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Correspondence between sexual isolation and allozyme differentiation: A test in the salamander *Desmognathus ochrophaeus*

(gene frequencies/sexual isolation/genetic differentiation/speciation)

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ABSTRACT **Ethological reproductive isolation and genetic divergence across 26 protein loci were measured among populations of the salamander *Desmognathus ochrophaeus* in the southern Appalachian Mountains. Levels of ethological isolation varied from none to complete and were statistically significant for all but two pairings between populations inhabiting different mountain ranges. When geographic and genetic distances were treated as independent variables in multiple correlation analyses, they accounted for about half the variance in levels of ethological isolation. When genetic distance is held constant, the remaining relationship between ethological isolation and geographic distance is still statistically significant. When geographic distance is held constant, the remaining relationship between genetic distance and levels of ethological isolation is nonsignificant, as is the relationship between geographic distance and genetic distance when ethological isolation is held constant. Ethological isolation and genetic divergence evidently both reflect the gradual divergence of allopatric populations, but genetic distance is a poor predictor of ethological isolation in these salamanders.**

A survey of ethological (defined as sexual) isolation among populations of a single species can illuminate the manner in which reproductive isolation evolves. By testing multiple pairs of populations for sexual compatibility, we can observe critical early stages in the origin of reproductively isolated species. Despite their value, such surveys are difficult to accomplish because of the technical problems of conducting and scoring a large number of mating trials. Consequently, surveys have been conducted in only a few animal taxa and rarely in vertebrates (1–3). Plethodontid salamanders of the genus *Desmognathus* are ideal material for such a survey because large-scale testing of mating compatibility is feasible and because insemination can be unambiguously scored for several hours after mating trials (4–5).

In this paper we survey levels of ethological isolation among populations of the salamander *Desmognathus ochrophaeus*. In the southern Appalachian Mountains of the eastern United States this species consists of many disjunct populations among which there is considerable genetic differentiation, striking variation in body size, life history, and color pattern and, for two of them, significant, though incomplete, ethological isolation (4, 6). We here report on the extent and nature of variation in levels of ethological isolation among southern Appalachian *D. ochrophaeus* populations and specifically address two questions: is the extent of ethological isolation correlated with the degree of allozyme divergence among populations, and how do these two manifestations of evolutionary divergence relate to geographic distances among populations?

A variety of stochastic and deterministic processes should generate both ethological isolation (7–10) and allozyme differentiation (11–13) among populations. Both aspects of differentiation should correlate with geographic separation of populations, because more distant allopatry should reflect lower rates of contemporary gene flow, longer histories of isolation, and more divergent selective regimes. Thus, a correlation between ethological isolation and allozyme differentiation might result from both these variables reflecting overall genetic divergence, rather than from a functional relationship between them. However, founder effects and drift should obscure their relationship with geographic distance by causing some populations to be more genetically divergent than geography would predict. Dissecting the relationships among ethological isolation, genetic divergence, and geographic distance might, thus, illuminate tempo and mode in the evolution of reproductive isolation.

Allozyme data have been used to diagnose the species status of allopatric populations in the absence of direct evidence on reproductive isolation (14–16). If species are, in fact, to be treated as reproductively isolated entities, such inference from allozyme data depends on the magnitude of the correlation between allozyme distance and reproductive isolation. Coyne and Orr (3) report positive correlations between both pre- and postzygotic isolation and allozyme differentiation in allopatric *Drosophila* species. We know of no other quantitative estimates of the correlation between allozyme differentiation and reproductive isolation.

MATERIALS AND METHODS

Population Sampling. Sexually mature salamanders of both sexes were collected from populations at 11 localities (see Fig. 1) during five field trips undertaken from May 1986 through May 1988. All populations were included in a previous survey of allozymic variation in *D. ochrophaeus* and *Desmognathus imitator* (6). We chose populations representing a wide spectrum of genetic differentiation with respect to the 12 loci studied by those authors. Most of the major mountain ranges of the southwestern Blue Ridge Physiographic Province are represented by one or more samples, which extend along a northeast to southwest axis from Mount Rogers in southwestern Virginia to John's Knob in the Unicoi Mountains of southwestern North Carolina.

