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Journal

Molecular Ecology, 2(3)

ISSN

0962-1083

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Publication Date

1993

DOI

10.1111/j.1365-294X.1993.tb00103.x

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Cytonuclear genetic architecture in mosquitofish populations and the possible roles of introgressive hybridization

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Abstract

Spatial genetic structure in populations of mosquitofish (*Gambusia*) sampled throughout the south-eastern United States was characterized using mitochondrial (mt) DNA and allozyme markers. Both sets of data revealed a pronounced genetic discontinuity (along a broad path extending from south-eastern Mississippi to north-eastern Georgia) that corresponds to a recently recognized distinction between the nominal forms *G. affinis* to the west and *G. holbrooki* to the east. However, several populations from the general contact region exhibited unusual allelic associations in high frequency, suggestive of evolutionary processes within a zone of introgressive hybridization. These involve: (i) cytonuclear profiles representing combinations of nuclear and mitochondrial genotypes that tended to be more nearly species-specific and concordant elsewhere; and (ii) significant nuclear gametic disequilibria, perhaps attributable to positive assortative mating and/or differential fitnesses of homospecific vs. recombinant genotypes. However, outside this suspected hybrid region, 'heterospecific' genetic markers also appeared in low frequency, thus complicating interpretations. These discordant alleles on a broader geographic scale may reflect: (a) the retention of polymorphisms from an ancestral gene pool; (b) occasional evolutionary convergence (especially with respect to electrophoretic mobility of allozyme alleles); (c) the 'footprints' of a moving hybrid zone; or (d) differential introgressive penetrance across the current hybrid region.

Keywords: allozymes, *Gambusia*, hybrid zone, mitochondrial DNA, genetic structure

Received 18 September 1992; revision received 13 January 1993; accepted 22 January 1993

Introduction

Considerable attention has been focused recently on ecological, demographic and evolutionary factors that mould observed associations between genotypes and geographic distributions (Avise 1989). Renewed interest in this area has been motivated in part by refinement of molecular techniques capable of resolving microevolutionary variation in genetic markers with contrasting modes of inheritance. Concordance in spatial distributions and depths of genealogical topologies across multiple genetic markers, and among evolutionarily independent taxa, has provided strong evidence linking contemporary population

structure to historical processes (Wilson *et al.* 1985; Avise *et al.* 1987). Joint examinations of nuclear and cytoplasmic (e.g. mitochondrial) genes allow further assessment of how sexual asymmetries in gene flow, mating preferences, or other demographic factors can influence population genetic structure, particularly in hybrid settings (Arnold *et al.* 1988; Asmussen *et al.* 1989; Asmussen & Arnold 1992).

Secondary hybrid zones offer favourable settings to study evolutionary forces that may have affected genetic distributions (Hewitt 1988). Taxa involved are likely to exhibit numerous genetic differences that evolved in allopatry and that can serve as convenient markers in the secondary contact region. Genotype-specific differences in mating preference, fertility, or viability are often more readily identified in hybrid settings, making their effects on population genetic structure easier to evaluate. Finally, the shape and width of a hybrid zone, and the ability of genetic material to penetrate it, may be functions of the

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magnitude and direction of gene flow relative to the intensity and direction of selection (Barton & Hewitt 1985).

Mosquitofish (*Gambusia*) occur over a large area of the south-eastern USA (Lee *et al.* 1980) and exhibit considerable spatial variation in gene frequencies (Wooten *et al.* 1988). Mate choice experiments (Scribner, in press), studies of species-specific differences in life-history traits (Reznick 1981; Scribner 1992, 1993) and preliminary analyses within a putative hybrid zone in nature (Wooten & Lydeard 1990; Lydeard *et al.* 1991), all suggest that mating between two nominal species — *G. affinis* (Baird and Girard) and *G. holbrooki* (Girard) — is not random. Furthermore, fertility and viability differences among parental and hybrid genotypes have been shown to lead to changes in population rates of recruitment and mortality within experimental mixed populations (Scribner 1993), and concordantly to changes in population gene frequencies (Scribner & Avise, in press). Because of initial reports indicating extensive sympatric-parapatric association — based on both genetic (Wooten *et al.* 1988; Lydeard *et al.* 1991) and morphological (R. Angus, personal communication) criteria — and hybridization between these species (Wooten & Lydeard 1990), and because predictions about the nature of selection against specific parental and hybrid genotypes are available, *Gambusia* offer an excellent opportunity for studying processes governing introgressive hybridization in nature. Furthermore, a considerable literature exists on population structure and taxon distributions of other fishes in the region (Avise & Smith 1974; Swift *et al.* 1985; Wiley & Mayden 1985; Bermingham & Avise 1986; review in Avise 1992), thus providing a further historical backdrop against which to interpret results for *Gambusia*.

