888 77789 67911 diiii ACAAC 	11111 88889 99990 23588 iiiii TTAGO CC	11111 99999 91124 35732 11111 3GGTA -A-A- -A-A- A- A-	111111 999999 155566 201369 11111 ATATAT A-GTF A-GTF A-GTF	1111 9999 7888 3357 .iiii ATCG 	11122 99900 89900 94507 iiiii ATTAC 	22222 00000 22222 2345 2444 2444 2444 2444 2444 244	222222 00000 222223 567890 ddddd 3GGTA	22222 00000 33333 1234 1dddd FAGC2 (((22222 00000 33333 45678 ddddd ATATC G GA GA GA
Base position Virilis-0 Virilis-8 Virilis-9 Virilis48 Lummei-1 Lummei-1 Lummei-8	11111 7777 22222 45677 ddddd TCCAi G 	1111111 7777777 2233333 8901234 ddddddd AATGTGC 	111111 777777 33334 567890 ddddddd A***** 	11111 77777 44444 123455 dddddd	111111 777777 4444555 6789017 dddddii ****GAA *GC *GC *GC	11111 77777 667777 740123 11111 AATGAG A - GATA - GA 	111111 78888 845555 591234 11dddd CG**** - TGGA T-TGA T-TTGA T-TTGA TATTGA	11111 88888 55555 ddddd TCCT1 TCCT1 TCCT1 TCCT1 TCCT1	1111111 8888888 6666666 0123456 ddddddd ****** CTATAAA CTATAAA CTATAAA CTATAAA CTATAAA	111111 88888 667777 790245 diiiii *TGAAC TC TC TC T TC - T TC - T TC - T	11111 88888 77789 diiii ACAAC - TC - (- TC - (11111 88888 99990 23588 iiiii TTAGO CC	11111 99999 91124 85732 11111 3GGTA -A-A- A- A- A- A-	L11111 999999 155566 201369 111111 ATATAT A-GTA A-GTA A-GTA A-GTA	1111 19999 7888 3357 .1111 ATCG 	11122 99900 89900 94507 iiiii ATTAC 	22222 00000 22222 2345 24ddd 2AATC	222222 00000 22222 56789 ddddd 3GGTA	22222 00000 3333 01234 1dddd FAGC2 ((((22222 00000 33333 45678 ddddd ATATC G GA GA GA GA
Base position Virilis-0 Virilis-8 Virilis-9 Virilis48 Lummei-1 Lummei-2 Lummei-4 Lummei-8 Amer-0	11111: 77777 2222: 45671 ddddd TCCAi 	1111111 777777 2233333 8901234 ddddddd AATGTGC	111111 777777 333334 567890 dddddd A***** 	111111 77777 44444 12345 dddddd	111111 777777 4444555 6789017 dddddii ****GAA *GC *GC *GC	11111 77777 667777 40123 11111 ATGAG A 	111111 788888 845555 591234 iidddd CG**** - TGGA T-TGA T-TTGA T-TTGA T-TTGA T-TTGA T-TTGA	11111 88888 55555 56789 1000000	1111111 8888888 6666666 ddddddd ctataaa ctataaa ctataaa ctataaa ctataaa ctataaa	111111 888888 667777 790245 diiiii *TGAAC TC TC T TC - T TC - T TC - T TC - T	11111 888888 77789 67911 diiii ACAAC - TC - (- TC - (- TC - (11111 88888 99990 23588 iiiiii TTAGO CC	11111 99999 01124 35732 11111 3GGTA -A-A- A- A- A- A- A-	L11111 999999 155566 201369 111111 ATATAT A-GTA A-GTA A-GTA A-GTA	1111 19999 7888 3357 1111 ATCG 	11122 999900 899900 11111 ATTAC 	22222 00000 22222 2345 24ddd 2AATC	222222 00000 222223 667890 1dddd GGGTA	22222 0000 3333 0123 dddd FAGC2 	22222 00000 33333 45678 ddddd ATATC G G GA GA GA GA
