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Kin Recognition and Incest Avoidance in Toads[1]

BRUCE WALDMAN AND JOHN E. RICE
Department of Organismic and Evolutionary Biology, Harvard University,
Cambridge, Massachusetts 02138

AND

RODNEY L. HONEYCUTT
Department of Wildlife and Fisheries Sciences, Texas A&M University,
College Station, Texas 77843

SYNOPSIS. Toads, like many amphibians, display breeding site fidelity. Individuals that return to natal ponds to breed are likely to encounter siblings as potential mates. We examined the genetic structure of *Bufo americanus* breeding populations at five localities within a 1 km radius. Mitochondrial DNA (mtDNA) haplotypes significantly differed among the breeding populations, but haplotype distributions did not differ from year to year within breeding sites. Natal philopatry may account for these results. Although incest is thus possible, siblings rarely mate. Comparing haplotypes of toads captured while mating, we found only 2 of 86 pairs that could possibly be siblings. We suggest that toads might recognize and actively avoid close kin as mates. Advertisement vocalizations given by males serve as potential cues by which females might recognize their kin. We recorded calls of males, in the field, and later obtained rank estimates of their relationships to one another by DNA fingerprinting of the nuclear genome. The resemblance of males' calls was positively correlated with the similarity of their fingerprints. Significant differences in call characteristics were also found among breeding populations. Temporal parameters, rather than frequency components, encode kinship information. Recently developed techniques in molecular genetics facilitate studies of the heritability of behavioral traits in natural populations.

INTRODUCTION

Anuran amphibians (frogs and toads) were among the first vertebrates shown to recognize collateral kin. Speculation that the conspicuous coloration and distastefulness of toad larvae (genus *Bufo*) evolved through kin selection (Wassersug, 1973; after Fisher, 1930; Hamilton, 1964) provided the initial impetus for studies of kin recognition (Waldman and Adler, 1979; Waldman, 1982). Further work established that kin-recognition abilities were not necessarily linked to aposematic traits. Larvae of numerous species—many palatable and cryptic—were found to recognize and associate preferentially with their siblings and other close kin (see reviews in Blaustein, 1988; Waldman, 1991; Blaustein and Waldman, 1992). A general functional model for kin recognition in anurans has proven elusive, but several adaptive scenarios have been proposed. Tadpoles may cooperate with siblings to facilitate growth and development, or they may more effectively deter or respond to predators (*e.g.*, Blaustein, 1988; Waldman, 1991). Yet tadpoles represent just the first stage in the complex life cycle of anurans, and the ability to discriminate between kin and non-kin might confer selective advantages during later stages. Indeed, experimental evidence that newly metamorphosed frogs associate with their siblings suggests that kin-recognition abilities might be retained through metamorphosis, later to be expressed as adults (Blaustein *et al.*, 1984; Cornell *et al.*, 1989; but see Waldman, 1989).

Natal philopatry can promote interactions among close kin, and may be common among amphibians (reviewed by Waldman and McKinnon, in press). The abilities of frogs, toads and salamanders to home after displacements over long distances are legendary (*e.g.*, Twitty, 1966). Grubb (1973*a*, *b*) showed that breeding Fowler's toads (*Bufo woodhousei fowleri*) and Mexican toads (*B. valliceps*) oriented toward odors from their home ponds in preference to those from other ponds, sometimes only a few meters

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away. Post-metamorphic dispersal and subsequent recruitment to breeding populations have been studied rigorously only during the past few years, however. Available evidence suggests that individuals often breed in the pond in which they metamorphosed. Most Fowler's toads, *Bufo woodhousei fowleri,* that Breden (1987) marked at metamorphosis and recaptured two years later, returned to their natal ponds to breed. Any migrants were usually found in adjacent ponds, and only a very few dispersed over longer distances. Similarly, Berven and Grudzien (1990) found that the largest number of marked wood frogs, *Rana sylvatica,* returned as breeding adults (79% of males and 87% of females) to their natal ponds. The remaining individuals usually dispersed to nearby ponds, but a few were recaptured as far as 2.5 km away (Berven and Grudzien, 1990).

Unless effective population sizes are huge, individuals that return to their natal pond to breed should encounter close relatives as potential mates. If breeding with close kin incurs genetic costs (inbreeding depression), then the ability to recognize siblings might be favored as a mechanism to avoid incest. The possible consequences of inbreeding on offspring viability have yet to be studied in amphibians, but many deleterious effects are associated with close inbreeding in other ectothermic vertebrates (Waldman and McKinnon, in press). In contrast, heterozygosity that results from outbreeding evidently confers important advantages. For example, Samallow and Soulé (1983) found that heterozyotes increased in frequency from autumn to spring in a natural population of western toads (*Bufo boreas*), presumably because they were better able to survive the winter. But outbreeding, like inbreeding, in principle can incur genetic costs: the breakup of coadapted gene complexes might lead to reduced viability and to the loss of local adaptations (Partridge, 1983). A preference for mates of intermediate relatedness then would be selected (Shields, 1982; Bateson, 1983). Philopatry, coupled with behaviorally mediated inbreeding avoidance, could effectively result in "optimal outbreeding."

