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Empirical evaluation of cytonuclear models incorporating genetic drift and tests for neutrality of mtDNA variants: data from experimental *Gambusia* hybrid zones

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Abstract

Statistical tests of genetic drift and of the neutrality of mtDNA are presented using empirical time-series data on multi-generational changes in cytonuclear disequilibria within replicated experimental hybrid populations of two species of live-bearing Poeciliid fishes (*Gambusia holbrooki* and *G. affinis*) which were monitored over a period of two years (three generations). Cytonuclear disequilibria D_1 and D_2 (which measure departures from random associations of cytoplasmic and nuclear genotypes) over the three generations of the experiment were non-zero for all replicate populations. For each of five nuclear loci, the observed measures of D_1 and D_2 were highly concordant between replicates during each generation. Significant departures from expectations were observed after one and two generations. A statistical measure of goodness of fit of observed changes in cytonuclear disequilibria (and implicitly of the neutrality of the mtDNA markers) was calculated for each nuclear locus. When the results for the replicates were combined into an overall test of neutrality, the fit to the random union of zygotes (RUZ) model was rejected for four of the five nuclear loci ($P < 0.05$). A simple genetic drift model does not explain the temporal changes in composite cytonuclear genotypic frequencies. Frequencies of parental *G. holbrooki* mitochondrial alleles and nuclear genotypes exceeded expected values during most time periods, implying some selective advantage of offspring produced by *G. holbrooki* females. Expansion of cytonuclear models to explicitly address questions of genetic drift and neutrality have general relevance to studies of natural populations.

Introduction

Recent advances in molecular technology and methods of statistical inference have expanded greatly our understanding of evolutionary processes generating patterns of genetic variation. Accumulating evidence suggests that within species, variation at the molecular level has evolved under both adaptive and neutral mechanisms. Accordingly, considerable attention has been focused on the development and application of novel tests of the selective neutrality of DNA polymorphisms. Such scrutiny is timely as analyses of DNA variation have become increasingly popular as tools for making evolutionary and ecological inferences.

Mitochondrial (mt)DNA has been used widely in evolutionary studies because of its uniparental mode of inheritance, high rate of evolution, and ease of isolation and characterization. It has also been employed extensively in population genetic studies (e.g. estimation of migration rates (Hudson et al., 1992) and effective population size (DiRienzo & Wilson, 1991)) under the assumption that variation in haplotype frequencies is governed primarily by migration and drift

effects. However, recent evidence (Ballard & Kreitman, 1994, 1995; Nachman et al., 1994, 1996) argues against strict neutrality.

Several independent approaches have been used to address questions of the neutrality of DNA polymorphisms. Experimental population studies have monitored the trajectories of multi-generational changes in gene frequency (MacRae & Anderson, 1988; Clark & Lyckegaard, 1988; Rand et al., 1994; Kilpatrick & Rand, 1995; Hutter & Rand, 1995). Other inferences about the adaptive nature of DNA polymorphisms have been made retrospectively, utilizing comparative analyses of DNA variants within and between species (Ballard & Kreitman, 1994; Nachman et al., 1994; Nachman et al., 1996). These comparative methods have taken several forms including examination of nucleotide variation in terms of pairwise estimates of nucleotide diversity (Tajima, 1989), comparisons of the frequencies of mutations of various classes (Fu & Li, 1993; Fu, 1996), comparisons of levels of polymorphism and inter-specific divergence of a specific gene with another locus (Hudson et al., 1987), and comparisons of inter-specific ratios of synonymous to replacement changes to intra-specific ratios (McDonald & Kreitman, 1991).

Alternative methods have focused on joint examinations of bi-parentally inherited nuclear and uniparentally inherited cytoplasmic genotypes, and of biological conditions that generate nonrandom cytonuclear associations. Clark and Lyckegaard (1988) documented cytonuclear interactions of second chromosomes with different cytoplasmic backgrounds in *Drosophila melanogaster*. Other studies have tested the selective neutrality of mtDNA variants by allowing mtDNA haplotypes to compete in different nuclear genetic backgrounds (e.g. Hutter & Rand, 1995; Kilpatrick & Rand, 1995). Results of experimental and comparative studies cast considerable doubt on the assumption of selective neutrality of mtDNA polymorphisms.

