

MITOCHONDRIAL DNA AND PROTEIN DIFFERENTIATION BETWEEN HYBRIDIZING CYTOTYPES OF THE WHITE-FOOTED MOUSE, *PEROMYSCUS LEUCOPUS*

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Abstract.—Restriction-enzyme analysis of mitochondrial DNA and protein electrophoresis were used to document patterns of gene flow across a hybrid zone between chromosomal races of *Peromyscus leucopus*. Chromosomal markers (three inversions) are such that individuals can be classified as potential F_1 's, backcrosses, or parental types. Allozymic characterization of the hybrid zone is congruent with the chromosomal data (Stangl, 1986) and indicates an asymmetrical distribution of markers, with the northeastern markers being distributed at a higher frequency into southwestern populations. Restriction patterns of mtDNA suggest that the two cytotypes may have had different evolutionary histories, and the distribution of haplotypes is concordant with other genetic markers used to identify the hybrid zone. Concordant changes in chromosomes, allozymes, and mtDNA suggest that the most viable hypothesis for the origin of the zone is secondary contact. A unique aspect of this study is that the same individuals were used for protein electrophoresis, mtDNA analysis, and chromosomal analysis. Thus, patterns of genetic variation can be interpreted free of any historical bias associated with samples collected at different times.

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Because zones of hybridization between differentiated populations or taxa offer a unique opportunity to study introgression, maintenance of genetic integrity, and the process of speciation, students of evolutionary biology have spent considerable effort studying both the dynamics of hybrid zones and the genetic interactions that occur in such zones (see Endler [1977], Moore [1977], Barton and Hewitt [1981, 1985], Shaw [1981], and Woodruff [1981] for reviews). One general conclusion from hybrid-zone studies is that information concerning the ecogeographic aspects, as well as the patterns and degree of genetic interactions characterizing a hybrid zone, are complex. Not only are the general zoogeographic and ecological factors maintaining a zone difficult to discern, but the dynamics of hybrid zones in terms of patterns of introgression have been shown to differ with respect to the genetic or morphological markers used (Moore, 1977; Barton and Hewitt, 1981, 1985). These factors point to the need to understand both the overall evolutionary history of the zone as well as the consequences of interactions within the hybrid zone.

Baker et al. (1983) and Stangl and Baker

(1984) have described two chromosomal races of the white-footed mouse, *Peromyscus leucopus*, which hybridize along a transect in Oklahoma. Initial characterization of this hybrid zone has revealed that the distribution of the cytotypes of *P. leucopus* occurs in a fairly continuous habitat, in which relatively large numbers of individuals are obtainable. The chromosomal markers (three inversions) are such that individuals can be classified as potential F_1 's, backcrosses, or parental types, thus providing a background (chromosomal constitution) for each individual against which variation in allozymes and mitochondrial DNA can be assessed.

In this paper, we report on patterns of mitochondrial-DNA (mtDNA) and allozyme variation, and we relate these patterns to the degree of chromosomal variation associated with individuals from the zone of hybridization and away from the zone (Baker et al., 1983; Stangl and Baker, 1984; Stangl, 1986). A unique aspect of this study is that the same individuals were used for protein electrophoresis, mtDNA analysis, and chromosomal analysis. Thus, patterns of chromosomal, protein, and mtDNA variation can be interpreted free of any historical bias associated with samples collected at different times.

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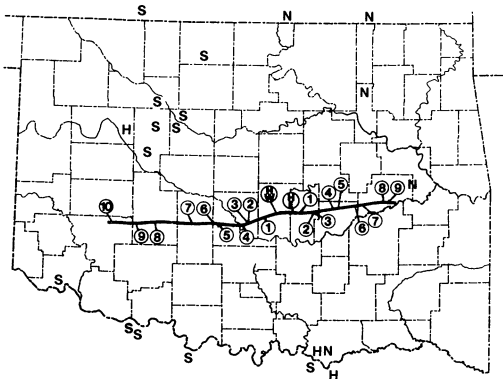


FIG. 1. Distribution of cytotypes of *Peromyscus leucopus* in Oklahoma, excluding the panhandle and including border localities in Kansas and Texas. N designates localities containing only the northeastern cytotype, S designates localities containing only the southwestern cytotype, and H designates localities where hybrid individuals were taken. The line across central Oklahoma represents the study transect and sampling localities. To the right of HE are populations containing individuals of the northeastern cytotype ("1E," "2E," etc.) and to the left of HW are populations containing individuals of the southwestern cytotype ("1W," "2W," etc.). Hybrid-zone locality numbers are based on the chromosomal analysis of Stangl (1986).