In this paper, we provide an overview of our work on the genetic structure of natural breeding populations of the American toad, *Bufo americanus,* conducted over the past five years. Our goals have been to determine (1) the extent to which close kin are present together at breeding aggregations, (2) expected and observed frequencies of matings between siblings or other close relatives, and (3) possible mechanisms by which close kin might avoid mating with one another. Our results reveal that close kin are accessible as mates, but siblings rarely mate. Temporal characters of males' advertisement vocalizations appear genetically encoded or constrained. We suggest that females might choose non-siblings as mates based on their calls. Our findings demonstrate the versatility of recently developed molecular techniques in addressing questions concerning the heritability of behavioral traits in natural populations.

THE GENETIC STRUCTURE OF *BUFO AMERICANUS* BREEDING POPULATIONS

We studied populations of toads at five breeding ponds bordering the Estabrook Woods, a 650 acre mixed oak-birch forest in Concord, Massachusetts (Fig. 1). Although suitable breeding habitats are absent within the woods, toads are abundant there during the spring, summer and fall. During late April and May, depending on weather conditions, individuals migrate to these breeding sites at the edge of the woods. Population and breeding structure were intensively studied at three primary localities: Beecher Pond, Concord Center, and Mink Pond. Calling behavior was studied principally at Beecher Pond, Evans Pond, and Freeman Pond, with some additional recordings at Mink Pond. Distances between localities range from 0.8 to 2.2 km.

Breeding ponds were monitored nightly during April and May each year (1986–1990). We collected a total of 200 amplectant pairs of toads from the three primary localities, most after pairs had begun laying eggs. Because we sampled mated pairs, both sexes are equally represented in our data. To compare levels of genetic variation within and among populations, and to estimate the number of matings that occurred