Base position Virilis-0 Virilis-9 Virilis-9 Virilis48 Lummei-1 Lummei-2 Lummei-4 Lummei-8 Amer-0	11111: 7777' 2222: 4567' dddd TCCA G 	1111111 7777777 2233333 8901234 ddddddd AATGTGC	111111 777777 333334 567890 ddddddd A***** 	111111 777777 44444 123450 dddddd ***** 	1111111 777777 4444555 5789017 dddddi ****GAA 	11111 77777 667777 40123 111111 AATGAG 	111111 788888 845555 591234 11dddd CG**** TGGA T-TGA T-TTGA T-TTGA T-TTGA T-TTGA T-TTGA	11111 88888 55555 56789 64dddd ***** TCCTT TCCTT TCCTT TCCTT TCCTT TCCTT	111111 8888888 6666666 ddddddd ****** CTATAAA CTATAAA CTATAAA CTATAAA CTATAAA CTATAAA CTATAAA CTATAAA	111111 888888 667777 790245 diiiii *TGAAC TC TC TC - T- TC - T- TC - T- TC - T- TC - T- TC - T-	11111 88888 77789 67911 diiii ACAAC - TC - (- TC - (- TC - (- TC - (11111 88889 99990 23588 iiiii TTAGC 	11111 99999 01124 35732 11111 3GGTA -A-A- -A-A- A- A- A- A-	L11111 999999 155566 201369 111111 ATATAT A-GTA A-GTA A-GTA A-GTA A-GTA	1111 9999 7888 3357 .iiii ATCG 	11122 999900 899900 94507 iiiii ATTAC 	22222 00000 02222 2345 1ddd CAATO	222222 000000 222222 567890 1ddddd 3GGTA	22222 0000 3333 1234 1dddd FAGC2 	22222 00000 33333 45678 45678 45678 45678 45678 45678 45678 45678 457884 457884 457884 457884 45788578 457884 457884 457885678 4578676678
Base position Virilis-0 Virilis-8 Virilis-8 Virilis48 Lummei-1 Lummei-1 Lummei-4 Lummei-8 Amer-0 Amer-1	11111: 77777 2222: 45677 ddddd TCCAJ G 	1111111 7777777 2233333 8901234 dddddd AATGTGC	111111 777777 33334 567890 dddddd A***** * * * - - - - - - - - - - - - -	111111 77777 44444 12345 dddddd ***** GAACC' GAACC' GAACT'	1111111 777777 4444555 6789017 dddddi ****GAA *GC 	11111 77777 67777 40123 11111 AATGAG - A - A - A - A - A - A - A - A - A - A	111111 788888 845555 591234 11dddcG**** TGGA T- TTGA T- TTGA T- TTGA T- TTGA T- TTGA T- TTGA T- TTGA	11111 88888 55555 56789 ddddd ***** TCCTT TCCTT TCCTT TCCTT TCCTT TCCTT TCCTT	1111111 8888888 6666666 0123456 dddddd ****** CTATAAA CTATAAA CTATAAA CTATAAA CTATAAA CTATAAA CCATAAA CCATAAA	111111 888888 667777 790245 di1i1i *TGAAC TC TC TC T TC - T TC - T TC - T TC - T TC - T	111111 888888 77789 67911 diiii ACAAC - TC - (-	11111 888889 99990 23588 iiiii TTAGC CC	11111 9999 01124 35732 iiiii GGTA - A - A A A A A A A	L11111 999999 155566 201369 L11113 ATATAT A-GTA A-GTA A-GTA A-GTA A-GTA	1111 9999 7888 3357 iiii ATCG 	11122 99900 89900 94507 iiiii ATTAC 	22222 00000 2222 2345 1dddd CAATO	222222 000000 222222 567890 1ddddd 3GGTA	22222 00000 3333 1234 dddd (22222 00000 33333 45678 ddddd ATATC GA GA GA GA GA GA GA GA GA GA GA
