

Tests for Sexual Isolation in Plethodontid Salamanders (genus *Desmognathus*)

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ABSTRACT.—This study establishes the feasibility of measuring sexual isolation between populations of plethodontid salamanders. Sexual isolation between Virginia and Tennessee populations of *Desmognathus ochrophaeus* and between *D. ochrophaeus* and *D. fuscus* was studied by staging mating trials in the laboratory. The two populations of *D. ochrophaeus* showed statistically significant but incomplete sexual isolation. In contrast, we found nearly complete isolation between *D. ochrophaeus* and *D. fuscus*.

In recent years the systematics of the more than 250 extant species of plethodontid salamanders have been intensively studied using the techniques of protein electrophoresis, microcomplement fixation analysis of albumins, and comparative osteology (e.g., Wake, 1966; Tilley et al., 1978; Highton and Larson, 1979; Wake et al., 1980; Larson et al., 1981; Larson, 1983*a, b*; Karlin and Guttman, 1986). These biochemical and morphological studies have yielded two major discoveries concerning the relationship between genetic (electrophoretic) and morphological variation within this diverse family of salamanders. One major new development is the repeated discovery of cryptic species. In many cases protein electrophoresis has revealed sympatric species that are virtually indistinguishable on morphological grounds (Highton, 1979; Highton and Larson, 1979; Hanken, 1983; Lynch et al., 1983; Highton and Webster, 1986). A second major discovery is that allopatric populations with highly similar morphologies often show great differences at allozyme loci (Wake, 1981).

Despite these advances, our understanding of reproductive isolation in plethodontids is incomplete. Direct assays of reproductive isolation have only recently been made (Dawley, 1986) and experimental work has not played a role in reaching systematic decisions. Because morphology is often conservative, species boundaries have sometimes been erected solely on the basis of electrophoretic differentiation. For

arrays of morphologically similar allopatric populations, reproductive isolation has been inferred by comparison with the electrophoretic differentiation that is characteristic of sympatry or zones of parapatry (Highton and Larson, 1979). This practice has been challenged by Wake (1981), who cautions that a given minimum value of electrophoretic differentiation does not necessarily correspond with a speciation event. Conversely, the lack of significant electrophoretic variation between two populations might not be indicative of conspecific status (see Templeton, 1980). Measures of sexual isolation and other forms of reproductive isolation could be a useful diagnostic tool, therefore, particularly in the troublesome case of allopatric populations.

In other taxa, direct assays of reproductive isolation are routinely used in systematic studies. In *Drosophila* systematics, for example, tests of sexual isolation and interfertility have been used for many years to help diagnose species (e.g., see review by Parsons, 1973).

We studied two species, *Desmognathus ochrophaeus* and *D. fuscus*, testing for sexual isolation between them as well as between two populations of *D. ochrophaeus*. Many detailed systematic and ecological studies have been made on these two species (Dunn, 1916; Hairston, 1949, 1973; Organ, 1961; Tilley, 1973, 1980; Tilley et al., 1978; Tilley and Schwerdtfeger, 1981). *Desmognathus ochrophaeus* is a montane form with a range contained within the distribution

of *D. fuscus*, which is primarily a lowland form. Karlin and Guttman (1981) reported electrophoretic evidence of hybridization between *D. fuscus* and *D. ochrophaeus* in Ohio, New York, and Pennsylvania, but hybrids were rare. One of our study populations of *D. ochrophaeus* (Unaka) is situated on a mountain top where no *D. fuscus* occur. We sampled both species at a second site (Mt. Rogers, 134 km NE) where they occur in sympatry and where, despite intensive observation and collecting, no hybrids have ever been discovered (Organ, 1961; and pers. obs.).