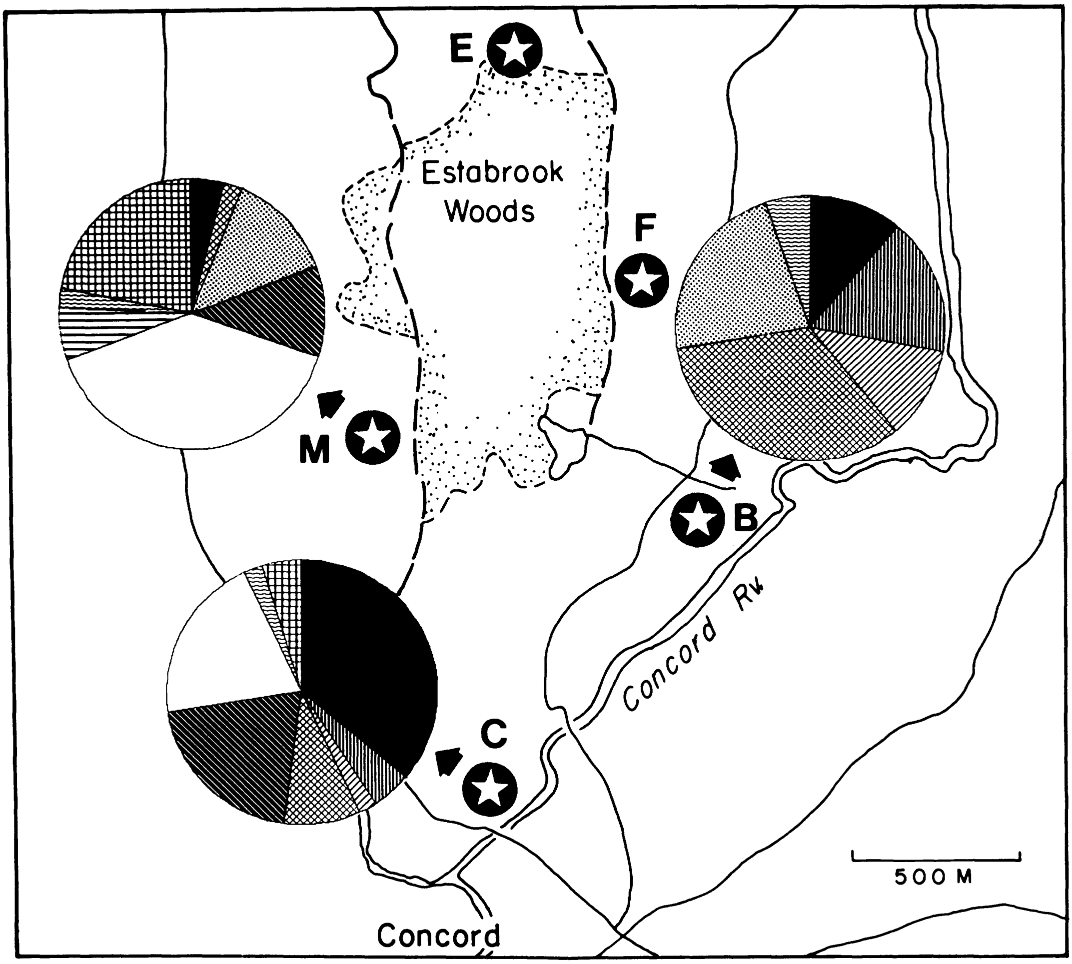

FIG. 1. Distribution of common mtDNA haplotypes among breeding individuals at three primary breeding sites surrounding the Estabrook Woods (*B*, Beecher Pond; *C*, Concord Center; *M*, Mink Pond). Advertisement vocalizations of males were recorded at Beecher and Mink Ponds, and two additional localities (*E*, Evans Pond; *F*, Freeman Pond). Frequencies of haplotypes (each denoted by a unique pattern) are shown in pie charts. Haplotypes were determined as composite restriction fragment length polymorphisms, based on digests with four restriction enzymes. See text for additional details.

between siblings, we conducted analyses of mitochondrial DNA (mtDNA) extracted from the pairs.

Many aspects of social behavior and population structure can be inferred by examination of restriction fragment length polymorphisms of mtDNA (Harrison, 1989). MtDNA is maternally inherited and is not subject to recombination (Dawid and Blackler, 1972), so siblings, as well as more distant matrilineal relatives, share identical fragment patterns. If individuals differ in mtDNA haplotypes, barring mutations, they cannot be siblings. The application of mtDNA to studies of population structure would be limited if particular haplotypes predominated, but often this is not so. In many vertebrates, mtDNA undergoes more rapid evolution than nuclear DNA. Consequently, mitochondrial markers can vary extensively among individuals, both within and between populations. Fishes, frogs, and reptiles have particularly variable mitochondrial genomes (Bermingham *et al.*, 1986; Avise *et al.*, 1989; Waldman *et al.*, in preparation), sometimes approaching hypervariable minisatellite regions of the nuclear genome in their diversity (used for genetic "fingerprinting"; Jeffreys, 1987).

Molecular methods.—MtDNA was isolated from fresh or frozen (stored at −75°C) liver tissue by ultracentrifugation in a cesium chloride-ethidium bromide gradient (Densmore *et al.*, 1985, as modified by Honeycutt *et al.*, 1987). Purified mtDNA was digested with a series of restriction endonucleases that cleave the mitochondrial genome at different recognition sequences. Based on a preliminary survey of 21 different restriction endonucleases (Waldman *et al.*, in preparation), six (*Acc*I, *Dde*I, *Hin*PI, *Mbo*I, *Msp*I, and *Taq*I) were chosen because of the high levels of genetic variation that they revealed in our populations. The digested mtDNA was end-labeled with $[^{32}P]$dNTPs using the Klenow large fragment DNA polymerase I (Brown, 1980). Labeled fragments were separated by electrophoresis on 1.2% agarose and 3.5% polyacrylamide gels. The fragment sizes were made visible by autoradiography as series of bands, and were measured by comparison to known standards run on the same gels.

Data analysis.—We assigned each individual a haplotype based on the composite patterns revealed by each enzyme. All six enzymes were used with 1986 and 1987 samples. Little additional variation was revealed by *Hin*PI and *Msp*I digests, so samples from subsequent years were digested only with *Acc*I, *Dde*I, *Mbo*I, and *Taq*I. For analyses of population structure, we include in this report only those 125 individuals for which we have unambiguously established haplotypes for digests with each enzyme. In examining mating, we include cases in which differences were evident between mates though haplotypes were incompletely resolved. Our inferences are conservative, while molecular analyses of the remainder of the data set still are underway.

We examined genetic differentiation among localities, and sex ratios of particular haplotypes within localities, by contigency-table analyses using chi-square tests. Due to the large number of haplotypes represented in our populations, expected frequencies were often too low to conduct standard tests. This problem is not uncommon in geographic studies of mtDNA variation, and the usual solution is to lump haplotype categories. Instead, we computed exact probabilities of goodness of fit, using the Monte Carlo simulation technique proposed by Roff and Bentzen (1989). Expected distributions of chi-square for each statistical test were derived by generating 1,000 randomizations of the haplotype distributions, holding row and column totals equal to those of the observed data. Statistical inferences then were made by comparing test statistics computed from the observed data with those computed from the repeatedly generated matrices (Roff and Bentzen, 1989).