Ethological isolation coefficients are not available for all possible pairs of all the populations we sampled. Our analyses are, therefore, based on composite Mount Mitchell (MM) and Highlands Plateau (HP) samples obtained by averaging data for populations 9 and 10 (MM) and 21 and 22 (HP) of ref. 6 and omit the Waterrock Knob (WR) and John's

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Abbreviations: Populations are indicated as follows: MR, Mt. Rogers; UN, Unaka Mountain; MM, Mt. Mitchell; WR, Waterrock Knob; RB, Rough Butt Bald; HP, Highlands Plateau; WA, Wayah Bald; SI, Standing Indian Mountain; JK, John's Knob.

Knob (JK) populations. These procedures generated a total of 7 "populations" for use in the multiple correlation analysis described below.

Conditions of maintenance of salamanders in laboratories at both Smith College and the University of Chicago were similar and are described in detail elsewhere (5).

Measurement of Ethological Isolation. Individual salamanders will mate repeatedly over periods of several months (17–19). The experimental design used in all crosses was modified from that used in a previous study (4) and comprised an incomplete Latin square. Because details of our design are available elsewhere (5), we provide only a brief description here. To stage a cross between a pair of populations (designated *A* and *B*), 10 males and 10 females from each were placed with one another to produce a total of 30 of each of the following types of heterosexual pairings: male *A* × female *A* and male *B* × female *B* (60 homotypic pairings), and male *A* × female *B* and male *B* × female *A* (60 heterotypic pairings). With this design, each salamander encountered six individuals of the opposite sex, three from its own population and three from the other population. No individuals encountered one another more than once, ensuring that all pairings were unique. Male–female pairs were left in isolation overnight for ≈15 hr (which allows ample time for courtship), after which time they were separated, and females were checked for a sperm mass in the cloaca.

A total of 3720 (31 crosses × 120 trials per cross) single male–single female mating trials involving the nine populations were conducted at Smith College and Chicago from May 1986 through May 1988. Data on the numbers of inseminations obtained during homotypic and heterotypic encounters were used to calculate an overall coefficient of the strength of sexual isolation between each pair of populations. The coefficient we employed is simply the sum of proportions of inseminations in homotypic encounters minus the sum of proportions of inseminations in heterotypic encounters. It effectively ranges from zero (when heterotypic proportions equal homotypic proportions) to two (when every homotypic encounter results in insemination but none of the heterotypic encounters are successful). The results of McCullagh and Nelder (20) were used to compute the sampling variance (squared SE) of this coefficient.

Calculation of Geographic Distances. Geographic distances were calculated as the great circle distances among the localities.

Electrophoresis. Our results are based on 25 allozyme loci and 1 blood protein locus (transferrin). Data for the transferrin locus were extracted from previous work (6). The remaining loci were scored using standard methods of horizontal starch gel electrophoresis in tissue samples from specimens used in our survey of ethological isolation. The loci and their EC numbers were as follows: aconitate hydratase (4.2.1.3), aspartate aminotransferase 1 and 2 (2.6.1.1), adenylate kinase (2.7.4.3), creatine kinase, (2.7.3.2), fumarate hydratase (4.2.1.2), glucose dehydrogenase (1.1.1.47), glutamate dehydrogenase (1.4.1.3), glucose-6-phosphate isomerase (5.3.1.9), glyceraldehyde-3-phosphate dehydrogenase (1.2.1.12), glycerol-3-phosphate dehydrogenase (1.1.1.8), 3-hydroxybutyrate dehydrogenase (1.1.1.30), isocitrate dehydrogenase 1 and 2 (1.1.1.42), L-lactate dehydrogenase 1 and 2 (1.1.1.27), malate dehydrogenase 1 and 2 (1.1.1.37), malate dehydrogenase (oxaloacetate-decarboxylating, 1.1.1.38), mannose-6-phosphate isomerase (5.3.1.8), phosphogluconate dehydrogenase (1.1.1.44), phosphoglucomutase 1 and 2 (5.4.2.2.), leucylglycylglycine peptidase, superoxide dismutase (1.15.1.1), and serum transferrin.