The usefulness of laboratory tests for isolation depends in part upon the consistency with which sexual isolation is measured (e.g., see Gilbert and Starmer, 1985). In the commonly used multiple choice testing method (where multiple males and multiple females are put together), two potentially confounding factors are differences in sample sizes of available mates in the mating pools and differences in the length of each courtship trial. In addition, aggressive interactions among males may confound the interpretation of subsequent mating scores. This is the case for many plethodontid salamanders (Arnold, 1976), including *Desmognathus ochrophaeus*, where male-male interactions can result in competitive exclusion from potential mates and in the disruption of courtships by rival males (Houck, 1988). To avoid these problems, we used a sequential choice protocol in which, for example, an individual female is paired first with a male from her own population and, on another night, with a male from a different population (see Materials and Methods). This protocol could be readily applied to a large number of populations and species of plethodontids for which electrophoretic, morphological or immunological information already is available.

MATERIALS AND METHODS

The *D. ochrophaeus* denoted UN were collected on the crest of the Unaka Mountains, Unicoi Co., Tennessee (36°07'32"N, 82°18'41"W), 1463 m elev., in August 1980. *Desmognathus ochrophaeus* and *D. fuscus* denoted MR were collected on Mount Rog-

ers, Grayson and Smyth counties, Virginia (36°39'22"N, 81°34'15"W), 1450 m elev., in August 1980.

In the laboratory, animals were individually housed in plastic shoe boxes (9 × 17 × 31 cm) with a damp paper towel substrate and a crumpled towel retreat. The boxes were kept at 15–16°C on a natural (Chicago) photoperiod. Each animal was supplied with an active *Drosophila* culture vial so that it could feed ad libitum on adult and larval flies.

Mating Trials.—For mating trials a single male and a single female were introduced into a new plastic shoe box with damp paper towel substrate (but no retreat) and left there overnight. Each box was visually isolated from other boxes. Pairs were put together 30–60 min before sunset and results were scored the next morning, 15–17 h later. After scoring, each animal was returned to its own maintenance box.

Eight animals of each sex from each population were used in the trials. Each animal engaged in nine mating trials, four days apart, beginning on 13 January and ending on 14 February 1981. The trials were balanced so that each animal encountered all three types of potential mates. Thus each MR *fuscus* female encountered three MR *fuscus* males, three MR *ochrophaeus* males, and three UN *ochrophaeus* males. The trials were blocked in time so that, in each of three successive blocks of three trials, each female encountered one of each of the three types of mates (in random order). Type of mates was not so precisely balanced for each male but, nevertheless, on the average each male encountered three females of each type over the nine trials (range = 2–4).

On the morning after each trial, each box was checked for the presence of a spermatophore or a spermatophore base. In addition, insemination was scored by putting each female on the clear plastic side of the mating box and checking for a sperm mass in her cloaca. A sperm mass is large (2–3 mm in diameter) and provides an unambiguous sign of insemination. Spermatophore deposition does not occur unless the female as well as the male exhibits courtship receptivity. We define a successful

TABLE 1. Results of staged courtship encounters between two allopatric populations of *Desmognathus ochrophaeus* (O) and one population of *D. fuscus* (F). Population sites are UN (= Unaka Mountains, Tennessee) and MR (= Mt. Rogers, Virginia). Values are the number of courtships in which a spermatophore was deposited, with the corresponding number of inseminations listed in parentheses. For each possible cross, the sample size was 24 male-female encounters.

Females	Males		
	MR-F	MR-O	UN-O
MR-F	8 (8)	0 (0)	1 (1)
MR-O	0 (0)	21 (16)	10 (9)
UN-O	0 (0)	6 (5)	21 (18)

mating as one in which a spermatophore was deposited.

Measures of Sexual Isolation.—We used Merrell's (1950) measure of sexual isolation:

$$I = \frac{AB + BA}{AA + BB}$$

where AB is the number of successful matings between males from Population A and females from Population B, BA is the number of matings between males from Population B and females from Population A, and where AA and BB are the respective number of homopopulation matings. For this index, complete isolation between populations is represented by $I = 0$ and random mating occurs when $I = 1$. We used Merrell's index, rather than those of Stalker (1942) or Levene (Levene, 1949; Malogolowkin-Cohen et al., 1965) because those indices require a decision about which sex exercises discrimination. We considered that decision to be arbitrary in *Desmognathus*.

