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Evolution

Directional introgression of mitochondrial DNA in a hybrid population of tree frogs: The influence of mating behavior

(interspecific hybridization/restriction enzymes/*Hyla*)

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ABSTRACT **A total of 305 individuals from a hybrid population of North American tree frogs was characterized for allozyme and mitochondrial DNA (mtDNA) genotype. Species-specific mating behaviors had suggested the potential for directional hybridization, in which matings between *Hyla cinerea* males and *Hyla gratiosa* females numerically predominate over the reciprocal combination. Such directional bias leads to predictions about expected distributions of the female-transmitted mtDNA markers in F_1, backcross, and later-generation hybrids. These predictions were fully confirmed by the observed distributions of mtDNA genotypes among these allozymically inferred hybrid classes. Results exemplify the significance of stereotyped mating behaviors in determining the genetic architecture of a hybrid population.**

Rapid nucleotide-sequence evolution and maternal inheritance characterize the mitochondrial genomes of higher animals (1, 2). Thus, genetic markers provided by mitochondrial DNA (mtDNA) can facilitate determination of the female parentage of organisms in nature, a goal that has previously proved impossible to achieve from analyses of nuclear genes or their products. For example, Brown and Wright (3) used patterns of mtDNA restriction-site variation to show that the whiptail lizard *Cnemidophorus tigris* was the maternal parent for the parthenogenetic derivatives *Cnemidophorus neomexicanus* and *Cnemidophorus tesselatus*. mtDNA has been similarly applied to other hybridization systems involving sexually reproducing (4) and hybridogenetic (5) species.

One potentially fruitful area for utilization of mtDNA data involves study of mating behaviors in hybrid zones where interspecific matings may be difficult to observe directly. Reproductive behaviors are often species specific and sufficiently stereotyped (26) to allow predictions on the direction of interspecific crosses. The major purpose of this investigation is to use genetic information to analyze mating behavior in a well-known hybrid population where mating bias has been proposed to exert an influence on direction of introgression.

Hyla cinerea and *Hyla gratiosa* are distributed widely and sympatrically throughout the southeastern United States. They exhibit a certain degree of habitat isolation with respect to breeding sites, as *H. gratiosa* utilizes temporary ponds extensively while *H. cinerea* is generally associated with permanent lentic systems. Nonetheless, many breeding sites are shared, and hybridization between *H. cinerea* and *H. gratiosa* is known to occur at least sporadically (6). Hybrid locales have been correlated with recent environmental disturbance (6), and in one series of artificial ponds near Auburn, Alabama, extensive introgressive hybridization has continued (7) for >20 years since its initial discovery (8).

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General behavioral observations at the Auburn site (7, 8) suggest the potential for a numerical bias in the direction of interspecific matings. During the breeding season, *H. gratiosa* males call from the water surface at various distances from shore, whereas *H. cinerea* males typically call from elevated perches in shoreline vegetation. Much vegetation has been eliminated at the Auburn site by regular mowing around the pond margins, and many *H. cinerea* now issue calls at ground level within the maintenance perimeters. Gravid females of both species approach the ponds from surrounding woods and initiate amplexus (9, 10). Therefore, most interspecific matings might involve *H. cinerea* males and *H. gratiosa* females: the latter must encounter a "barricade" of *H. cinerea* males before reaching conspecific partners, while most *H. cinerea* females are likely to be in amplexus upon entering the ponds.

To genetically characterize the magnitude and pattern of hybridization and introgression between *H. cinerea* and *H. gratiosa* at this locale, we have studied restriction-site variation in mtDNA as well as multilocus allozyme genotypes in 305 individuals. Do most interspecific matings indeed involve *H. cinerea* males and *H. gratiosa* females? If so, what effect can such a mating bias have on the cytoplasmic and nuclear gene architectures of an introgressed population?

MATERIALS AND METHODS

Field research was conducted at the Auburn Fishery Research Ponds where four collecting sites were chosen from a complex of 38 man-made ponds. Sites were visited 3–7 nights each week from April to mid-August 1984. Frogs were collected by hand and the date, location, and call (or amplexus) site were recorded. Calling males composed the majority of our samples, although amplectant pairs were taken occasionally.

Serum and kidney samples were taken from doubly pithed frogs and stored at −80°C for allozyme analysis. Five marker loci, previously established for the Auburn site (7), were assayed by using horizontal starch-gel electrophoresis with modifications by Gerhardt *et al.* (6). Serum consistently provided the best results for albumin (*Alb*) and phosphoglucoisomerase (*Pgi-2*), while kidney was more effective for lactate dehydrogenase (*Ldh-2*), malate dehydrogenase (*Mdh-1*), and peptidase (*Pep-1*).