Population differentiation

Thirty-one distinct mtDNA haplotypes were represented in the three breeding sites from which we collected mated pairs. Genetic differentiation among these populations was evident (for the 10 most common haplotypes, $\chi^2 = 74.94$, 18 df, $P < 0.0001$; see Fig. 1). Variation among years within localities was not significant (*e.g.*, for the 18 haplotypes comprising Mink Pond breeding populations over three years, $\chi^2 = 41.10$, 34 df, $P = 0.17$). Certain haplotypes consistently appeared in large numbers, year after year, at some localities but not others. Over three-quarters of 44 individuals present in temporary pools at Concord Center, south of the woods, belonged to one of three haplotypes (*A1, B2, B3*), and the remaining individuals comprised five other haplotypes. Of 18 toads spawning in the Beecher Pond due east of the woods (less than 1 km northeast of Concord Center), 6 haplotypes were represented. Neither haplotypes *B2* nor *B3*, two of the most common at Concord Center, were present in Beecher Pond. West of the woods, in Mink Pond (less than 1 km from Beecher Pond and Concord Center), 8 haplotypes were represented among 46 individuals, but again in proportions that differed from the other localities. Haplotype *C1*, for example, was found in Mink Pond but nowhere else.

The genetic differentiation evident among the three breeding populations results neither from an inability nor a disinclination of individuals to traverse distances such as those separating the ponds. The common European toad *Bufo bufo* sometimes migrates 3 km or further to breed (Heusser, 1969; Sinsch, 1987). Dispersal patterns of adult *Bufo americanus* appear similar (Blair, 1943; Oldham, 1966; Ewert, 1969). Maynard (1934) tracked an individual *B. americanus* that dispersed more than 4 km across land and water, and Blair (1943) recaptured one subject after it had swum across rapid currents to reach its breeding site. In our study, individuals inhabiting central areas of the woods needed to migrate comparable distances to reach a suitable breeding pool.

Natal philopatry might lead to genetic variation among breeding populations as demonstrated by our analyses of mtDNA haplotypes. In the absence of strong selection for local adaptation, even small numbers of migrants between populations can prevent particular alleles from becoming fixed within populations (*e.g.*, see Falconer, 1989; Hartl and Clark, 1989). Yet considerable levels of overall genetic differentiation should persist, especially in populations of small to moderate size (Wright, 1969; Breden, 1987). Most of our breeding populations ranged from 25 to 100 individuals, and effective population sizes were considerably less. Moreover, breeding success at some localities fluctuated enormously from year to year due to climatic and biotic effects on larval survivorship. These conditions should further amplify levels of genetic differentiation among nearby populations (Crow and Morton, 1955), in agreement with our molecular findings.

The large number of haplotypes that exist in our populations exceeds the typical number of females that mate at any of the primary localities. Differential recruitment to breeding sites, due simply to random sampling among the haplotype pool, might account, at least partially, for the observed patterns of microgeographic differentiation. Our failure to find significant differences in haplotype distributions within localities among years, however, suggests that toads disperse nonrandomly, and are likely to return to the same ponds to breed year after year. This breeding site fidelity probably contributes substantially to the patterns of variation observed among ponds. Increased numbers of matings between close kin are also expected due to philopatry.

Over larger geographic areas, genetic differentiation among populations increases. We found numerous novel haplotypes, not represented in any of the Concord breeding sites, when surveying *Bufo americanus* breeding populations in South Hadley, Massachusetts and Cranberry Lake, New York (100 and 300 km, respectively, from Concord) from 1987 through 1990. Divergence in haplotype patterns observed over these distances is consistent with the well-defined local population structure revealed by microgeographic studies.

Inbreeding

We computed the number of matings between close relatives (specifically, those sharing haplotypes) that would be expected if pair formation were random. Expected numbers of consanguineous matings were determined, for each locality and year, from the observed frequencies of haplotypes of individuals captured in amplexus. Factors that reduce the likelihood of matings between close relatives, such as skewed sex ratios (see below), thus are already reflected in these random expectations. Should mates share haplotypes, they might be siblings, but they also could be related through a more distant female ancestor.

Despite the imprecision with which we can determine ancestry, we conclude that incest is exceedingly rare. Of 86 mated pairs collected in the primary breeding populations, members of only two pairs had identical haplotypes. Randomly generated pairings of males with females, summed over ponds and seasons, led to a null expectation of 12 matings between individuals bearing identical haplotypes. Thus, fewer individuals mated with close relatives than would be expected if pairing were random ($P <$ 0.02, binomial test), but the expected frequencies are low.

The low incidence of matings between close relatives suggests that siblings recognize and avoid mating with one another. Even in the absence of kin recognition, however, few incestuous matings are expected. Curiously, individuals of like haplotype within ponds during any season more often than not were members of the same sex ($\chi^2 = 21.07$, 5 df, $P < 0.0001$; Table 1). Consequently, potential mates were less genetically similar, on average, than members of the population sampled at random. This result might come about if the sexes disperse asymmetrically, as in many other vertebrate groups (Greenwood, 1983; Pusey, 1987). Our data on spatial heterogeneity, while consistent with models of natal philopatry, provide evidence only of genetic partitioning among localities by females, as dispersing males do not pass mitochondria to their progeny. Available mark-and-recapture data on other anurans, however, fail to reveal any differences between tendencies of males and females to return to natal ponds to breed (*e.g.*, *Rana sylvatica*, Berven and Grudzien, 1990).