MATERIALS AND METHODS

Specimens Examined.—The transect across central Oklahoma runs from Mountain View (Kiowa Co.) on the west to Vivian (McIntosh Co.) on the east and spans a distance of approximately 263 km. Twenty-one collecting localities were located along the transect (Fig. 1). Locality designations follow those established by Stangl (1986). Localities labeled "E" are samples in which some individuals were of the northeastern parental cytotype, those labeled "W" had individuals of the southwestern cytotype, and localities "HE" and "HW" contain only chromosomal hybrid individuals. Specimens were obtained with Sherman live-traps during collections made in June, August, September, November, and December of 1983. A list of exact collecting localities is available from K.N. upon request.

A total of 332 *Peromyscus leucopus* were used in the electrophoretic analyses. G- and C-band karyotypes (Stangl, 1986) were available for 279 of these individuals. Eighteen of the above individuals from six localities (10W, 1W, HW, HE, 1E, 9E) were used in the mtDNA analysis. All specimens

are deposited as voucher skins and skeletons in The Museum, Texas Tech University.

Protein Electrophoretic Analysis.—Heart, kidney, liver, muscle, spleen, serum, and red-blood-cell tissue samples were collected from each individual. Samples of blood were obtained from mice by retro-orbital sinus punctures with heparinized hematocrit tubes and centrifuged to separate the serum and red-cell components. Tissue samples were stored at -90°C . Tissue preparation and electrophoretic techniques primarily followed the methods described in Selander et al. (1971).

Enzyme-staining techniques were as in Selander et al. (1971) and Harris and Hopkinson (1977). The following enzymes were examined: acid phosphatase (ACP-3), aconitase (ACON-2), adenylate kinase (AK-1, AK-3), alcohol dehydrogenase (ADH), aldolase (ALD), NADH diaphorase (DIA), esterase (ES-1, ES-2), fumarate hydratase (FH-1, FH-2), glucose-6-phosphate dehydrogenase (GD), glucose phosphate isomerase (GPI), L-glutamate dehydrogenase (GLUD), glutamate-oxaloacetate transaminase (GOT-1, GOT-2), glyceraldehyde-phosphate dehydrogenase (GAPDH), hemoglobin (HEM), isocitrate dehydrogenase (ICD-1, ICD-2), lactate dehydrogenase (LDH-1, LDH-2), malate dehydrogenase (MDH-1, MDH-2), malic enzyme (ME), peptidase (PEP-A, PEP-B), phosphoglucomutase (PGM-1, PGM-2, PGM-3), phosphogluconate dehydrogenase (PGD), purine nucleoside phosphorylase (NP), red blood cell general protein (RBCGP-1, RBCGP-2), sorbital dehydrogenase (SORDH), superoxide dismutase (SOD-1, SOD-2), xanthine dehydrogenase (XDH).

When multiple isozymes of an enzyme were present, the isozyme migrating the farthest anodally was designated numerically as "1," and isozymes migrating progressively cathodally were given increasing numerical designations. For each presumptive locus, alleles were designated alphabetically with "a" representing the allele corresponding to the electrophoretic variant migrating farthest anodally.

Rogers' (1972) genetic similarity value was calculated for each pairwise comparison of the populations. Clustering analyses

(unweighted pair group method using arithmetic means [UPGMA]) were performed using these matrices (Sneath and Sokal, 1973). Hardy-Weinberg statistics and average individual heterozygosity values (H) were calculated for each population using BIOSYS-1 (D. L. Swofford and R. B. Selander, unpubl. [available from D. L. Swofford, Univ. Illinois, Urbana]).

Mitochondrial DNA Analysis.—Mitochondrial DNA from frozen livers of each individual was isolated by CsCl-propidium iodide gradient centrifugation (Brown, 1980; Densmore et al., 1985). The isolated mtDNA's were digested with the following eight restriction enzymes: 1) *Bam*H I, 2) *Bgl* II, 3) *Bst*E II, 4) *Eco*R I, 5) *Hinc* II, 6) *Hind* III, 7) *Hpa* II, and 8) *Xba* I. Digested fragments were end-labeled with 32 P- α NTP using the large (Klenow) fragment DNA polymerase I (Brown, 1980). The labeled fragments were separated by electrophoresis in 1.2% agarose gels and 3.5% polyacrylamide gels. Digestion patterns were visualized by autoradiography (Brown, 1980). *Hind* III digests of lambda DNA and *Hinf* I digests of SV40 DNA were used as molecular-weight markers.