Base position Virilis-0 Virilis-8 Virilis-9 Virilis48 Lummei-1 Lummei-2 Lummei-4 Lummei-8 Amer-0 Amer-1 Amer-3	11111 7777 2222 4567 dddd TCCA G G G G G **** **** ****	1111111 7777777 233333 9901234 ddddddd AATGTGC	111111 777777 333334 567890 dddddd A***** 	111111 77777 44444 12345 dddddd ***** GAACC' GAACC' GAACC'	1111111 777777 4444555 6789017 dddddi ****GAA 	111111 777777 677777 740123 111111 111111 ATGAG 	111111 788888 845555 591234 11dddd CG**** TGGA T-TTGA T-TTGA T-TTGA T-TTGA T-TTGA T-TGGA T-TGGA	11111 88888 55555 56789 ddddd ***** TCCTT TCCTT TCCTT TCCTT TCCTT TCCTT TCCTT	111111 888888 6666666 ddddddd ****** CTATAAA CTATAAA CTATAAA CTATAAA CTATAAA CTATAAA CTATAAA CCATAAA CCATAAA CCATAAA	111111 888888 667777 790245 diiii +TGAAC TC TC T TC - T	111111 888888 77789 67911 ACAAC - TC - (- TC - (- TC - (- TC - (- T - (T - T - (11111 88889 99990 23588 iiiii TTAGC 	11111 9999 91124 35732 11111 3GGTA - A - A A A A A A A A A A	111111 999999 155566 201369 11111 ATATAT A-GTA A-GTA A-GTA A-GTA A-GTA A-GTA A-GTA A-GTA A-GTA	1111 9999 7888 3357 .1111 ATCG - A-A - A-A - A-A - A-A - A-A	11122 999900 94507 iiiii ATTAC 	22222 0000 2222 2345 24dd 2AATC	222222 00000 22222 66789 144444 366787	22222 00000 3333 1234 1ddd (22222 00000 33333 45678 ddddd ATATC GA GA GA GA GA GA GA GA GA GA
Base position Virilis-0 Virilis-8 Virilis-9 Virilis48 Lummei-1 Lummei-1 Lummei-4 Lummei-8 Amer-0 Amer-3 Amer-3	11111 77777 22222 45677 ddddd **** **** **** **** **** *	1111111 777777 223333 9501234 dddddd AATGTGC	111111 777777 333334 567890 dddddd A***** 	11111: 77777 44444 12345 dddddd ddddd ddddd ddddd ddddd ddddd dddd	1111111 777777 444455 6789017 dddddi ****GAA *GC *G	111111 77777 67777 40123 111111 AATGAG A A A 	111111 788888 845555 591234 11dddd CG**** - TGGA T - TGGA T - TTGA T - TTGA T - TTGA T - TTGA T - TGGA T - TGGA T - TGGA	11111 88888 55555 56789 ddddd ***** TCCT1 TCCT1 TCCT1 TCCT1 TCCT1 TCCT1 TCCT1 TCCT1 TCCT1	111111 888888 6666666 0123456 dddddd CTATAAA CTATAAA CTATAAA CTATAAA CTATAAA CTATAAA CCATAAA CCATAAA CCATAAA CCATAAA	111111 888888 667777 790245 d111111 *TGAAC TC TC T - TC T - TC - T - TC - T - TCT - T - TCT - T - TCT - T -	111111 88888 77789 67911 diiii ACACAC - TC - (- TC - (- TC - (T - T - (T	11111 888889 99990 23588 iiiiii TTAGO 	11111 99999 9124 5732 11111 GGTA - A - A - A - 	111111 999999 155566 201369 111111 ATATAT A-GTA A-GTA A-GTA A-GTA A-GTA A-GTA A-GTA A-GTA A-GTA A-GTA A-GTA A-GTA A-GTA A-GTA	1111 9999 7888 3357 1111 ATCG 	11122 99900 89900 94507 iiiii ATTAC 	22222 0000 2222 2345 CAATO	222222 00000 22222 56789 1dddd 3GGTA 	22222 00000 33333 01234 1dddd FAGC2 (((+++++) (+++++)	22222 00000 33333 45678 ddddd ATATC GA GA GA GA GA SA SA SA SA SA