For natural or experimental populations in which cytoplasmic and nuclear genetic markers have been characterized for each individual, measures of cytonuclear association (i.e. cytonuclear disequilibria) can also be employed to quantify departures from random associations between these genes (Asmussen et al., 1987). Analyses of multi-generational changes in population cytonuclear genotypic composition provide a novel means of assessing the neutrality of mtDNA polymorphisms through explicit tests for departures from expected values under genetic drift (Fu & Arnold, 1992; Datta et al., 1996; Datta & Arnold, 1996). Previously, this theory has not been applied rigorously to empirical data.

Herein we present statistical tests of genetic drift and of the neutrality of mtDNA using empirical time-series data on multi-generational changes in cytonuclear disequilibria within experimental hybrid populations of live-bearing Poecillid fishes (*G. holbrooki* and *G. affinis*) studied by Scribner and Avise (1994a). Previous work provided descriptive characterizations of the trajectories of cytonuclear disequilibrium over time and goodness-of-fit tests comparing observed cytonuclear data to expectations under competing models of random and assortative mating. This paper expands previous analyses by explicitly incorporating dynamics of cytonuclear drift into predictions of generational changes in genotypic composition, and by providing a novel test of the null hypothesis (mtDNA neutrality). Quantitative tests of the alternative hypothesis (i.e. at least one population does not comply with neutrality) are of particular interest since all experimental populations, although statistically independent, are expected to be biologically similar (i.e. cytonuclear genotypic frequencies change in a similar direction and rate in response to the same evolutionary pressure(s)).

Our specific objectives were to: (1) use recently derived moments of genotypic disequilibria D_1 and D_2 to determine whether observed temporal changes in measures of cytonuclear disequilibrium deviated significantly from expectations under a 'null' model of genetic drift; and (2) examine the neutrality of mtDNA markers. Under a strictly neutral model, we expect that the magnitude and direction of temporal changes in measures of cytonuclear disequilibria would fall within bounds estimated simply as a function of effective population size, and the nuclear and cytoplasmic gene frequencies and allelic disequilibrium (D) in the initial (founding) population.

Materials and methods

Sampling design

Details of the construction of replicate aquatic communities and population sampling were as described in Scribner (1993) and Scribner and Avise (1994a). Briefly, three virgin females and three males of *G. holbrooki* and *G. affinis* (total $N = 12$) were placed into each of 12 replicate pools, approximately 2.4 m in

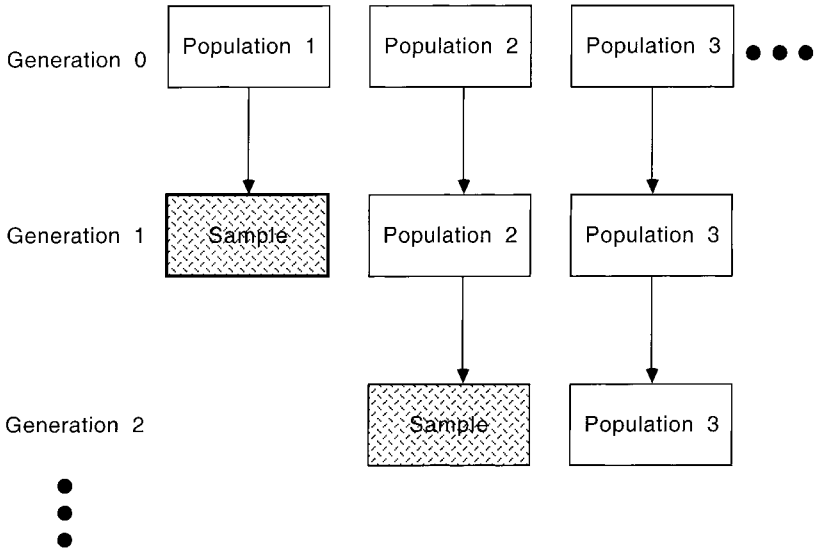

Figure 1. Sampling design for *Gambusia* experimental populations. During each of three generations two replicate populations were sampled completely for genetic characterization.