Numbers of sib matings also might be decreased by sex-biased aberrations in the age structure of breeding populations. In some anurans, including *Bufo* species, males first breed at a younger age than females, often a year earlier (*e.g.*, Hemelaar, 1981; Gittins, 1983). Substantial mortality between breeding seasons would then reduce the likelihood that siblings have an opportunity to mate with one another. In addition, environmental sex determination mechanisms have been reported in amphibians (Bull, 1983). If sex determination occurs early in development, siblings exposed to a common environment may be more likely than non-siblings to be of the same sex. Environmental sex determination thus may enforce some level of outbreeding in populations.

VOCALIZATIONS AS POTENTIAL KIN-RECOGNITION CUES

To the extent that the advertisement calls of *B. americanus* males are heritable, and thus encode information about the caller's genotype, they serve as potential kinship cues. Mate choice to avoid incest or to optimally outbreed might be possible if females can (1) detect this information and (2) evaluate their genetic relationship to the caller. Female mating preferences based on intraspecific variation in acoustic components of mating calls are widespread in anurans (reviewed in Gerhardt, 1988).

TABLE 1. *Sex ratios of common haplotypes (1987).*

Haplotype*	Males	Females
S1	2	5
S2	2	6
T1	8	2
T2	2	11
T3	8	0
U2	5	5

* Composite restriction fragment length polymorphisms (digests with full complement of six restriction enzymes).

Bufo americanus females appear often to have the opportunity to choose their mates (Licht, 1976; Howard, 1988; Sullivan, 1992; Waldman, personal observation). Male toads call, or clasp indiscriminately for females on the surface. Females are silent, and often swim underwater, out of reach of the clasping males. When approached by males, females often elude amplexus by diving. Females frequently orient toward particular males, especially when the males are calling. Subsequently, females may initiate amplexus by touching or even jumping onto males. Some variation may exist in the potential for female choice among populations or breeding localities, but to view females in explosive mating systems as having no chance to choose a mate (*e.g.*, Wells, 1977) is incorrect.

In analyzing variation in advertisement vocalizations within and among breeding sites, we addressed two questions. First, we examined variation in call structure among breeding sites to determine whether calls reflected the genetic divergence we had noted among populations. Second, we studied whether genetically more similar males, within ponds, give more similar advertisement vocalizations than non-relatives or distant relatives.

Sound analyses.—Advertisement calls of male toads were recorded at four breeding localities during 1989. Field recordings were made with a Marantz PMD420 tape recorder and a Sennheiser ME80 microphone (K3 power module), fitted when necessary with

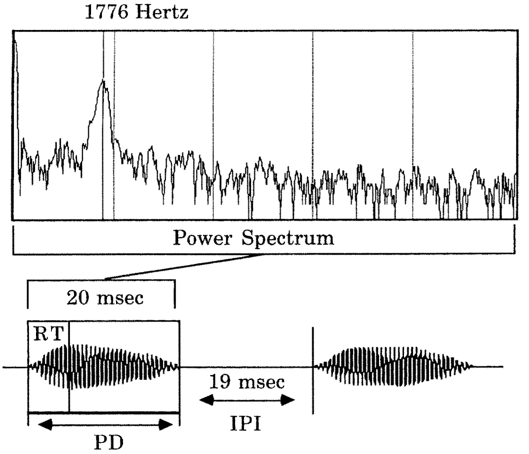

FIG. 2. Components of typical *Bufo americanus* advertisement call. Top: Power spectrum taken from 20 msec section, showing narrow frequency bands; 1776 Hz dominant frequency. Bottom: Oscillogram showing 2 pulses (energy *vs.* time); *RT,* rise time; *PD,* pulse duration; *IPI,* interpulse interval.

a windscreen. Calls were recorded at distances of 40 cm or less without any apparent disturbance to the calling toad. In many anurans, variation in temporal and frequency components of advertisement calls is attributable, in part, to ambient temperature and body size (see Sullivan, 1992, for a review of these effects in *Bufo americanus*). Thus, we measured cloacal temperatures of calling males with a Weber quick-reading thermometer immediately following each recording. Water and air temperatures at the calling site, and snout-vent length of each calling male, were also measured.

Three calls were analyzed for each male, and call parameters were measured at beginning, middle and end segments of each call. Fine temporal properties of the calls (pulse duration, interpulse interval, and pulse rise time; see Fig. 2) and dominant frequency (±1 Hz over 0.25-sec intervals) were analyzed using a DATA 6100 digital waveform analyzer, set at a 10 kHz sampling rate. Call durations also were measured with the waveform analyzer (±0.01 sec).