Base position Virilis-0 Virilis-8 Virilis-8 Virilis48 Lummei-1 Lummei-2 Lummei-4 Lummei-8 Amer-0 Amer-3 Amer-3 Amer-5	11111 7777 2222 4567 dddd TCCA G G 	1111111 777777 223333 8901234 dddddd AATGTGC	111111 777773 33334 567890 dddddd A**** 	11111: 77777 44444 12345 dddddd ***** GAACC' GAACC' GAACT' GAACC'	1111111 777777 4444555 5789017 dddddii ****GAR 	11111 77777 67777 40123 11111 AATGAG A - GA - GA - GA 	111111 788888 845555 591234 11dddd CG**** - TGGA T - TGGA T - TTGA T - TTGA T - TTGA T - TGGA T - TGGA T - TGGA T - TGGA	11111 88888 55555 56789 ddddd TCCT1 TCCT1 TCCT1 TCCT1 TCCT1 TCCT1 TCCT1 TCCT1 TCCT1	111111 888888 6666666 ddddddd ****** CTATAAA CTATAAA CTATAAA CTATAAA CTATAAA CTATAAA CTATAAA CCATAAA CCATAAA CCATAAA CCATAAA	111111 888888 667777 790245 diiiii *TGAAC TC TC T - TC - T - TC - T - TC - T - TC - T - TCT - T - TCT - T - TCT - T - TCT - T -	11111 88888 677789 67911 ACAAC 	11111 88889 99990 23588 iiiii TTAGC CC	11111 99999 91124 35732 11111 3GGTA - A - A - A - 	111111 999999 155566 201369 11111 ATATAT A-GT A-GT A-GT A-GT A-GT A-GT A-	1111 199999 7888 3357 .1111 ATCG 	11122 99900 89900 94507 iiiii ATTAC 	22222 00000 2222 2345 2444 2444 2444 2444 2444 2444	222222 00000 22222 66789 ddddd GGGTA 	22222 00000 3333 1234 1dddd 7AGC2 (((++++) (++++)	22222 00000 33333 45678 ddddd GA G
Base position Virilis-0 Virilis-8 Virilis-9 Virilis48 Lummei-2 Lummei-2 Lummei-4 Lummei-8 Amer-0 Amer-1 Amer-3 Amer-5 Amer-6	11111 7777 2222 4567 dddd TCCA 	1111111 7777777 223333 3901234 ddddddd AATGTGC	111111 777777 333334 567890 dddddd A***** 	11111: 77777 4444 12345 ddddd ***** Gdddd Gddd Gaact Gaact Gaact GaacC GaacC	1111111 777777 4444555 6789017 dddddi ****GAA 	11111 77777 67777 40123 11111 ATGAG 	111111 788888 845555 591234 11dddd CG*** - TGGA T- TTGA T- TTGA T- TTGA T- TTGA T- TTGA T- TGGA T- TGGA T- TGGA T- TGGA T- TGGA T- TGGA	11111 88888 55555 56789 ddddd 55555 56789 ddddd 55555 56789 TCCT1 TCCT1 TCCT1 TCCT1 TCCT1 TCCT1 TCCT1 TCCT1	1111111 888888 6666666 ddddddd ****** CTATAAA CTATAAA CTATAAA CTATAAA CTATAAA CTATAAA CCATAAA CCATAAA CCATAAA CCATAAA CCATAAA	111111 888888 667777 790245 diiiii *TGAAC TC TC TC T TC - T	11111 88888 77789 67911 ACAAC - TC - (- TC - (- TC - (- T - (T - - (T - (T	11111 888889 99990 23588 11111 TTAGC 	11111 99999 91124 35732 11111 3GGTA - A - A A - A - 	11111 999999 155566 201369 111111 ATATAT A-GTA A-A-GTA A-A-A-A-A-A-A-A-A-A-A-A-A-A-A-A-A-A-A	1111 19999 7888 3357 .1111 ATCG - A - A - A - A	11122 99900 89900 94507 iiiii ATTAC 	22222 00000 22227 2345 dddd CAATC	222222 00000 22222 66789 1dddd GGGTA 	22222 00000 3333 01234 dddd FAGC (((*****) (*****) (*****)	22222 00000 33333 45678 ddddd ATATC GA G
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FIGURE 3.—Continued