We also measured mating propensity (equivalent to Gilbert and Starmer's [1985] *vigor*) according to Merrell (1950):

$$M_f = \frac{AA + BA}{BB + AB} \quad \text{and} \quad M_m = \frac{AA + AB}{BB + BA}$$

where M_f is the female mating ratio and M_m is the male mating ratio.

Statistical significance of measures of isolation and of mating propensities were evaluated using Chi-square tests where, for each test, expected values for both obser-

vations (numerator and denominator) were equivalent. Our design involved multiple trials for each individual and technically violated the assumption of independent observations for Chi-square tests. Courtship trials are independent, however, in that they involve unique male-female pairs. Also, because individuals of each sex in each sample concurred in their tendencies to mate with different types of partners, the violation was not a serious one.

RESULTS

Spermatophore deposition and insemination rates reveal nearly complete sexual isolation between *Desmognathus fuscus* from Mt. Rogers and both sympatric and allopatric *D. ochrophaeus* (Tables 1 and 2). In tests with sympatric populations, no inseminations occurred in 48 interspecific trials. In tests between allopatric populations, one trial in 48 resulted in an insemination. The incidence of interspecific courtships is very low and there is no evidence for a significant difference between sympatric and allopatric pairings. Significant differences in spermatophore depositions between *D. fuscus* and *D. ochrophaeus* (Table 1; Table 2, male and female mating ratios) indicate a greater mating propensity for *D. ochrophaeus*.

The tests revealed significant sexual isolation between Unaka and Mt. Rogers *D. ochrophaeus* (Tables 1 and 2). Isolation was evident when either Unaka or Mt. Rogers males were paired with females from the other population. There was no indication of population differences in propensity to mate, however, since neither the female mating ratio nor the male mating ratio was statistically significant. On the average, 71% (34 of 48) of control trials resulted in insemination, but only 29% (14 of 48) of interpopulation trials resulted in insemination.

DISCUSSION

Houck et al. (1985) found that courtship and insemination rates were higher in the spring than in the fall in a North Carolina population of *D. ochrophaeus*. The current experiment was staged in the winter and yielded courtship rates for *D. ochrophaeus*

TABLE 2. Tests for sexual isolation between *Desmognathus* from Unaka Mountain, Tennessee (UN); and Mt. Rogers, Virginia (MR). Results are based on the relative number of spermatophore depositions or inseminations per 24 courtship opportunities for each possible cross. *D. ochrophaeus* (O) occurs at both sites, but *D. fuscus* (F) occurs only at Mt. Rogers. I = Merrell's isolation value, M_f = female mating ratio, and M_m = male mating ratio. Statistical probability values (P) are NS = not significant, * ≤ 0.05 , ** ≤ 0.01 , or *** ≤ 0.001 .

	I	χ^2	P	M_f	χ^2	P	M_m	χ^2	P
Allopatric <i>D. ochrophaeus</i> (UN-O \times MR-O)									
Spermatophore deposition	0.38	11.66	***	0.87	0.28	NS	1.15	0.28	NS
Insemination	0.41	8.33	**	0.92	0.08	NS	1.29	0.75	NS
Allopatric <i>D. ochrophaeus</i> \times <i>D. fuscus</i> (UN-O \times MR-F)									
Spermatophore deposition	0.03	26.13	***	2.33	4.80	*	2.75	6.53	*
Insemination	0.04	23.15	***	2.00	3.00	NS	2.38	4.48	*
Sympatric <i>D. ochrophaeus</i> \times <i>D. fuscus</i> (MR-O \times MR-F)									
Spermatophore deposition	0.00	29.00	***	2.63	5.83	*	2.63	5.83	*
Insemination	0.00	24.00	***	2.00	2.67	NS	2.00	2.67	NS

intermediate between the fall and spring values reported by Houck et al. (1985).