Molecular methods.—We estimated levels of genetic similarity among males within breeding localities by DNA "fingerprinting" of the nuclear genome. Fingerprinting involves the simultaneous detection of marker alleles at many loci, by use of multilocus probes that recognize tandemly repeated DNA sequences (minisatellite regions) dispersed throughout the nuclear genome. While the resultant fingerprint bands typically are inherited in Mendelian proportions (Jeffreys, 1987), Lynch (1988) has pointed out several problems with the use of DNA fingerprinting to obtain estimates of relatedness: (1) the upward bias of fingerprint similarity compared with relatedness due to limited numbers of marker alleles; (2) the need to establish empirically how fingerprint similarity corresponds to relatedness for each new population studied due to individual specificity in this bias; and (3) the sampling variance caused by confusion between bands identical by descent and those identical in state. These problems are inherent with the methodology, as contrasted with problems in sample preparation or analysis which can be resolved by refining experimental protocols (Lander, 1989). As Lynch (1988) emphasizes, however, these considerations do not reduce the usefulness and power of fingerprinting as an analytical tool to provide rank-order estimates of relatedness. Rank-order estimates are sufficient for the goals of this study.

Total genomic DNA was isolated from frozen (−75°C) liver tissue in CTAB buffer (Saghai-Maroof *et al.,* 1984) following procedures of Westneat (1990). In brief, DNA was extracted using Proteinase K followed by phenol-chloroform extractions and ethanol precipitation. The purified DNA was digested with *Hin*fI, the fragments were separated by electrophoresis on a 1% agarose gel, and then transferred to nylon membranes (Gene Screen Plus) by the Southern blot method (Southern, 1975). The DNA on the membranes was hybridized with Jeffreys 33.6 and 33.15 probes labeled with [^{32}P]dCTP by random priming (Feinberg and Vogelstein, 1984). Bands were visualized by autoradiography using intensifying screens.

Fifteen individuals were run on each gel, all from the same breeding locality. Between 20 and 50 bands typically were resolved for each individual, revealing extensive genetic variation. DNA fingerprint similarity between each pair of individuals was deter-

TABLE 2. *Mean call parameters in breeding localities* (±SD).

Pond	N	Pulse duration (ms)	Inter-pulse interval (ms)	Rise time (ms)	Call duration (s)	Dominant freq (kHz)
Beecher	15	22.2 (3.6)	22.6 (3.8)	7.8 (1.3)	14.3 (4.4)	1.60 (0.12)
Evans	16	23.9 (4.0)	23.7 (5.6)	7.9 (1.4)	12.2 (3.4)	1.56 (0.08)
Freeman	16	18.9 (3.2)	18.1 (3.8)	6.9 (0.9)	10.4 (3.4)	1.73 (0.12)
Mink	8	20.7 (2.3)	21.8 (3.8)	7.1 (1.2)	14.0 (3.0)	1.66 (0.09)

mined as the fraction of bands they shared. Matches were assigned only when bands overlapped exactly (using a grid system in which each band was marked to the nearest mm). Both probes yield similar indexes of genetic similarity, so combined estimates were used in the analyses presented in this paper.

Statistical methods.—Variation in each call parameter within and among individuals was evaluated by univariate analysis of variance for each breeding population. Variation in these parameters within and among breeding populations was analyzed by nested analysis of covariance, treating temperature and body size as covariates. Analyses were conducted with the General Linear Models procedure of SAS (releases 5.18, 6.03). Where significant regressions of call parameters on either covariate were found, these effects were removed from further investigations into the genetic correlates of call parameters (Mantel analyses; see below) by transforming data using the appropriate regression equation.

Comparisons between inter-individual differences in call parameters and DNA fingerprints were made following Mantel's (1967) method. For each comparison, two 15 × 15 matrices were constructed and compared, one describing the genetic relationships among all pairs and the other describing similarities in their calls. Genetic similarities were represented by S_{xy} values, computed as the number of shared fragments divided by the average number of fragments expressed for each pair of individuals. The resemblance of each pair's calls was represented, for each call parameter, as the absolute difference between their calls divided by their average. The statistical association of genetic relationships and call similarities was tested by comparing the sum of the cross products of the corresponding elements of the two matrices with those expected under a null hypothesis of random permutations using the Student's t-distribution (Douglas and Endler, 1982). Schnell *et al.* (1985) provide further details of the methods and applications of these statistical procedures.

Genetic similarity and call variation

Consistent with the genetic differences that we observed among localities, temporal properties of advertisement calls differed significantly among ponds (pulse duration, interpulse interval, pulse rise time, and call duration; see Tables 2 and 3) when temperature and size were held constant. Dominant frequency, however, did not significantly differ among ponds. Interpulse interval significantly differed among ponds only during the beginning segments of calls, whereas rise time significantly differed only during the end segments of calls.

Males had individually distinctive calls.

TABLE 3. *Analysis of covariance of call parameters among breeding localities.*

Dependent variable	Pulse duration	Interpulse interval[a]	Rise time[b]	Call duration	Dominant frequency
Pond	$P < 0.0001$	$P < 0.03$	$P < 0.02$	$P < 0.006$	ns
Temperature	ns	ns	ns	$P < 0.0005$	$P < 0.0002$
Body size	ns	$P < 0.05$	$P < 0.007$	$P < 0.012$	$P < 0.03$

[a] Interpulse interval: variation among ponds, and regression on body length, significant only during beginning call segments.