sample; or they may have once been polymorphic in *D. americana* but are not now. Interestingly, the collection sites of the six *D. novamexicana* lines do not show a geographic pattern of separation between groups A and B (Table 1, Figure 1).

DNA sequence variation summary: If DNA sequence variation is neutral, then the patterns of DNA sequence variation can be used to estimate relative historical population sizes. Figure 3 shows all of the variable sites, and Table 2 lists the types of variation found in each group. Two estimates of variation are shown in Table 3: π , the average pairwise difference, and θ , calculated from the number of segregating sites (see MATERIALS

AND METHODS, Measuring variation). Both *D. virilis* and *D. lummei* have levels of variation similar to the range observed at the *per* locus in *D. melanogaster* (KLIMAN and HEY 1993). In contrast, *D. americana* shows a level of variation twice that of *D. virilis* and *D. lummei*, (this is true whether the *americana* and *texana* subspecies are grouped together or not), suggesting a large historical effective population size. *D. novamexicana*, when divided into groups A and B, contains very low levels of variation, which is consistent with small population size (Table 3).

These measures of variation can also be used to examine the history of natural selection. Both π and θ have

1020

TABLE 1List of lines sequenced

Species name	Line no.	Location
D. virilis	1051.0	Pasadena, CA
	1051.8	Truckee, CA
	1051.9	Sendai, Japan
	1051.48	Texmelucan, Mexico
D. lummei	1011.1	Moscow, Russia
	1011.2	Overhalix, Sweden
	1011.4	Kukkola, Finland
	1011.8	Sakata, Japan
D. A. americana	0951.0°	Anderson, IN
	0951.1°	Poplar, MT
	0951.3°	Millersburg, PA
	0951.4°	Keelers Bay, VE
	0951.5°	Jackson, MI
	0951.6°	Chadson, NE
	0951.9^{c}	Myrtle Beach, SC
D. A. texana	1041.0°	St. Francisville, LA
	1041.22°	New Orleans, LA
	1041.23°	Morrilton, AR
	1041.25	So. Richmond, VA
	1041.26	Tallahassee, FL
	1041.27°	Goldenhead Branch, FL
	1041.29	Jamestown, SC
	1041.31	Hollandale, MS
D. novamexicana	1031.0^{a}	Grand Junction, CO
	1031.4^{b}	Moab, UT
	1031.7^{a}	Patagonia, AZ
	1031.8^{a}	San Antonio, NM
	1031.11^{b}	Gila, NM
	1031.12^{b}	Antlers, CO

All lines are from the National Drosophila Species Resource center.

^a A member of group Nova-A.

^bA member of group Nova-B.

^c These lines were checked for the appropriate metaphase chromosome compliment; see text for details.

the same expected value, however θ is more influenced by low frequency polymorphic bases. This is because a single rare segregating base contributes little to the average pairwise differences (π), but it is counted as an additional segregating site in the calculation of θ . A measure of the discrepancy between π and θ , TAJIMA's D, is proportional to the difference between these two measures of variation (TAJIMA 1989). When there is an excess of low frequency polymorphisms (as expected with purifying selection or selective sweeps), θ will be bigger than π , and TAJIMA's D will have a negative value. A positive value is expected with balancing selection or population subdivision (TAJIMA 1989). Tajima's D is slightly negative in *D. virilis, D. lummei*, and *D. americana*, but these values are not significant and neutrality cannot be rejected (Table 3). Also, the power of Tajima's D is low with the small sample sizes used here (SI-MONSEN *et al.* 1995). In *D. novamexicana* Tajima's D is significant and positive, suggesting that the subdivision into two distinct groups is appropriate.

The Fu and Li test (FU and LI 1993) is similar to that of TAJIMA (1989) and can be used to explore the same selective forces as Tajima's D. This test compares the numbers of mutations that occur in external branches of a genealogy to those that occur on internal branches. Under some types of selection, the number of external mutations deviate from the expectation based on numbers of internal mutations. Fu and Li's D will be negative when there is an excess of external mutations (suggestive of purifying selection or selective sweeps) and positive when there is an abundance (suggestive of balancing selection or population subdivision). Fu and Li's D is slightly negative in D. lummei and D. americana, and slightly positive in D. virilis (Table 3). These values are not significant and neutrality cannot be rejected. In D. novamexicana the value of Fu and Li's D is significant and positive (Table 3), in accordance with the findings of Tajima's D for the group.

A third way to look for evidence of natural selection is to compare the numbers of substitutions that result in amino acid replacements with those that do not. If natural selection is acting to fix amino acid replacement mutations within species, we may expect a higher proportion of replacement differences in interspecific contrasts than in intraspecific contrasts. Alternatively, natural selection may be preventing the fixation of

TABLE 2

The number of polymorphic sites within species

			Exons			Intron	s
	n	Synonymous	Replacement	No. bases	Base	Length	No. bases ^a
D. virilis	4	3	2	1367	14	1	681
D. lummei	4	3	5	1367	9	0	678
D. americana	15	29	8	1367	44	1	700
D. novamexicana	6	7	3	1367	16	3	690
Nova-A ^b	3	0	0	1367	1	1	711
Nova-B ^b	3	1	0	1367	1	0	700

n is the number of DNA sequences in the sample. Under introns, base refers to base substitutions at the sequence level and length refers to differences in sequence length.

^a Intron lengths are an average because of length polymorphisms.

^b Nova-A and Nova-B are two subdivisions of D. novamexicana.