The patterns of mtDNA digestion for each restriction enzyme were given a letter designation. Where possible, digestion patterns were compared to published data for *P. leucopus* (Avisé et al., 1983), and the same letter designations were used. A composite mtDNA haplotype from all digestion patterns was constructed for each individual.

RESULTS

Allozyme Variation.—Thirty-eight presumptive loci were examined for each individual. Fourteen loci were monomorphic for all individuals: *Acon-2*, *Ak-3*, *Ald*, *Fh-1*, *Gpi*, *Gapdh*, *Hem*, *Ldh-2*, *Mdh-1*, *Mdh-2*, *Pep-A*, *Pgm-2*, *Rbcgp-1*, and *Rbcgp-2*. Five loci showed single allelic variants found in one individual at a single locality (*Fh-2^a*, locality 10W; *Gd^b*, locality 8W; *Icd-2^b*, locality 2W; *Me^a*, locality 6E; *Sordh^b*, locality 7W). The remaining 19 loci (50%) showed two or more alleles at two or more localities. Allele frequencies of polymorphic loci for each locality are available from K.N. upon request.

Several loci showed patterns of variation

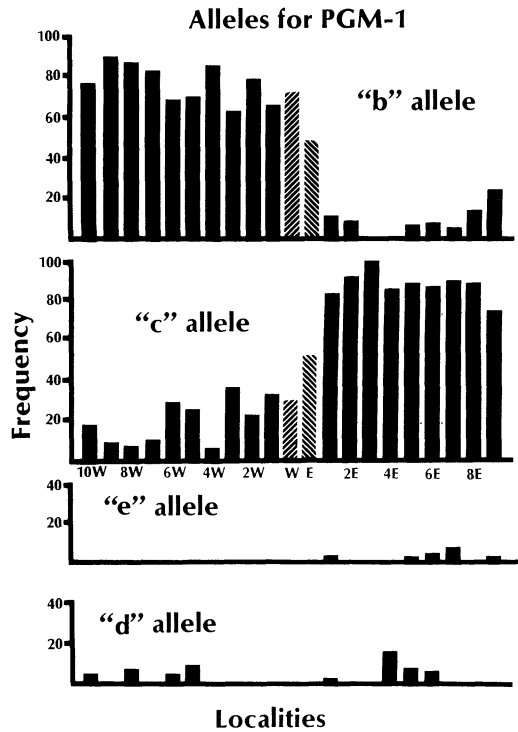


FIG. 2. Frequency of *Pgm-1* alleles across the hybrid zone. Slanted bars are the two localities (HW and HE) where only chromosomal hybrid individuals were taken. To the left of HW and HE are western localities, and to the right are eastern localities. Not shown is allele *a* which occurs at low frequency in localities 7W and 4W.

associated with the two parental cytotypes. However, there were no fixed allelic differences between the cytotypes. The most notable pattern of variation was seen for two loci, *Pgm-1* (Fig. 2) and *Adh*. For these two loci, the frequency of alternative alleles shifted across the zone, with one allele more common in the western populations and a second allele more common in the eastern populations (Fig. 2). The shift in the frequency of these alleles occurred between the populations containing parental cytotypes and near the central hybrid populations (HE and HW). *Pgm-1* shows an asymmetric distribution across the zone, with the northeastern marker (allele *Pgm-1^c*) occurring in a greater frequency in the southwestern populations than does allele *Pgm-1^b* in the northeastern populations.

Twenty-three loci displayed one or more uncommon alleles (total of 38). Uncommon

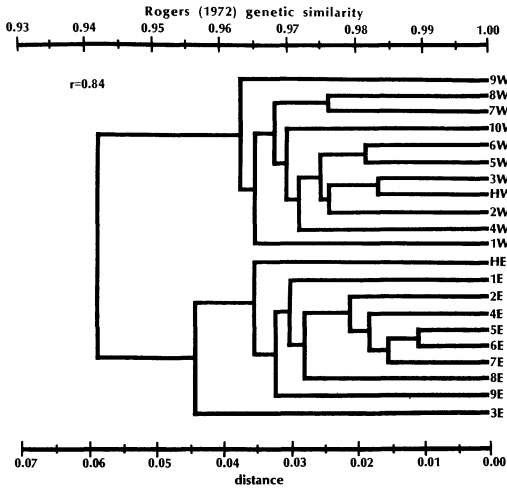


FIG. 3. Phenogram of Rogers's (1972) similarity values calculated from the allozyme data for the 21 populations of *Peromyscus leucopus*. Clustering is by UPGMA. Cophenetic correlation is 0.84.