In the present experiment *D. fuscus* showed lower rates of spermatophore deposition than *D. ochrophaeus* (homopopulation matings in Table 1). Additional work is needed to establish whether this is a species difference or a reflection of differing seasonal schedules for sexual activity.

We observed nearly complete sexual isolation between *D. fuscus* and *D. ochrophaeus*. Although we found one instance of insemination between allopatric *D. fuscus* and *D. ochrophaeus* and no instances between sympatric populations, a much larger experiment would be required to show that sexual isolation is enhanced in sympatry.

The degree of sexual isolation we observed between two *D. ochrophaeus* populations falls in the middle range of sexual isolation documented between races of widespread semi-species of *Drosophila* (Stalker, 1942; Levene, 1949; Ehrman, 1965; Malogolowkin-Cohen et al., 1965; Zouros and d'Entremont, 1980). This supports the idea that behavioral differences contributing to sexual isolation can evolve in allopatry.

Sexual isolation between races or species has rarely been studied in salamanders. Qualitative observations by Noble and Bra-

dy (1930) and Arnold (1976) suggested that species-specific odors from the skin of females was important in male courtship disinterest in heterospecific plethodontids. Recent tests for odor choice and courtship interest in *Plethodon* spp. support the contention that conspecific odors are important cues in sexual isolation (Dawley, 1984, 1986). In other work, Arnold (1976) demonstrated nearly complete sexual isolation between *Ambystoma maculatum* and *A. tigrinum* (Ambystomatidae). Kawamura and Sawada (1959) and Sawada (1963) conducted the most thorough investigation, studying isolation among geographic races of the Asiatic newt *Cynops pyrrhogaster* (Salamandridae). They found a full spectrum in degree of isolation, ranging from unencumbered intermating to nearly complete isolation. Kawamura and Sawada used the *Drosophila* protocol of mass mating trials in which males from both populations are simultaneously paired with females from both populations. In plethodontids, including *Desmognathus*, sexually active males are prone to aggressive interaction and female mimicry (Arnold, 1977; Houck, 1988), and consequently the "sequential choice" protocol may be more appropriate.

The protocol described here could be used to help resolve a number of issues in

the systematics and evolutionary biology of plethodontids. (1) Tests of sexual isolation could be a valuable adjunct to electrophoretic analysis of hybrid zones. It would be useful to know, for example, whether the hybridizing populations of *D. ochrophaeus* and *D. fuscus* studied by Karlin and Guttman (1981) show less sexual isolation than do sympatric, nonhybridizing populations. (2) Because numerous trials can be completed in a few nights, the protocol could be used to survey isolation among arrays of populations. Thus it should be possible to test for reinforcement of isolation in sympatric populations. (3) By combining assays of isolation with behavioral observations, it should be possible to unravel the causes of sexual isolation in plethodontids. Dawley (1984, 1986) has pioneered this approach and has shown that chemoreception is implicated in the sexual isolation of species of *Plethodon*. If chemoreception is likewise important in mate identification in *Desmognathus*, one would expect that sexual interactions between *D. ochrophaeus* and *D. fuscus* would cease after the initial chemoreceptive investigation of the female by the male.

Ideally, the contributions to reproductive isolation from various pre- and post-zygotic isolation should be assessed (Dobzhansky, 1937). To date it has not proved possible to assess post-zygotic isolation in plethodontids (due to the difficulty of inducing oviposition and of rearing embryos). We have, however, been able to implement a test for sexual isolation. While such tests do not give a complete picture of reproductive isolation, they do resolve one important mechanism. If broadly applied, tests for behavioral isolation could add another perspective to studies of reproductive isolation and its evolution in plethodontids.

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