Measurement of Genetic Differentiation. Modified Rogers and Nei (22) unbiased genetic distance coefficients were calculated for all population pairs using release 1.7 of the BIOSYS-1 program adapted for use on IBM-PCs and compatibles and provided by David L. Swofford (Illinois Natural History Survey, Champaign, IL). Routines SIMDIS, WRIGHT-78, and CLUSTER were used to calculate genetic distances, *F* statistics, and the unweighted pair-group arithmetic average (UPGMA) (31) phenogram.

Data Analysis. Our data set consists of matrices of Nei unbiased genetic distance coefficients and geographic distances for all possible pairs of nine populations and absolute ethological isolation coefficients for seven of these pairs. Each of the resultant three 7 × 7 subdiagonal half matrices contains 21 entries.

Standard correlation analyses are inappropriate for these data because (*i*) each population enters into the determination of several matrix entries, and (*ii*) spatial autocorrelation contributes to the covariance among cell entries (23–27). We employed a variant of the Mantel (23) method developed by Smouse *et al.* (27), which generates simple and partial correlation coefficients and evaluates their statistical significance by comparing them with values generated by repeatedly randomizing the entries in one of the matrices. This procedure was performed by the NEWMAN3R program provided by R. R. Sokal (State University of New York at Stony Brook), using 9999 randomizations. Because we are dealing with allopatric populations of a subdivided species, both common ancestry (28) and spatial autocorrelation (24) contribute to covariation among data points. The randomization procedures account for both sources of covariation (J. Felsenstein, personal communication).

RESULTS

Ethological Isolation Among Populations. Twenty-eight ethological isolation coefficients were obtained in pair-wise crosses between nine allopatric salamander populations. One of these combinations involved two populations in the same mountain range near Mount Mitchell, North Carolina (populations 9 and 10 of ref. 6). As might be expected, this cross yielded an isolation coefficient that differs nonsignificantly from 0 (0.20, SE = 0.16). As explained above, these two populations and the two from the Highlands Plateau were pooled for subsequent analyses. The isolation coefficients for these crosses ranged from 0.20 [for Standing Indian Mountain (SI) × JK] to 1.50 [for Mount Rogers (MR) × HP, indicating essentially complete ethological isolation]. For those 21 crosses that provide the data set we used in matrix correlation analysis, isolation coefficients ranged from 0.33 [SI × Wayah Bald (WA)] to 1.50 (MR × HP) (mean coefficient = 0.76, SD = 0.28).

Several crosses involving the two HP populations allow us to evaluate the consistency of our ethological isolation coefficients. Population Rough Butt Bald (RB) was paired with the Whiteside Mountain HP population three times at different times of the year, yielding isolation coefficients and their SE values of 0.47 (0.08), 0.50 (0.15), and 0.70 (0.15). When population RB was paired with the Cashiers HP population, we obtained an isolation coefficient of 0.50 (0.14). The Whiteside Mountain HP and Cashiers HP populations were both paired with the SI populations, yielding coefficients of 0.73 (0.16) and 0.90 (0.15), respectively. Taken as a whole these values suggest relatively robust estimation of ethological isolation across years and seasons.

Other than the cross involving the two Mount Mitchell area populations, there were only two crosses where we were unable to detect statistically significant ethological isolation. These are WA × SI (0.33, SE = 0.17) and SI × JK (0.20, SE = 0.15).

Allozyme Differentiation. Southern Appalachian *D. ochrophaeus* populations exhibit high levels of genic differentiation; this can be quantified by the fixation index, F_{st}, a measure of the proportion of overall genetic variation attributable to differentiation among local populations (29). This

index equals 0.643 when averaged across all loci, indicating a high level of genetic differentiation (29). However, this is not an especially high level for conspecific populations of plethodontid salamanders (30).

Genetic distances were relatively low for comparisons between populations in the same mountain ranges (0.091 for the HP populations and 0.043 for the two MM populations). Comparisons of populations in different mountain ranges yielded a wide spectrum of distances, ranging from 0.116 for Unaka Mountain (UN) × MM to 0.637 for MR × MM.

Fig. 1 shows a dendrogram constructed from a matrix of modified Rogers genetic distances (29) by the UPGMA method (31). The clusters of populations on the dendrogram correspond to geographic groupings, and, in general, the more deeply differentiated two populations are, the farther apart they are geographically. The MR population, however, is quite differentiated from all other populations (Nei distances range from 0.384 to 0.637), and genetic distances for comparisons with MR show no tendency to increase with geographic distance.