Fresh heart, liver, and abdominal musculature were combined to yield sufficient quantities of mtDNA. Closed circular mtDNA was isolated from tissue homogenate by differential centrifugation and purified by CsCl/ethidium bromide gradient centrifugation (11). Four marker restriction endonucleases (*Ava* I, *Hin*dIII, *Nde* I, *Xba* I) were selected from 15 previously used to examine sequence divergence among certain North American *Hyla* (12). The digestion profiles produced by these enzymes are diagnostic for *H. cinerea* and *H. gratiosa*, differing by two or more restriction-site changes for each endonuclease. At least two of the four endonucleases were used to assay any mtDNA sample. Digestion

fragments were end-labeled with the appropriate α-^{32}P-labeled nucleotide(s) and electrophoresed through 1% agarose gels (13). Digestion profiles were revealed by autoradiography (13). Fragment sizes were compared against the 1-kilobase ladder standard available from the Bethesda Research Laboratories.

RESULTS

For the collection of 305 tree frogs considered as a unit, allozyme genotype frequencies show significant excess homozygosity, relative to Hardy–Weinberg expectations, at all five loci (*Alb*, $\chi^2 = 95.3$; *Ldh*-2, $\chi^2 = 89.1$; *Mdh*-1, $\chi^2 = 82.9$; *Pep*-1, $\chi^2 = 203.2$; *Pgi*-2, $\chi^2 = 90.9$; all χ^2 values associated with $P < 0.001$). These results are attributed to the persistence of both parental species despite hybridization and are in accord with previous morphological (8) and electrophoretic (7) observations. Nonetheless, hybrid frogs are clearly common at the Auburn site. For example, 20 individuals (6.5% of the population) appear to be F_1 hybrids, and a total of 142 frogs (46.5%) exhibit some evidence of mixed ancestral parentage (Table 1).

On the basis of multilocus allozyme genotype, each frog was provisionally assigned to one of six parental or hybrid categories: (*i*) pure *H. cinerea*, (*ii*) pure *H. gratiosa*, (*iii*) F_1 hybrid, (*iv*) *H. cinerea* backcross, (*v*) *H. gratiosa* backcross, and (*vi*) later-generation hybrid. Group criteria and the number of individuals in each category are presented in Table 1. For example, presumptive F_1 hybrids are heterozygous at all five marker loci; and presumptive *H. gratiosa* backcrosses (backcross generation unspecified) are heterozygous at some loci and homozygous for *H. gratiosa* alleles at others. Category *vi*, later-generation hybrids, is composed of individuals alternately homozygous for *H. cinerea* and *H. gratiosa* alleles at various loci. These individuals may represent F_2 or later-generation hybrids, or products of matings among heterozygous backcross tree frogs. We recognize that these categories may inevitably include a few misclassifications. For example, the probability that a presumptive F_1 hybrid (heterozygous at five unlinked marker loci) is truly a first-generation backcross in either direction is 1/32 (0.031).

Table 1. Allozyme category and classification criteria

Provisional category	*n*	% of total population	Group criteria
Pure *H. cinerea*	103	33.8	Homozygous for *H. cinerea* alleles at all marker loci
Pure *H. gratiosa*	60	19.7	Homozygous for *H. gratiosa* alleles at all marker loci
F_1 hybrid	20	6.5	Heterozygous for *H. cinerea*/*H. gratiosa* alleles at all marker loci
H. cinerea backcross (generation unspecified)	58	19.0	Homozygous for *H. cinerea* alleles at 1 to 4 loci, heterozygous *H. cinerea*/*H. gratiosa* alleles at remaining loci
H. gratiosa backcross (generation unspecified)	53	17.4	Homozygous *H. gratiosa* alleles at 1 to 4 loci, heterozygous *H. cinerea*/*H. gratiosa* alleles at remaining loci
Later-generation hybrid	11	3.6	Homozygous for *H. cinerea* alleles at one or more loci, homozygous for *H. gratiosa* alleles at one or more other loci

Nonetheless, as we will show, this classification scheme is conservative and does allow relevant biological interpretations of the mtDNA data.

Some individuals in each backcross category probably represent later generations of backcrossing, as suggested by the data in Table 2, which documents an observed excess of single-locus heterozygotes (and a corresponding deficit of three-locus and, in the case of *H. gratiosa* backcrosses, four-locus heterozygotes) relative to expectations. For this reason as well, we cannot critically utilize the backcross categories to establish genetic maps for the marker loci. [The genes are probably not tightly linked because recombinant genotypes for all possible pairs of loci were observed in high frequency (ranging from 35% to 69%) among the presumptive backcross individuals.]