-	n	s	π	θ	Tajima's D	Fu and Li's D
D. virilis	4	19	0.0057	0.0058	-0.195	0.322
			(0.0033)	(0.0033)		
D. lummei	4	17	0.0049	0.0051	-0.484	-0.189
			(0.0029)	(0.0030)		
D. americana	15	81	0.0109	0.0136	-0.894	-0.928
			(0.0051)	(0.0051)		
D. novamexicana	6	26	0.0077	0.0059	1.891*	1.656*
			(0.0041)	(0.0030)		
Nova-A	3	1	0.0004	0.0004	NA	NA
			(0.0004)	(0.0004)		
Nova-B	3	2	0.0007	0.0007	NA	NA
			(0.0006)	(0.0006)		

	ТА	BLE 3	
DNA	sequence	variation	summary

n is the number of DNA sequences. S is the number of polymorphic sites within groups, π and θ were calculated using expressions (1) and (2), respectively, and then these quantities were divided by the number of base pairs in the DNA sequences. The standard errors of the estimates, per base pair, are in parentheses. To calculate these, first the variances were determined using expressions (4) and (13) in TAJIMA (1993) for θ and π , respectively. For each variance, the square root was taken and then this quantity was divided by the number of base pairs sequenced. Tajima's D (TAJIMA 1989) compares the similarity of measures of π and θ ; it requires at least four sequences to perform the test. Fu and Li's D (FU and Li 1993) also requires four sequences to perform the test. The D values of D. novamexicana are significant at the 0.05 level. NA, not available.

replacement polymorphisms. In this case the proportion of replacement polymorphisms, relative to synonymous polymorphisms within species, may be higher than expected on the basis of interspecific fixed differences. MCDONALD and KREITMAN (1991) formulated a test that compares the numbers of sites that are polymorphic within species to those fixed between species for replacement vs. synonymous sites. We tested several different species pairs and found no evidence of selection (Table 4).

Recombination and genealogical inference: HUDSON and KAPLAN (1985) described a way to estimate the minimum number of recombination events that are consistent with the polymorphism patterns in a sample of four or more DNA sequences. In general, this estimate is expected to be larger with larger sample sizes, and to be far lower than the actual number of recombination events (HUDSON and KAPLAN 1985). We found that *D. virilis* and *D. lummei*, each with four sequences, must have had recombination occur at least once. *D.*

TABLE 4

McDonald-Kreitman tests

Species pair	$\chi^2_{1 m df}$
virilis-lummei	0.733
virilis-americana	0.248
lummei-americana	0.970
americana-Nova-A	0.094
americana-Nova-B	1.625

 χ^2 tests are for differences between replacements and synonymous sites, within and between species (all contrasts not significant, 1 df). MCDONALD and KREITMAN (1991). See text for details.

americana, with 15 sequences, has experienced recombination at least 13 times. There is no recombination seen within *D. novamexicana*, however this is not surprising, given the pattern of variation of two distinct types with no intermediate forms.

Recombination makes the process of gene tree estimation not only problematic but it also causes any particular estimate to be "not real." When there has been recombination within a gene, each piece of the nonrecombined DNA has its own gene tree (HUDSON 1990). Although these different gene trees are not independent, the history of multiple trees means that there is no true bifurcating tree for the gene as a whole. Despite these limitations on their usefulness in the face of recombination, gene tree estimates can still be informative in the case of the presence of deep branches that separate widely divergent taxa. In addition, tree estimates for sequences with a history of recombination share certain structural characteristics. For example, when there has been a lot of recombination scrambling the relationships among different sequences, a tree estimate is expected to have short internal branches relative to the terminal branch tips.

In our tree estimates an outgroup was not used, although the large divergence between *D. virilis* and *D. lummei*, as well as other information (THROCKMORTON 1982; SPICER 1991b, 1992), strongly suggest the root is along this branch. Distance matrices were created using the program DNADIST (PHYLIP 3.5; FELSENSTEIN 1989). A neighbor-joining tree (SAITOU and NEI 1987) was produced by using the PHYLIP program NEIGHBOR (Figure 4A). Neighbor-joining bootstrap trees were produced by using NEIGHBOR in conjunction with the programs SEQBOOT, DNADIST, and CONSENSE. The majority



FIGURE 4.—Neighbor joining trees. (A) A standard distance tree. (B) A consensus tree based on bootstrapping; branches that appeared in <60% of trees were collapsed.

rule consensus tree based on 100 replicates is shown in Figure 4B. Branches with bootstrap values of <60% were collapsed. Figure 4 reveals that the relationships of *D. virilis, D. lummei* and the americana complex flies (*D. a. americana, D. a. texana* and *D. novamexicana*) are consistent with other phylogenetic analyses (THROCKMORTON 1982; SPICER 1992).

The effect of recombination can be seen in the two trees in Figure 4. Figure 4A shows that the branches connecting the sequences sampled from the americana complex are joined by short internal branches. This pattern is reminiscent of a star phylogeny and could be taken as evidence of recent population bottleneck and expansion. However, the method of HUDSON and KAPLAN (1985) has revealed multiple recombination events in the history of these sequences. The effect of this scrambling is to distribute the variation among sequences uniformly so that all sequences are about equally different from all others. Most of the short internal branches collapse in the consensus tree (Figure 4B), revealing that various *D. americana* lines are all related to about the same degree and that Nova-A and Nova-B both arise out of this.