Associations Between Ethological Isolation, Genetic Distance, and Geographic Distance. Scatterplots of ethological isolation against geographic distance and genetic distance (Fig. 2 *A* and *B*) indicate positive correlations for both sets of variables. Likewise, genetic distance increases with geographic distance (Fig. 2*C*). Table 1 summarizes the results of the multiple correlation analysis. Simple correlation coefficients indicate that genetic distance and ethological isolation are both significantly correlated with geographic distance ($P < 0.05$), whereas ethological isolation and genetic distance are not significantly correlated ($P = 0.077$).

The partial correlation coefficients indicate that the relationship between isolation and geographic distance remains significant when genetic distance is held constant ($P = 0.027$), but neither of the other partial correlation coefficients is significant ($P > 0.05$).

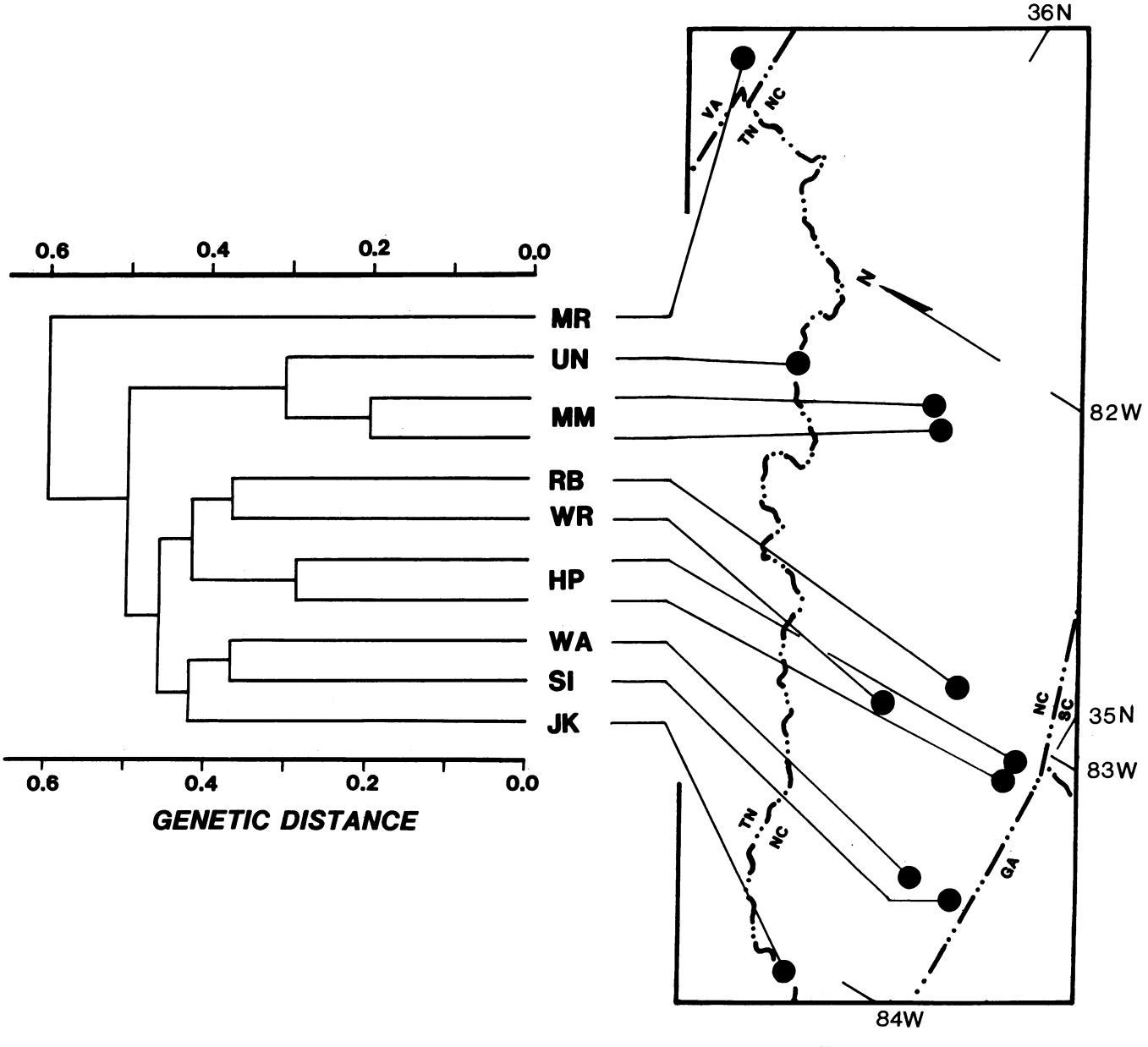

FIG. 1. Dendrogram expressing levels of genetic distance (using modified Rogers distances, ref. 29) among allopatric populations of *D. ochrophaeus* shown on the accompanying map. Populations correspond to locality numbers in ref. 6 as follows: MR (Mount Rogers), 2; UN (Unaka Mountain), 6; MM (Mount Mitchell), 9 and 10; WR (Waterrock Knob), 15; RB (Rough Butt Bald), 17; HP (Highlands Plateau), 21 and 22; WA (Wayah Bald), 26; SI (Standing Indian Mountain), 28; JK (John's Knob), 30. The adjacent populations near MM and on the HP are pooled in the remaining analyses.

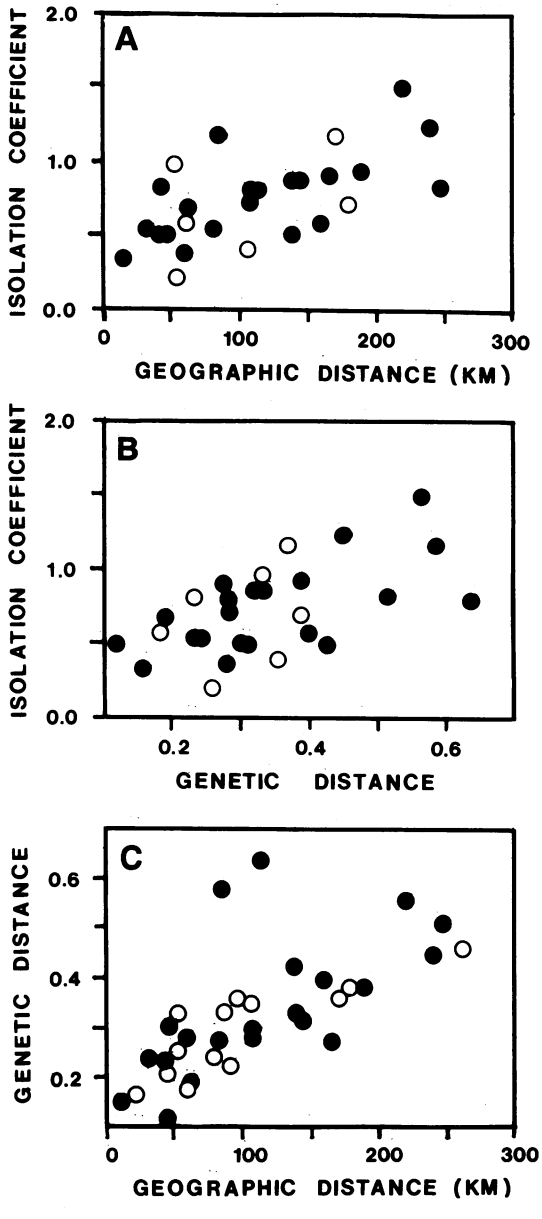

FIG. 2. Scatterplots showing the relationships between ethological isolation coefficient and geographic distance (n = 27) (*A*), ethological isolation coefficient and Nei genetic distance (n = 27) (*B*), and Nei genetic distance and geographic distance (n = 36) (*C*). Filled circles indicate the 21 points used in the multiple correlation analysis.

These analyses suggest that ethological isolation is correlated with geographic distance between populations. Information on genetic distance between populations appears to be of no additional value in attempting to predict levels of ethological isolation between populations. The correlation between genetic distance and geographic distance falls to insignificance when variation in ethological isolation is held constant. The apparent (though nonsignificant) relationship between isolation and genetic distance indicated in Fig. 2 simply reflects covariation of both of these variables with geographic distance, rather than an independent relationship between them.