Hyla cinerea and *H. gratiosa* have been shown to be highly divergent in mtDNA, with estimated nucleotide sequence divergence of $P \cong 0.19$ (12). mtDNA digestion profiles produced by the four "marker" restriction endonucleases in the Auburn population appear identical to those previously reported in *H. cinerea* and *H. gratiosa* from South Carolina (12). These are pictured diagrammatically by Kessler (13), and an example of the gel pattern for *Nde* I is shown in Fig. 1. For convenience, the mtDNA genotypes characteristic of *H. cinerea* and *H. gratiosa* are hereby designated *C* and *G*, respectively. Species assignments based on mtDNA digestion profiles were in all cases perfectly concordant across enzymes. Furthermore, there was no evidence of paternally derived heteroplasmy in any of the individuals from the four hybrid categories. Although large-scale size heteroplasmy was observed in both "pure" parentals as well as hybrids (28), none of the heteroplasmic profiles reflected a combination of *C* plus *G* mtDNAs. These data lend support to previous studies that dismiss paternal leakage as a probable source of heteroplasmy (15, 27).

Associations between electromorph category and mtDNA genotypes are shown in Table 3. Of the four hybrid classes, the F_1s provide the most critical test for predictions on the nature and direction of interspecific matings. All 20 F_1 hybrids possessed *G* mtDNA (Table 4). These results are in complete agreement with the prediction that most interspecific matings involve *H. cinerea* males and *H. gratiosa* females.

In light of these findings, mtDNA genotype distributions can be predicted for the backcross and later-generation hybrids. Progeny from backcrosses to *H. gratiosa* should possess exclusively *G* mtDNA, since either cross combination (F_1 ♂ × *H. gratiosa* ♀ or F_1 ♀ × *H. gratiosa* ♂) will yield offspring with *H. gratiosa* mitochondria. However, progeny from backcrosses to *H. cinerea* could exhibit the mtDNA of either parental species. Under the simplest assumption, that reciprocal matings (F_1 ♂ × *H. cinerea* ♀ vs. F_1 ♀ × *H. cinerea* ♂) are equally likely, ≈50% of the *H. cinerea*

Table 2. Frequency distributions of heterozygous allozyme loci in presumptive backcross progeny

	No. of marker loci heterozygous				
	1	2	3	4	χ^2
	H. cinerea backcross ($n = 58$)				
Observed	21	16	8	13	21.5
Expected*	9.7	19.3	19.3	9.7	$P < 0.001$
	H. gratiosa backcross ($n = 53$)				
Observed	23	16	7	7	29.9
Expected*	8.8	17.7	17.7	8.8	$P < 0.001$

*Under the assumption that all individuals are first-generation backcrosses, and corrected for the absence of zero- and five-locus heterozygotes, which would have been scored as "pure" parental species or F_1 hybrids, respectively.

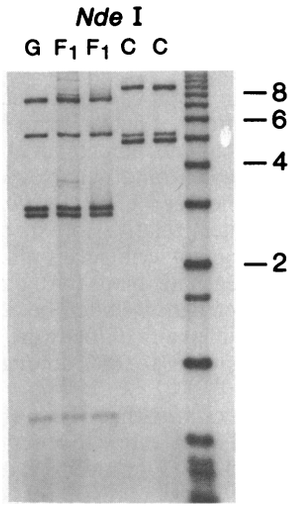

FIG. 1. Example of *Nde* I fragment profiles for mtDNA in *H. cinerea* (lanes C), *H. gratiosa* (lane G), and F_1 hybrids. Selected sizes (in approximate kilobase pairs) of fragments in the molecular weight standard are indicated to the right.

backcrosses should carry *G* mtDNA. Among later-generation hybrids, both mtDNA types should also be observed in frequencies generally biased toward *G* but numerically dependent on exact mode of hybrid production.

All of the genetic results are remarkably consistent with the expectations based on suspected mating propensities (Table 3). Thus, 52 of 53 (98%) presumptive *H. gratiosa* backcross individuals exhibit *G* mtDNA, as do 22 of 58 (38%) presumptive *H. cinerea* backcrosses. Among later-generation hybrids, 9 of 11 individuals (82%) possess *G* mtDNA. Overall, among the total of 142 hybrids, 103 (72%) carry *G* mtDNA, evidencing the collective genetic effects of apparent directional hybridization in the Auburn population.