diameter and 30 cm deep. Each replicate was sampled at regular intervals (weeks 6, 12, and 18 during 1990 and weeks 52, 58, and 64 during 1991), covering a period of three generations (generation length was estimated based on life history data collected concurrently; Scribner, 1993). Total population size was estimated for each replicate at each time period based on serial removals of fish. For each sampling period, two replicate pool populations (arbitrarily assigned 1 and 2) were chosen randomly, and all individuals were sampled destructively for genetic characterization (see Figure 1 for sampling design). Effective population sizes for replicate populations 1 and 2 for each of three generations were estimated to be 12, 23, and 41 and 12, 51, and 43, respectively.

The design of replicate populations is consistent with earlier studies of genetic drift. Replicate populations provide more information than would be obtained by following one population over time. An unusual feature of the experimental design is that the populations were completely sampled so that there was no sampling variation.

Molecular procedures

Details of mitochondrial DNA extraction and characterization of species-specific restriction site polymorphisms are described in Scribner and Avise (1994a). Five nuclear loci that exhibit large differences in allele frequency between *Gambusia* species (Wooten and Lydeard, 1990) also were analyzed – adenosine deaminase (E.C. 3.5.4.4; Ada), aspartate aminotransferase-1 (E.C. 2.6.1.1; Aat-1), malate dehydrogenase (E.C. 1.1.1.37; Mdh-1), peptidase-A (E.C. 3.4.11 or 3.4.13; Pep-A, leucyl alanine as substrate), and aconitate hydratase-1 (E.C. 4.2.1.3; Ah-1), using electrophoretic conditions described in Wooten and Lydeard (1990). Counts of the number of individuals possessing each of six cytonuclear genotypes (two mtDNA cytotypes and three nuclear genotypes at each allozyme locus) were made for each replicate population, sampled during each period.

Statistical analysis

Each replicate hybrid population was characterized by an effective population size (N_e) each sampling period (each 6-wk period corresponds to approximately 0.5 generations; see Scribner and Avise (1994a) for discussion). The number of males and females of breeding age was determined. Sexual maturity for females was assessed based on the presence of pigmented lateral brood spots (G. Meffe, pers. comm.). Sexual maturity for males was determined based on gonopodium morphology (Kallman & Schreibman, 1973). In the absence of definitive data on parameters that may influence effective population size (i.e. variances of male and female reproductive success), effective population size for each replicate during each period was estimated simply as a function the number of adult