alleles are defined as those that occur at an average frequency of less than 20% in all populations. Few uncommon alleles occurred at the hybrid localities; however, one allele unique to the hybrid localities, *Adh^d*, was found in a single individual from HW. Forty-five percent (17 of 38) of the alleles were unique to one side of the hybrid zone but not the other side and thus showed an association with one parental type. Nine of these alleles occurred on the western side of the hybrid zone, and eight occurred on the eastern side.

Mean individual observed heterozygosity for all populations ranged from 0.054 to 0.092 and averaged 0.075. No significant differences for *H* were found between localities, and there was no increase in heterozygosity at the hybrid localities.

Rogers' (1972) similarity values between all pairs of populations were calculated based on the 38 loci examined. Relationships among the 21 localities are summarized in a phenogram (Fig. 3) based on UPGMA analysis of Rogers' similarities. All western populations cluster together, and all eastern populations cluster together. These two major subdivisions then cluster at the 0.94 level of genetic similarity.

mtDNA Variation.—Eight restriction enzymes were used to analyze mtDNA variation in eighteen individuals of known

karyotype (Tables 1, 2) and where possible, letter designations for digestion patterns follow those established by Avise et al. (1983), who reported on geographic variation of mtDNA in *Peromyscus leucopus*. Their geographic study consisted of 14 individuals, which represented 12 localities, none of which was within 200 km of the hybrid zone. Four restriction enzymes (*Bgl* II, *Hinc* II, *Hind* III, and *Xba* I) revealed a single mtDNA digestion pattern for all individuals within the hybrid zone. At least two new mtDNA restriction patterns not revealed by Avise et al. (1983) were found near or within the hybrid zone and occurred in chromosomal hybrid individuals (Tables 1, 2). These new patterns are *Bam*H I "C" and *Eco*R I "B." In addition, digestion patterns from two restriction enzymes, *Bam*H I and *Hpa* II, were not directly relatable to those described by Avise et al. (1983). This was due primarily to the number of different restriction patterns for each enzyme and the similarity between the number of bands and size of bands for several patterns. An additional problem arose because of the appearance of small fragments (less than 400 base pairs) on the polyacrylamide gel which are not easily detected using only agarose gels for fragment visualization as in Avise et al. (1983).

Estimates of nucleotide-sequence divergence between mtDNA haplotypes were calculated from restriction-site data using the method of Nei and Li (1979). Mean sequence divergence between haplotypes ranged from 0.0020 to 0.0109 (Table 3). Phylogenetic relationships among haplotypes based on cladistic analysis of restriction-site data using parsimony are depicted in Figure 4. Seven unique haplotypes exist for the individuals sampled and there were seven polymorphic sites for these haplotypes. Therefore, one must use caution when interpreting relationships based on these preliminary mtDNA data.

Two restriction enzymes, *Eco*R I and *Bam*H I, showed a distribution of digestion patterns consistent with the distribution of parental cytotypes and allozymes (Figs. 4, 5). *Eco*R I "E" occurred in localities 10W and 1W and *Eco*R I "C" in localities HW, HE, 1E, and 9E (Table 1). The distribution of *Bam*H I patterns is similar to that of

TABLE 1. Mitochondrial-DNA digestion patterns for 18 individuals of *Peromyscus leucopus* for which the chromosomal phenotype is known. Only chromosomes 5, 11, and 20 distinguish the two races (Stangl, 1986). S = chromosomal morph characteristic of the southwestern race; N = chromosomal morph characteristic of the northeastern race.