Table 1. Simple and partial correlation coefficients and estimates of their statistical significance from the matrix randomization procedure

Coefficient	Value	Probability
r_{ed}	0.670	0.008
r_{en}	0.632	0.077
r_{nd}	0.626	0.033
$r_{ed.n}$	0.455	0.027
$r_{en.d}$	0.367	0.139
$r_{nd.e}$	0.351	0.139

e, ethological isolation coefficient; n, Nei genetic distance; d, geographic distance; when symbol appears after the period in the subscript, that variable is held constant.

The multiple coefficient of determination (R^2, ref. 27) equals 0.52, indicating that about half the variance in ethological isolation remains unaccounted for by either geographic or genetic distances among populations. The comparisons among WA, SI, and MM are an interesting case in point. Although MM is virtually equidistant from the other two populations, sexual isolation is stronger with SI (0.87) than with WA (0.50). As might be expected from the multiple correlation data, genetic distances are of no additional predictive value; they are roughly of the same magnitude, but the more sexually isolated pair of populations (MM and SI) exhibits a lower genetic distance (0.323) than does MM and WA (0.423). This trio of populations illustrates another phenomenon: populations that are genetically similar and behaviorally compatible may exhibit quite different levels of sexual isolation from a third population.

DISCUSSION

Substantial ethological isolation has evolved among allopatric populations of *D. ochrophaeus*. Considering all crosses reported in this paper, coefficients of sexual isolation ranged from low to high values, from pairs of populations showing virtually no ethological isolation (e.g., JK × SI) to other pairs showing complete sexual incompatibility (e.g., MR vs. several populations). Continuous variation in indices of ethological isolation and genetic distance, and the mutual correlation of these variables with geographic distance suggest that both these indices evolve in a gradual manner as consequences of genetic divergence among allopatric populations.

Ethological isolation, genetic distance, and geographic distance are all positively correlated in the *D. ochrophaeus* data, but Nei distance was a weak and nonsignificant predictor of ethological isolation (r = 0.63, P = 0.077). This may reflect error in the estimation of genetic distance and isolation coefficients, variable rates of evolution, or both. In any event, we cannot support the view that levels of ethological isolation among these populations can be reliably inferred from allozyme data on 26 loci.

Partial correlation analysis enables us to test for the following associations between these variables (Table 1): (*i*) covariation of geographic distance and ethological isolation ($r_{ed.n}$), (*ii*) covariation of ethological isolation and genetic distance ($r_{en.d}$), and (*iii*) covariation of genetic distance and geographic distance ($r_{nd.e}$). Only the first of these effects is supported by our data. Presumably ethological isolation increases with geographic separation because geographically distant populations have undergone longer periods of divergence and because geographic separation disrupts gene flow. The absence of a significant partial correlation between ethological isolation and genetic distance implies that geography, rather than sexual incompatibility, is the primary inhibitor of gene flow among these populations; that (not surprisingly) sexual isolation and allozymes are determined by different genes; and that geographic distance may reflect overall genetic divergence more accurately than Nei distances based on 26 loci. The lack of a significant partial correlation between geographic separation and genetic distance is surprising because the corresponding simple correlation coefficient is significant (Table 1) and because associations between these variables are predicted by theory and have been reported elsewhere (24, 32–35). The most likely

explanation is that genetic distance and ethological isolation both reflect overall genetic divergence. Controlling for ethological isolation in the multiple correlation amounts to controlling for level of genetic divergence, which leaves little further effect of geographic distance on genetic distance.

This apparent primacy of geographic distance as a predictor of genetic divergence and ethological isolation suggests relatively minor roles for founder effects and drift as speciational mechanisms in these salamanders. However, ethological isolation and genetic distance are both subject to several sources of measurement error, whereas the determination of geographic distance is not, and our data should not be regarded as conclusive on this point.

We cannot speculate on levels of genic divergence and ethological isolation required to isolate sympatric populations of these salamanders. However, we must conclude that high levels of ethological isolation can evolve among allopatric populations and note that levels of genetic differentiation between some southern Appalachian *D. ochrophaeus* populations approach and even exceed those among certain sympatric species of *Desmognathus* (36). *D. ochrophaeus* appears to be a species in the process of fragmenting.

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