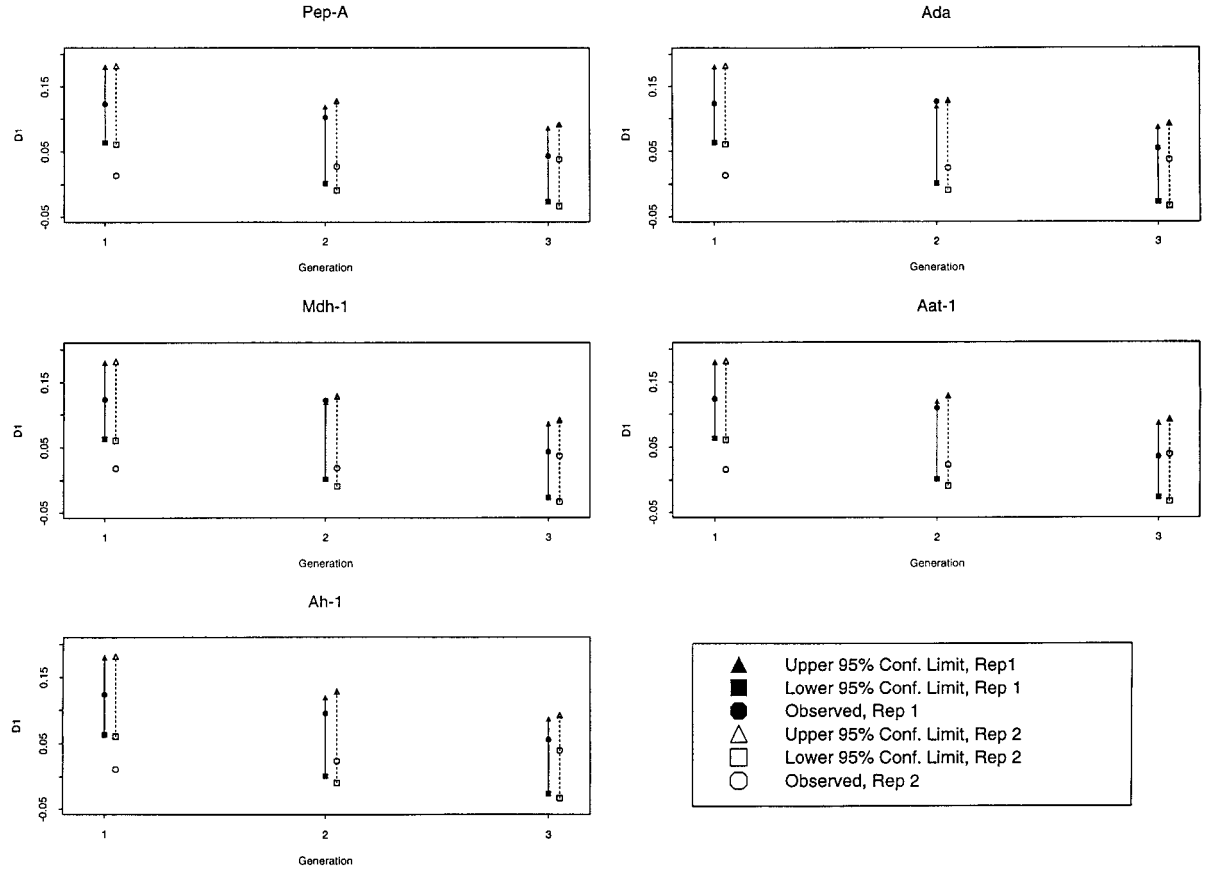

Figure 2. Observed values of genotypic disequilibium (D_1) and 95% confidence intervals for each of two replicate populations sampled during each of three generations.

males (N_m) and females (N_f), as $4N_mN_f/(N_m + N_f)$ (Crow & Kimura, 1970).

Statistical associations between cytoplasmic and nuclear loci were measured using genotypic cytonuclear disequilibria as defined by Asmussen et al. (1987). In brief, for a diploid population with two alleles at a mitochondrial locus (M and m) and two alleles (A and a) at a nuclear locus, genotypic cytonuclear disequilibria was quantified as the departure of the joint genotypic frequencies from expectations under random mating. In the context of the present analysis, data are presented for D_1 = freq. (AA/M) − [freq. (AA) × freq. (M)] and D_2 = freq. (Aa/M) − [freq. (Aa) × freq. (M)]. Replicate populations were closed to outside recruitment and were assumed to be random-mating. Deviations of observed genotypic cytonuclear disequilibria (D_1 and D_2) from expected trajectories over three generations were assumed (under the neutral model of genetic drift) to be due strictly to the finite mating pool of gametes. The joint distribution of gametic frequencies, conditional on their values in the previous generation, were specified assuming RUZ (Watterson, 1970). This procedure previously has been reported as being appropriate for cytonuclear systems (Fu & Arnold, 1992). Expected values of D_1 ($ED_{1(t+1)}$) and D_2 ($ED_{2(t+1)}$) are determined as in Datta et al. (1996, formulas 14 and 21, respectively). The second moments of D_1 and D_2 which were used to calculate the variances were determined under the RUZ model (Datta et al., 1996; formulas 26 and 27, respectively). Variances were used to calculate point-wise 95% confidence bands about the expected trajectories based on effective population sizes for each replicate and time period. Confidence intervals were estimated corresponding to $E(D_i) \pm 1.96\mathrm{SD}(D_i)$, where $E(D_i)$ is the expected value of either D_1 or D_2 and SD is the standard deviation. Approximate normality of the statistics was used and has been proven in Datta et al. (1996). Further, to check the effectiveness of this large sample