DISCUSSION

Selection at period: It does not appear that the pattern of variation in this 2.1-kb region of the per locus has been strongly affected by natural selection. First, a McDonald-Kreitman test showed no evidence of selection (Table 4, MCDONALD and KREITMAN 1991). Second, Tajima's D and Fu and Li's D were not significantly different from zero in D. virilis, D. lummei, and D. americana (Table 3, TAJIMA 1989; FU and LI 1993). These tests were significant in D. novamexicana, and this result will be discussed below. Third, recombination has occurred at *per*, reducing the length of tight linkage groups, which in turn reduces the probability that any particular portion of the sequence is tightly linked to a site under selection (MAYNARD-SMITH and HAIGH 1974). Similar observations were made by KLIMAN and HEY (1993) for a 1.9-kb portion of the *per* locus studied in the four species of the D. melanogaster complex. The region sequenced by KLIMAN and HEY (1993) in the D. melanogaster group ends ~ 150 bases upstream to our D. virilis sequence.

Within the region we sequenced in *D. virilis*, the homologous corresponding region of *D. melanogaster* group has a large Thr-Gly repeat of variable length (PEIXOTO *et al.* 1992). ROSATO *et al.* (1994) examined the Thr-Gly region in eight populations of *D. simulans* (a member of the *D. melanogaster* group) and found significant departures from neutrality based on Tajima's D, suggesting balancing selection in *D. simulans*. In the *D. virilis* sequence, there are just two pairs of Thr-Gly repeats (position 3044 of COLOT *et al.* 1988; position 185 of the region sequenced for this paper). We found no variation in this pattern among the lines we sequenced in any of the species. Our results reveal no evidence of selection acting on this very short Thr-Gly region in the *D. virilis* phylad.

D. a. americana/D. a. texana divergence: These two subspecies have been differentiated on the basis of a chromosomal fusion of the X and the fourth. Both subspecies share the fusion of chromosomes 2 and 3, which is not seen in the three other species of the phylad, so this fusion is presumably the derived state (PATERSON and STONE 1952; ALEXANDER 1976). However, we show that at the X-linked *per* gene there is no divergence between the two subspecies. A trivial explanation, that can be ruled out, is that the stocks were cross contaminated or misidentified with regard to subspecies. First, the subspecies designations were confirmed with mitotic chromosome squashes (see Table 1 and MATERIALS AND METHODS). Second, cross contamination of americana/texana stocks is expected to lead to the appearance of identical *per* sequences among different stocks. However, at the sequence level, all the lines were very divergent with the exception of texana.26 and texana.27, which were both collected in Florida.

Chromosomal changes may contribute to speciation in many groups (WHITE 1978). For example, two chromosomal types that differ by inversions or fusions may have different selective advantages in separate environments, and this could lead to speciation if the hybrids between the two are at a selective disadvantage. Low fitness in hybrids could be expected because recombination within the germ line of hybrid individuals that are heterozygous for different karyotypes will generate inviable gametes. However, the X-4 fusion in D. a. americana does not seem to lead to an increase in inviable gametes when mixed with D. a. texana. BLIGHT (1955) studied the karyotypic frequencies in several populations that contained hybridizing populations of D. a. americana and D. a. texana near St. Louis. He found hybrids and pure types existed in Hardy-Weinberg equilibrium and concluded that the subspecies distinction was not useful for his populations.

The dual observation of the presence of an X-4 fusion hybrid zone and a lack of divergence at the X-linked *per* locus may be the result of a combination of selection, gene flow and recombination. There may be some selection acting, with the X-4 fusion being advantageous in the north, and that advantage diminishes as one moves south. Within the hybrid zone, recombination within hybrids would lead to gene flow between types, which could swamp any effect of selection seen at *per*. Under this scenario, the site or sites of selection that maintain the X-4 fusion hybrid zone are not expected to be in tight linkage with the *per* locus.

The *per* locus data are consistent with a large historical effective population size in *D. americana*. This conclusion is based on the combination of two pieces of evidence. First, the level of variation is high (Table 3). Second, a large portion of the variation looks old, because it is well scrambled by recombination.