[b] Rise time: variation among ponds significant only during ending call segments; regression on body length significant only during middle call segments.

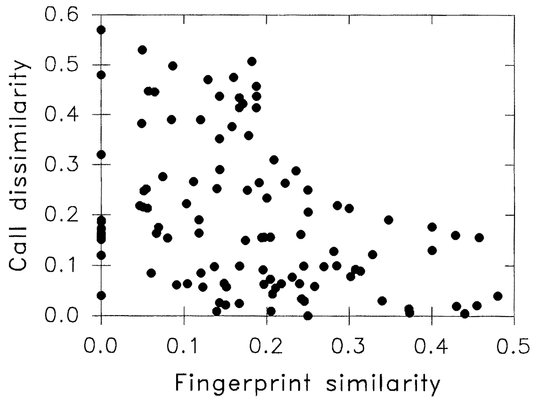

FIG. 3. Call dissimilarity in pulse duration as a function of genetic similarity among calling males sampled from Evans Pond. Identical calls have a dissimilarity value of 0, and increasingly dissimilar calls have higher values. Fingerprint similarity values increase with relatedness (r) and inbreeding (F) coefficients.

Within ponds, for every call parameter that we measured, variation among individuals was significantly greater than within individuals ($P < 0.0001$ for each comparison). Moreover, variation in temporal parameters of calls between males corresponded significantly with their genetic similarity. Genetically similar individuals (quite likely brothers) had calls that were similar in their temporal properties, whereas calls of genetically dissimilar individuals (non-relatives) were much more variable (Figs. 3–5).

Mantel analyses revealed that pulse duration, interpulse interval, pulse rise time, and call duration were nonrandomly associated with genetic similarity (Table 4). Pulse duration and interpulse interval were more similar among relatives in each of the three primary breeding sites. Pulse rise time and call duration showed significant statistical associations with genetic similarity in two of the three populations. No relationship was found at any locality between dominant frequency and genetic similarity. Further analyses of temporal parameters suggest that the same call features that significantly vary among populations are influenced by genetic differences within populations. For example, interpulse interval appeared most strongly influenced by genetic similarity during the beginning segments of calls, whereas genetic correlations with pulse rise time were strongest during the end segments of calls.

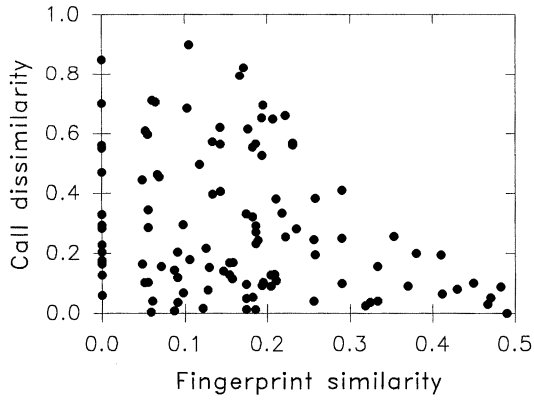

FIG. 4. Call dissimilarity in interpulse interval as a function of genetic similarity among calling males sampled from Freeman Pond.

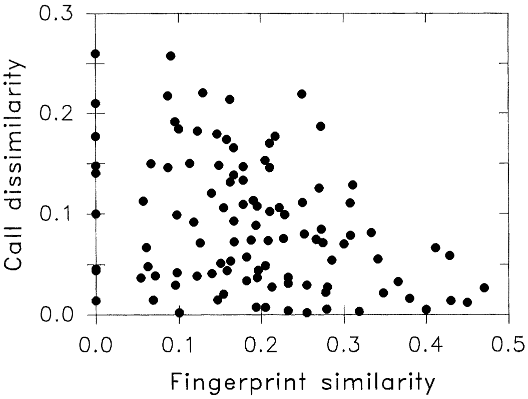

FIG. 5. Call dissimilarity in rise time as a function of genetic similarity among calling males sampled from Beecher Pond.

DISCUSSION

The tendencies of anuran amphibians to return to their natal ponds to breed can lead to substantial levels of genetic partitioning among nearby breeding sites, as we have shown here. Philopatry may confer selective advantages, for example, by ensuring that eggs are deposited in a hospitable environment or by fostering local adaptation. But philopatry also increases the likelihood that individuals will mate with close relatives and suffer consequences of inbreeding depression. Yet, our results demonstrate that matings between close relatives are surprisingly rare. Incest avoidance may be facili-

TABLE 4. *Genetic determinants of call differences (Mantel analyses).*

Pond	Pulse duration	Interpulse interval	Rise time	Call duration	Dominant frequency
Beecher	$P < 0.005$	$P < 0.03$	$P < 0.01$	$P < 0.05$	ns
Evans	$P < 0.03$	$P < 0.05$	$P < 0.05$	$P < 0.01$	ns
Freeman	$P < 0.03$	$P < 0.03$	ns	ns	ns

tated both by behavioral and population-level mechanisms. Even while toads avoid mating incestuously, they might be mating with more distant relatives, and thus may achieve benefits associated with "optimal outbreeding" (Bateson, 1983).