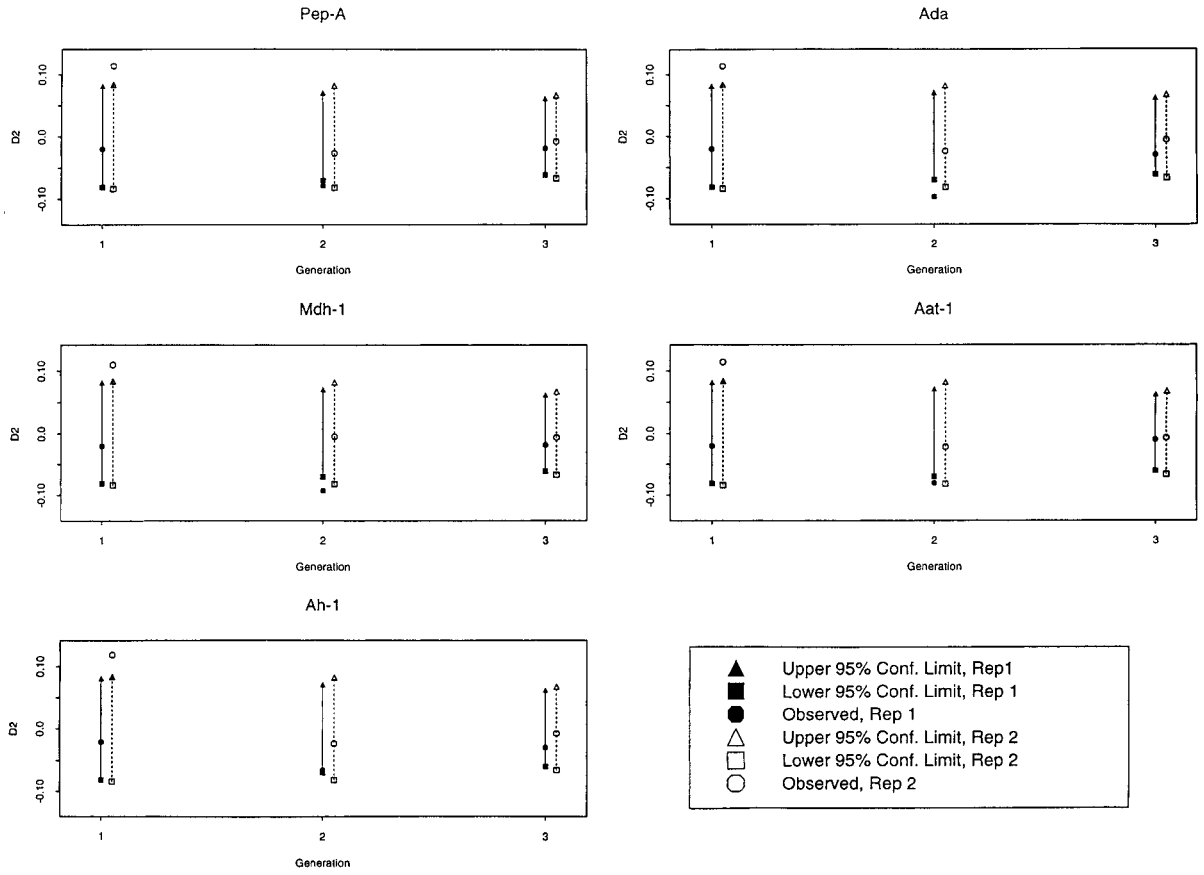

Figure 3. Observed values of genotypic disequilibium (D_2) and 95% confidence intervals for each of two replicate populations sampled during each of three generations.