Given the extensive genetic exchange at the Auburn site and the length of time over which hybridization has occurred (at least 25 years), one might expect some pure *H. cinerea* to contain the mitochondrial genome of *H. gratiosa*. The establishment of heterospecific mtDNA across species boundaries has been documented for other organisms under somewhat similar circumstances (5, 16). Yet for these *Hyla*, the mtDNA genotypes were in perfect agreement with the respective parental groups defined by allozyme genotype (*n* = 163). This fact, in conjunction with the limited number of later-generation hybrids, probably indicates that hybrids experience some selective disadvantage, although we have no direct evidence on this matter. Similar conclusions were drawn by Schlefer *et al.* (7) to explain the continued presence of pure parentals at the Auburn location.

Table 3. Associations between allozyme category and mtDNA genotype

Allozyme category	*G* mtDNA		*C* mtDNA		
	Observed	Expected*	Observed	Expected*	Total
Pure *H. cinerea*	0	—	103	—	103
Pure *H. gratiosa*	60	—	0	—	60
F_1 hybrid	20	20	0	0	20
H. cinerea backcross	22	29	36	29	58
H. gratiosa backcross	52	53	1	0	53
Later-generation hybrid	9	Some†	2	Some†	11

*Under the simple behaviorally motivated assumption that interspecific crosses are in the direction *H. cinerea* ♂ × *H. gratiosa* ♀ and that to a given backcross category F_1 hybrids of both sexes have contributed equally (see text).

†Both *G* and *C* mtDNA genotypes are expected among later-generation hybrids, but relative frequencies are probably dependent on many additional factors of unknown importance. Generally, some numerical bias in favor of *G* mtDNA would be anticipated.

DISCUSSION

Alternative Explanations. Although the F_1 mtDNA identifications offer strong support for the prevalence of crosses in the direction *H. cinerea* ♂ × *H. gratiosa* ♀, no such matings were observed directly. Is it possible that other phenomena, apart from mating behaviors, may account for our results? One alternative explanation is differential hybrid viability. In principle, direction of parental cross might influence hybrid development such that only the *H. cinerea* ♂ × *H. gratiosa* ♀ cross yields offspring with normal viability. However, in laboratory experiments, Mecham (8) found that viability is high in both interspecific crosses and equivalent to parental controls, although *H. gratiosa* ♂ × *H. cinerea* ♀ crosses appeared to have a slightly higher hatching success (≈98%) than did *H. cinerea* ♂ × *H. gratiosa* ♀ crosses (≈87%). These hatching differences are small and even in the wrong direction to account for the observed preponderance of *G* mtDNA among the F_1 hybrids.

Another alternative to account for the observed distribution of mtDNA genotypes involves chance sampling error from a small number of hybridization events. If most or all of the 20 F_1 hybrids are full sibs, general conclusions about mating behaviors producing these hybrids would be unwarranted. Two lines of evidence argue against this possibility. First, overall survival in anuran larvae is usually quite low (often <10%) in species without parental care (17–19). It thus seems unlikely that a large number of progeny would survive from any one mating. While factors including egg mass shape and oviposition site may increase the variance in egg and embryo survivorship across clutches in some species (20, 21), such an outcome is not likely for *H. gratiosa* because eggs are deposited singly about the pond (22). Second, we have utilized a fairly direct age determination process [counts of growth rings in phalanges (23)] to document the presence of several age classes among our sample of F_1s. Each phalangial ring corresponds to a period of arrested growth (such as a winter season), although the potential for ring resorption in older individuals makes absolute age assignments questionable (23). In any event, ring numbers ranged from 2 to 5 among our F_1 hybrids (Table 4). When other factors such as locality data (four ponds) and survivorship are included, a conservative estimate is that our 20 F_1 hybrids represent products of at least 10 independent matings.

Behavioral Explanations for Directional Hybridization. Call site preference in *H. cinerea* and *H. gratiosa* has been well documented (8–10) and apparently contributes to reproductive isolation in natural situations (10) by segregating calling males and minimizing interspecific contact. However, as already mentioned, regular mowing of the Auburn pond margins has eliminated the shoreline vegetation from which *H. cinerea* normally call. In response to their modified environment, the calling males assume one of two behavioral patterns. Some *H. cinerea* continue to select elevated perches located some distance (5–10 m) from shore. Others now choose shoreline sites, even though this necessitates calling from the ground. The latter response places *H. cinerea* and *H. gratiosa* in close proximity and increases the potential for physical contact. Mecham (7) viewed the artificial nature of the ponds as the primary factor in hybridization.