Population	Individual	Chromosomal phenotype			mtDNA digestion pattern							
		5	11	20	<i>Bam</i> H I	<i>Bgl</i> II	<i>Bst</i> E II	<i>Eco</i> R I	<i>Hinc</i> II	<i>Hind</i> III	<i>Hpa</i> II	<i>Xba</i> II
10W	1	SS	SS	SS	A	B	P	E	α	A	A	A
	2	SS	SS	SS	A	B	P	E	α	A	A	A
	3	SS	SS	SS	A	B	P	E	α	A	A	A
1W	4	SS	SS	SS	A	B	P	E	α	A	A	A
	5	SS	SS	SS	A	B	P	E	α	A	A	A
	6	NS	SS	SS	A	B	P	E	α	A	A	A
HW	7	NN	SS	NS	B	B	P	C	α	A	A	A
	8	NS	NS	NS	B	B	P	B	α	A	A	A
	9	NN	NN	NS	B	B	P	C	α	A	A	A
HE	10	SS	SS	NN	B	B	Q	C	α	A	C	A
	11	NS	NS	NS	B	B	Q	C	α	A	C	A
	12	NS	NS	NN	B	B	P	C	α	A	A	A
1E	13	NS	NN	NN	C	B	P	C	α	A	A	A
	14	NN	NN	NN	B	B	Q	C	α	A	A	A
	15	NN	NN	NN	B	B	Q	C	α	A	A	A
9E	16	NN	NN	NN	A	B	P	C	α	A	A	A
	17	NN	NN	NN	A	B	P	C	α	A	A	A
	18	NN	NN	NN	B	B	P	C	α	A	A	A

*Eco*R I except that the mtDNA's of two individuals from locality 9E have the *Bam*H I "A" digestion pattern common to the western populations. Both hybrid populations (HW and HE) have the common eastern digestion pattern for all individuals and therefore show a skewed distribution of markers, as do the chromosomes and allozymes.

Assuming that recombination between mitochondrial genomes is not a factor, the

two individuals from 9E may be explained either by 1) convergence of restriction site pattern for *Bam*H I or 2) maintenance of an ancestral mitochondrial lineage through stochastic processes (Avisé et al., 1984). Because *Bam*H I "A" differs by a loss of restriction site from *Bam*H I "B" (the pattern common for all eastern populations; see Table 1), it is certainly possible that the similarity of digestion patterns of 10W, 1W, and 9E are due to convergent loss of a re-

TABLE 2. Fragment sizes produced by digestion of mtDNA of *P. leucopus* with eight restriction enzymes.

Enzyme	Pattern	Fragment sizes (bp)										
<i>Bam</i> H I	A	6,225	5,236		3,361			1,074	388	210		
	B		5,236	4,400	3,361	1,599		1,074	388	210		
	C		5,236	4,400	3,361	1,599	1,525			210		
<i>Bgl</i> II	B	12,460	3,740									
	Q	12,000			3,844	793						
<i>Bst</i> E II	P		7,133	5,008	3,844	793						
<i>Eco</i> R I	B	5,480		2,661	2,570	2,071			1,194	1,050	814	462
	C		3,917	2,661	2,570	2,071	1,255		1,194	1,050	814	462
	E		3,917	2,661	2,570	2,071	1,255	1,200	1,194	1,050		
<i>Hinc</i> II	α	9,475	3,363	1,060	945	795						
<i>Hind</i> III	A	4,883	3,358	2,867	2,463	1,035	918	473				
<i>Hpa</i> II	A			4,863	4,322	2,440	2,322	999	714	495		
	B	7,150		4,863			2,322	999	714	495		
	C	7,150	6,000				2,322		714	495		
<i>Xba</i> I	A	7,900	7,350	1,350	437							