While we have established that information about the genetic relationships between males is encoded in their advertisement calls, we do not know whether females respond to these cues or possibly to other genetically based cues (*e.g.*, odors). The overall similarity of calls of males at a breeding site provides an indication of their average relatedness, which can influence levels of cooperation and competition in complex ways (Waldman, 1988). If males in a pond are indeed brothers, then offspring of different females will be cousins. Larval fitness can be enhanced by schooling with close relatives (Waldman, 1991), and when the benefits are sufficient, kin altruism might well extend to cousins. By mating in ponds in which male advertisement calls are relatively homogeneous, females increase the likelihood that their offspring will interact with close kin. Further, if kin altruism is important, we speculate that females should choose males with the most common call type. Frequency-dependent mate choice, in turn, might drive selection for philopatry, as males that associate with brothers or other close kin at mating ponds would most often be chosen by females. Convergence of advertisement calls on the most common type also would be expected among non-kin, although the extent to which genetically constrained calls can be influenced through social experience needs to be investigated (*e.g.*, see Wagner, 1989).

How females would assess their own relatedness to specific calling males is more problematic. First, although calls of close relatives have very similar temporal characteristics, so too do calls of some non-relatives (Figs. 3–5). Thus to avoid mating with close kin, females need to choose a male whose call differs as much as possible from those of their brothers. This conclusion must be considered tentative, however, as our analyses of particular call characters may fail to fully reveal differences among males. Even if two non-relatives share a particular call character, the overall structure of their calls may still differ considerably. Moreover, temporal parameters of the call are likely correlated. Multivariate comparisons of vocalizations of kin and non-kin are needed to determine how frequently calls of non-kin are as similar as those of close kin.

Second, we do not know whether females can compare males' calls either with a genetically based template or one acquired through learning. Females presumably use innate templates for species recognition. These templates, if specifically correlated with brothers' call characteristics, could act, in principle, to effect kin recognition. No animals studied to date, however, appear to use such a genetically based system to recognize their kin (Waldman, 1987). And female toads appear to lack a contextually reliable opportunity to learn characteristics of their brothers' calls. Tadpoles aggregate in sib groups (Waldman, 1982) and may even metamorphose with their siblings and depart the pond together with them. Yet at this stage, males do not vocalize, and if toadlets recognize kin (which is uncertain), kin recognition probably occurs by olfactory cues (*e.g.*, Blaustein *et al.*, 1984). Some anurans are known to produce spurious advertisement vocalizations in the autumn (*e.g.*, Zweifel, 1989), and if siblings disperse together, females might learn their brothers' calls during this period. Should siblings overwinter and migrate to breeding sites in the spring together, calls might be learned en route to the pond or upon arrival there. Yet these possibilities seem remote.

Perhaps siblings initially recognize one another through other cues, such as odors, that they had previously learned (*e.g.*, at metamorphosis) and then selectively learn their calls.

Males should be able to recognize their brothers and other close male kin by directly comparing those males' calls with their own ("phenotype matching"; see Getz, 1981; Waldman, 1981, 1987; Holmes and Sherman, 1982; Lacy and Sherman, 1983). Potentially, brothers might cooperate in attracting females or they may show restraint in competing for them. We envision several means by which calling males might benefit. Male toads often call synchronously, though this probably hinders the ability of females to make a choice (Schwartz, 1987). Yet brothers that call in unison may be more attractive together because the similar temporal characters of their calls reinforce each other in an additive manner (Roederer, 1975). Calling males might even attract females to particular locations within a pond where they are more likely to be clasped by the males' non-calling brothers. This tactic would be most effective when males are present in high densities because relatively fewer males call (Höglund and Robertson, 1988; Waldman, personal observation). Or by listening to each other's calls and moving away from calls that sound similar to their own, brothers might avoid competing with one another for access to mates.

Advertisement vocalizations differed significantly among our breeding populations in concordance with the genetic differentiation we observed. Although geographic variation in mating calls of anurans has long been noted (*e.g.,* Capranica *et al.,* 1973), only recently has such variation been investigated on a more fine-grained scale. Substantial differences in call characters between populations of cricket frogs (*Acris crepitans*) within 65 km of one another but in very different ecological settings were noted by Ryan and Wilczynski (1988). More detailed studies reveal variation among populations, some considerably closer, in virtually every call character measured (Ryan and Wilczynski, 1991). Our results suggest that calls may vary substantially on a microgeographic scale, over distances of 1 km or less, even under extremely similar environmental conditions. We are currently conducting female choice tests to determine whether individuals preferentially mate with members of their own population, or even more specifically, whether they discriminate between vocalizations of close kin and other members of their population. Such preferences would further restrict gene flow and reinforce genetic differentiation among nearby breeding sites.

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