result for populations of small to moderate size, simulation studies have been preformed illustrating that results were robust (see Datta & Arnold, 1996). Typically, confidence intervals are drawn around the estimated (or observed) statistics, as the expected values are populations parameters, and hence typically unknown. However, in the present experimental context, we knew the initial population values and in turn the expected values in all the subsequent generations. Therefore, in the present context, both are equivalent. A formal goodness-of-fit test of predicted dynamics of cytonuclear disequilibria under genetic drift was derived as

$$T = \sum (D(t) - ED(t))' S^{-1}(t)(D(t) - ED(t)) \quad (1)$$

(Datta & Arnold, 1996; formula 7) where $E(D)$ is the expected value in the t^{th} generation, and S^{-1} is the inverse of the variance-covariance matrix of D_1 and D_2. The test statistic 'T' is the total distance, summed across the three generations, between observed and expected disequilibria under the drift model, and is chi-squared distributed with $2k$ degrees of freedom, where k is the number of samples.

Results

Trajectories of the expected values of genotypic disequilibrium, as shown in Datta et al. (1996), do not depend strongly on effective population size, but instead resemble those in populations of infinite size. The measure D_1 is expected to decrease monotonically over time (Datta et al., 1996). Expected values for D_2 are generally quite small, as replicate populations were started with the same equal mix of the two parental types.

Cytonuclear disequilibria D_1 and D_2 over the three generations of the experiment were non-zero for all replicate populations (Figures 2 and 3, respectively) and represent the true values of these measures because the entire population was sampled. For each

Table 1. Neutrality test of the deviation between observed and expected cytonuclear disequilibria under the genetic drift model using the '*T*' test statistic (Datta & Arnold, 1996)

Locus	*T* values			*P*
	Replicate 1	Replicate 2	Entire experiment	
Pep-A	5.622	15.556	21.180	0.048
Ada	9.308	15.596	24.900	0.015
Mdh-1	7.814	13.859	21.670	0.041
Aat-1	5.661	15.227	20.900	0.052
Ah-1	4.883	16.213	21.100	0.049

of five nuclear loci, the observed measures of D_1 and D_2 were highly concordant between replicates during each generation, and generally fell within the 95% confidence bands about the expected trajectories, suggesting a reasonable fit to the drift model. Nonetheless, significant departures from expectations were observed after one and two generations (Figures 2 and 3).

To evaluate formally the test statistic, '*T*' (Datta & Arnold, 1996) was calculated for each nuclear locus (Table 1). Results of the goodness-of-fit of observed changes in cytonuclear disequilibria (and implicitly of the neutrality of the mtDNA markers), for the replicates were combined into an overall test of neutrality. The fit to the RUZ model was rejected for four of the five nuclear loci ($P < 0.05$) and the fifth test was marginally significant ($P = 0.052$). The simple genetic drift model does not explain the temporal changes in composite cytonuclear genotypic frequencies. Frequencies of parental *G. holbrooki* mitochondrial alleles and nuclear genotypes exceeded expected values during most time periods (Scribner & Avise, 1994a), implying some selective advantage to individuals of *G. holbrooki* maternal parentage.

Discussion

It is of interest to know the effects of genetic drift on the variance in associations of alleles (or genotypes), as this variation is one possible source of new gene complexes that might be acted upon by other evolutionary forces. Further, the ability to understand the behavior of cytonuclear disequilibria under drift is important for testing the neutrality of mtDNA markers, and for interpreting spatial patterns in measures of cytonuclear disequilibria or the trajectory of changes in disequilibria over time (e.g. in the context of hybrid zones).

In Scribner and Avise (1994a), several lines of evidence were forwarded which demonstrated that genetic changes within replicate experimental *Gambusia* hybrid zones did not result from random drift. Consistency in the pattern and direction of change in mitochondrial and nuclear allele frequencies, consistency in reduction in population genetic diversity owing to loss of *G. affinis* alleles, and lack of appreciable (or temporally increasing) levels of inter-replicate genetic variance implicated nonrandom evolutionary forces. We further compared the degree of departure from a 'no-association' hypothesis based on deviations of observed cytonuclear gene (and genotype) frequencies from expectations (Asmussen et al., 1987). Although departures of observed cytonuclear combinations from expectations under random and assortative mating systems (Arnold et al., 1989) were significant, no explicit test for cytonuclear drift was available at that time.