Nonetheless, opportunity for interspecific contact may be an insufficient condition for interspecific mating. Oldham and

Table 4. Allozyme and mtDNA genotypes for presumed F_1 hybrids from the Auburn ponds

Hybrid individual	Pond*	Snout–vent length, mm	Phalangial ring number	Allozyme genotype†					mtDNA genotype			
				Alb	*Ldh-2*	*Mdh-1*	*Pep-1*	*Pgi-2*	*Ava* I	*Hind-* III	*Nde* I	*Xba* I
1	F6	58	3	100/104	10/100	100/112	92/100	−100/−160	—	G	G	—
2	F6	55	2	100/104	10/100	78/100	92/100	−100/−160	—	G	G	—
3	F6	58	3	100/104	10/100	78/100	92/100	−100/−160	—	G	G	—
4	S19	67	4	100/104	10/100	100/112	92/100	−100/−160	G	—	G	—
5	S19	60	3	100/104	10/100	78/100	92/100	−100/−160	—	G	G	—
6	S19	53	2	100/104	10/100	100/112	92/100	−100/−160	—	G	G	—
7	F6	56	2	100/104	10/100	78/100	92/100	−100/−160	—	—	G	G
8	F6	56	2	100/104	10/100	78/100	92/100	−100/−160	G	—	G	—
9	F6	63	5	100/104	10/100	78/100	92/100	−100/−160	—	G	G	—
10	F6	57	2	100/104	10/100	78/100	92/100	−100/−160	—	G	G	—
11	F6	50	2	100/104	10/100	78/100	92/100	−100/−160	—	G	G	—
12	F6	56	2	100/104	10/100	78/100	92/100	−100/−160	—	G	G	—
13	S19	60	3	100/104	10/100	78/100	92/100	−100/−160	—	G	G	—
14	F6	56	3	100/104	10/100	78/100	92/100	−100/−160	—	G	G	—
15	S20	65	4	100/104	10/100	78/100	92/100	−100/−160	—	G	G	—
16	S19	62	3	100/104	10/100	78/100	92/100	−100/−160	—	G	G	—
17	F8	59	3	100/104	10/100	78/100	88/100	−100/−160	—	G	G	—
18	F8	59	3	100/104	10/100	78/100	88/100	−100/−160	—	G	G	—
19	F8	60	3	100/104	10/100	78/100	92/100	−100/−160	—	G	G	—
20	F8	57	3	100/104	10/100	100/112	92/100	−100/−160	—	G	G	—

*Official pond names designated by the Auburn Fishery Research Unit.
†Electromorphs are labeled by mobility relative to the *H. gratiosa* allelic product designated 100 (or −100 for cathodal migration).

Gerhardt (10) demonstrated mating call discrimination by females of both species. Using recordings in a series of two-stimulus playback experiments, they found that females consistently selected conspecific over heterospecific calls. A revised scenario that augments Mecham's habitat/call site model may better explain the observed levels and patterns of genetic exchange.

Satellite behavior is a relatively common mating strategy in *H. cinerea*, where noncalling "satellite" males sit next to calling males and attempt to intercept females (24). Perrill *et al.* (24) found that some attempts are met with success and that up to 18% of the calling males may have accompanying satellites. Furthermore, they observed that "the satellite male moved rapidly toward the female, presumably as soon as he detected her movements" and that satellites "also pursued other males which moved into the vicinity." Apparently, a quick decisive response toward approaching frogs is crucial to the satellite's success. As a byproduct of this strategy, it seems feasible that *H. cinerea* satellite males might also intercept *H. gratiosa* females en route to one of the Auburn ponds, with amplexus ensuing.

Censuses taken at one of the Auburn ponds indicate that satellite males are associated with ≈13% of the *H. cinerea* calling within the maintenance perimeter (25). No *H. gratiosa* males were seen enacting satellite roles, nor are there any accounts of such behavior in the literature. Thus, we suggest that habitat alteration establishes a requisite setting, while *H. cinerea* satellites may be the proximate force in directional hybridization responsible for the observed patterns of genetic interchange.

As an example of a general approach to the genetic analysis of hybrid zones, our results are significant in several respects. First the allozyme data document the magnitude of effective hybridization and introgression by tallying the numbers of probable hybrid categories of nonparental genotypes (Table 1). Second, the mtDNA data allow determination of the female ancestries of particular hybrids. Third, and most important for our purposes, the joint distributions of mtDNA/allozymes allow strong inferences about the mating behaviors responsible for hybrid production. In this case, a behaviorally based asymmetry in direction of interspecific hybridization has had dramatic consequences on the pattern of introgression that would not have been apparent from data on nuclear genes alone. The joint analysis of nuclear and mitochondrial genotypes should offer many novel opportunities for understanding animal behavior and its influence on the genetic structure of hybridizing populations.

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