The divergence of D. novamexicana from D. americana may give some insight into the history of chromosomal evolution. Considered together, the two groups of D. novamexicana have little per locus divergence from D. americana, yet they do have a distinct karyotype. D. novamexicana has the "ancestral" chromosomal type of no chromosomal fusions, while D. a. texana has the 2-3 fusion and D. a. americana has both the 2-3 fusion and the X-4 fusion. One explanation is that after D. novamexicana split off from ancestral D. americana, both the X-4 and 2-3 fusion occurred. Yet from the pattern of sequence data, in which D. americana is segregating variation that separates the two D. novamexicana groups (see below), it appears that ancestral D. americana's population size was large before the split of D. novamexicana. Therefore it seems likely that multiple chromosomal types, including the 2-3 fusion and possibly the X-4

fusion, were segregating before the origin of *D. novamexicana*. Alternatively, the origin of the 2-3 fusion may have been directly associated with the origin of *D. novamexicana*. An additional piece of evidence suggesting that a variety of chromosomal types existed in the ancestor to the americana complex is that *D. novamexicana*, although it has an "ancestral" chromosomal type, contains many of the inversions found in the americana complex relative to *D. virilis* and *D. lummei* (PATTERSON and STONE 1952; THROCKMORTON 1982).

D. novamexicana: The history of D. novamexicana seems to have been different than for D. americana. Based on per locus sequences, the species contains two groups that are divergent at the DNA level, but which have not diverged morphologically or chromosomally. The divergence in the sequence variation is confirmed by the significant results of Tajima's D and Fu and Li's D (Table 3). One explanation for this pattern is that balancing selection is maintaining two distinct "alleles" at high frequency within D. novamexicana. Under this model, our designations of Nova-A and Nova-B reflect the divergence of functional per locus alleles and are not representative of variation elsewhere in the genome. The most appropriate test of this hypothesis is to examine a second unlinked locus. The alternative explanation is that the per locus pattern reflects population level processes and not balancing selection. If this is so, similar patterns of variation are expected elsewhere in the genome. One piece of evidence that argues against balancing selection is that at D. americana, the per locus sequences reveal a history with considerable recombination. If D. novamexicana is a single species with balancing selection maintaining two functionally distinct per alleles, then a history of per locus recombination is also expected here. Thus the balancing selection model also requires an additional component to explain the absence of recombination in D. novamexicana.

Regardless of whether the pattern of *per* locus variation has been due to natural selection, or whether it is because *D. novamexicana* consists of two populations that are not exchanging genes (*e.g.*, "cryptic" species), the variation does show that *D. novamexicana* is very closely related to *D. americana*. Furthermore, it does appear that *D. novamexicana* probably arose from an ancestral species that had a large population size. This can be inferred from the 11 fixed differences between Nova-A and Nova-B that were found still segregating in *D. americana*.

If the pattern of variation at *per* is taken to be representative of the genome, then we can consider the kinds of processes that might have given rise to two groups within *D. novamexicana*. Both groups have in common a light mesothorax color, a chromosomal karyotype, and geographic range that is separate from that for *D. americana*. The two groups also share a single fixed difference, with respect to *D. americana*, in the *per* locus sequence. Though the two groups could have arisen independently, these shared characteristics suggest that *D. novamexicana* arose once and then split into two groups.

If a single origin of D. novamexicana is taken as a working hypothesis, then some other aspects of this speciation event and initial divergence can be explored. During the time between the origin of *D. novamexicana* and the divergence of Nova-A and Nova-B, D. novamexicana (1) acquired its lighter mesothorax color, (2) began living in a drier habitat, (3) did not lose much per locus variation, and (4) may have acquired one new substitution at per. However, the very low level of divergence between D. novamexicana (including both Nova-A and Nova-B) and D. americana suggests that it was soon after the origin of D. novamexicana that this new species split into two groups. If this model of two splitting events in rapid succession is correct, it follows that the evolution of the lighter mesothorax color was fairly rapid.

KLIMAN and HEY (1993) examined four closely related taxa in the *D. melanogaster* group for DNA sequence variation at *per*. They concluded that *D. simulans*, a large population still segregating very old lineages, gave rise to two island species, *D. mauritiana* and *D. sechellia*. They found that *D. simulans* was still segregating polymorphism fixed between *D. mauritiana* and *D. sechellia*. They felt it was appropriate to consider *D. simulans* a parent species to the two island species. In our analysis, we found that *D. americana* is a large population that is still segregating variation that predates the split of Nova-A and Nova-B. These two findings of large populations that still segregate old variation suggest that speciation may often proceed by the formation of daughter species that bud off of larger species.

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