Whereas expected disequilibria decay to zero for randomly mating populations, genetic drift will generate departures from this expectation in the form of new gene associations (Arnold, 1993). With genetic drift, cytonuclear disequilibria no longer are deterministic, but vary from generation to generation due to the finite mating pool of gametes (Fu & Arnold, 1992). Using recently derived moments for genotypic cytonuclear disequilibria measures under genetic drift (Datta et al., 1996), we report results from statistical tests that explicitly address the behavior of gene associations under drift in a hybrid zone.

Experimental results revealed that the variances about the expected values for D_1 and D_2 (as shown by 95% confidence bands) were large, reflecting the relatively small effective population sizes for each replicate during each period. Populations grew from initial starting effective sizes of 12 to between 41–51 throughout the course of the study. Stochastic processes could be pervasive, yet during many sampling periods levels of genotypic disequilibria differed significantly from expectations under genetic drift.

High levels of cytonuclear disequilibrium could conceivably result from the historical sampling of gametes in finite populations. Historical bottlenecks cannot be ruled out as alternative explanations to selection (Excoffier, 1990). Founder effects would be expected to be most pervasive during the early portion of the experiment due to low founding numbers. Indeed, the greatest deviations of observed from expected values of D_1 and D_2 were during the first

two generations. Variation between replicates was also greatest during this time.

The direction of deviation of genotypic disequilibria from expected values followed predictions based on genotype-specific variation in life history traits (Scribner, 1993; Scribner & Avise, 1993a) and were consistent with additional empirical characterizations of experimental (Scribner & Avise, 1994b) and natural (Scribner & Avise, 1993b) hybrid zones for these species. Laboratory breeding studies have revealed that progeny from parental *holbrooki* crosses and F_1 hybrid offspring with *holbrooki* maternal parentage exhibited larger sizes at birth, faster growth rates, and greater size and younger age at sexual maturity than progeny from pure *G. affinis* and F_1 hybrid offspring with *affinis* maternal parentage (Scribner, 1993). Such life history traits may place offspring of *holbrooki* maternal parentage at a selective advantage in environments characterized by high density, resource scarcity, and high juvenile mortality rates, all of which are environmental regimes characteristic of these experimental systems (and some natural environments as well). Although the proportions of AA/M parental and Aa/M hybrid cytonuclear genotypes varied between replicates during each generation, offspring of *G. holbrooki* maternal parentage were clearly present in frequencies significantly exceeding expectations (Figures 2 and 3). Significantly fewer numbers of parental *G. holbrooki* cytonuclear genotypes (AA/M), but significantly greater numbers of hybrids with *G. holbrooki* mtDNA (Aa/M cytonuclear genotypes) were observed in replicate 2 after one generation. Significantly greater numbers of parental *G. holbrooki* (AA/M) cytonuclear genotypes but significantly fewer than expected numbers of hybrid Aa/M genotypes with *G. holbrooki* mtDNA were observed in replicate 1 after two generations.

Interpretations of descriptive results of the spatial dispersion of genetic variants or of the magnitude of measures of disequilibrium often may be confounded, as results can be due to a multiplicity of factors (e.g. population subdivision (Asmussen & Arnold, 1991), mating systems (Arnold et al., 1989; Asmussen et al., 1989), hybridization (Lamb & Avise, 1986), or hitchhiking of neutral or near-neutral mtDNA with selected novel nuclear variants (Kilpatrick & Rand, 1995)). Positive disequilibrium also could be explained by drift alone or might be the by-product of founder events (Gyllensten & Wilson, 1987). Due to the lack of direct historical information on selective regimes, gene flow, or of genotype-specific life history traits, serious questions may be raised about the extent to which apparent patterns in nature bear witness to the evolutionary processes that are invoked to explain them. Clearly, multiple lines of evidence are needed. One such source of inference should involve further statistical tests of neutral expectations in appropriate experimental settings.

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