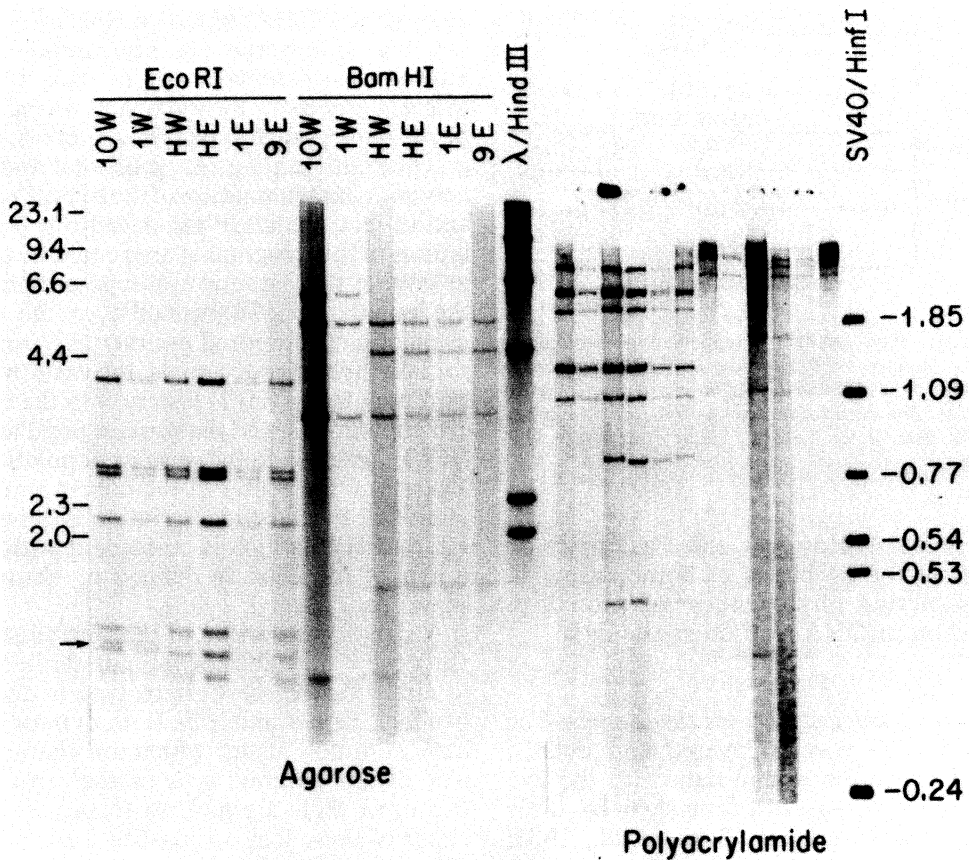


FIG. 5. *EcoR* I and *Bam*H I digestion patterns used as markers for the two cyotypes of *P. leucopus*. Localities 10W and 1W show patterns diagnostic of the southwestern race, whereas localities HW, HE, 1E, and 9E show patterns diagnostic of the northeastern race. Fragment sizes for the *Hind* III digest of lamda DNA are indicated for the agarose gel. Lane designations for the polyacrylamide gel are the same as for the agarose gel, except the last lane contains *Hinf* I digest of SV40 DNA as an internal molecular weight marker. Arrow indicates the fragment with a site gain for *EcoR* I.

allozymes is corroborated by the patterns of mtDNA variation seen across the zone. In terms of matriarchial lineages, the mtDNA variation suggests that the two cyotypes may have had different evolutionary histories. The separation between the two cyotypes is most apparent in the *EcoR* I digestion patterns. Additionally, chromosomal, allozymic, and mtDNA patterns of variation are concordant, in that a higher frequency of northeastern markers can be found in populations of the southwestern cyotype than vice versa, creating an asymmetry to the hybrid zone.

The first studies of mtDNA variation between hybridizing taxa reported a lack of concordance between these maternal mark-

ers and other genetic markers identifying the taxa (Ferris et al., 1983; Powell, 1983; Spolsky and Uzzell, 1984). However, subsequent studies, including this one, report concordance between genetic markers used to identify hybrid zones (Szymura et al., 1985).

The combination of chromosomal markers for identifying potential parental, F_1 , and backcross individuals and the maternal mode of inheritance of the mitochondrial genome provides a unique opportunity to study the interactions occurring in this zone of hybridization. The initial data from the mtDNA analysis (Table 1) suggest that reciprocal crosses between the two races may not be occurring with equal frequency with

respect to females. Lamb and Avise (1986) document the value of combined nuclear and mitochondrial markers in predicting the direction of crossing in hylid frogs. Future breeding studies and a more extensive study of mtDNA variation across the zone will be necessary to document the occurrence of nonreciprocal crosses.

Origin of the Zone. — The confirmation of whether the zone of hybridization has arisen as a result of primary or secondary contact is essential to our understanding of speciation, but this is difficult to determine (Barton and Hewitt, 1985; Littlejohn and Watson, 1985). In terms of primary contact, the zone would presumably represent clinal differentiation without geographic separation; however, secondary contact requires disjunct allopatry (Endler, 1977; Barton and Hewitt, 1985). Without a clear picture of past geographic events, the most powerful empirical evidence for primary or secondary contact resides with the distribution of markers used to identify the zone. For *Peromyscus leucopus*, we conclude that the most viable explanation for the origin of the zone is secondary contact, because it is difficult to envision how strong selection could cause rapid concordant changes in chromosomes, allozymes, and mtDNA.

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