The objectives of this study were:

- 1 to estimate levels and spatial patterns of mtDNA and allozyme divergence within and among populations of *G. affinis* and *G. holbrooki* throughout the southeastern USA;
- 2 to assess distributional patterns of uni- and biparentally inherited markers within and outside a suspected hybrid zone; and
- 3 to interpret results in the context of the contemporary natural histories of the species and the historical zoogeography of the region.

Materials and methods

Fish were collected using dip nets from 45 locations in seven states (SC, GA, FL, AL, MS, LA and TX) during the spring and summer of 1989, and the specimens were immediately frozen in liquid nitrogen for later protein analysis. Additional females were randomly chosen from field collections obtained from each site and returned live to the laboratory for mtDNA isolations.

MtDNA characterization

Mitochondrial DNA (mtDNA) was isolated from fresh tissues using standard procedures of CsCl density gradient centrifugation (Lansman *et al.* 1981). Because of low mtDNA yields, only large (> 30 mm standard length) females were used. Eleven restriction enzymes (listed in Table 1) were scored in $n = 4$ individuals for most localities. Restriction digests were conducted overnight under specifications recommended by the enzyme supplier (Boehringer, Mannheim). Digestion fragments were end-labelled with appropriate ^{35}S -radionuclides, separated on 1.0% agarose gels, and revealed by autoradiography (Brown 1980; Maniatis *et al.* 1982). Fragment sizes were compared against a 1-kb ladder standard (Gibco).

Allozyme characterization

Five allozyme loci which exhibit large allele frequency differences between *G. affinis* and *G. holbrooki* (Wooten & Lydeard 1990; K. T. Scribner, unpublished data) were assayed: adenosine deaminase (Ada; EC 3.5.4.4), aspartate amino transferase (M-Aat-A; EC 2.6.1.1), malate dehydrogenase (S-Mdh-A; EC 1.1.1.37), peptidase-B (Pep-B; EC 3.4.11 or 3.4.13; leucyl alanine as substrate), and aconitase-1 (Acon-1; EC 4.2.1.3). Each locus was scored for $n = 30$ individuals at most localities. Electrophoretic techniques follow those described previously (McClengahan *et al.* 1985; Wooten & Lydeard 1990). Alleles were designated by gel mobilities relative to a reference electromorph from *G. affinis* ('100').

Statistical analyses

All differences among mtDNA gel-fragment patterns could be interpreted as gains or losses of particular restriction sites. Multienzyme mtDNA genotypes were designated by lower-case letters, and estimates of nucleotide sequence divergence among composite genotypes were calculated using the 'site method' (Nei & Li 1979). Distance estimates were used to construct a UPGMA phenogram (Sneath & Sokal 1973). A data matrix of restriction site presence/absence was also used to generate Wagner parsimony networks (by the branch-swapping option) and a consensus network (using the majority-rule option) of PAUP (version 3.0; Swofford 1990). Bootstrapping (100 replicates; Felsenstein 1985) was conducted to allow confidence statements about particular branches in the Wagner network.

Frequencies of allozyme alleles normally characteristic of *G. affinis* and *G. holbrooki* were calculated for all locations, and a 'species index score' (number of *G. affinis* alleles — range 0–10) was computed for each individual. Multilocus genotypes were used to estimate composite measures of

gametic phase disequilibrium (Δ_{AB} ; Weir 1979; Weir & Cockerham 1989), and chi-square tests (Weir & Cockerham 1989) were used to evaluate whether these disequilibrium measures for each locale differed significantly from zero. Although comparisons of disequilibrium measures may be biased somewhat by differences in allele frequency across locations, suggested adjustments (Hill 1974; Hedrick 1987) typically assume random union of gametes, which is unlikely in our hybrid populations. Therefore only major qualitative points will be emphasized from the disequilibrium analyses.

Allelic frequencies were used to calculate genetic distances between population samples (Rogers 1972), and a UPGMA phenogram (Sneath & Sokal 1973) was constructed from the distance matrix. Because only taxonomically informative loci were assayed, differences among localities should not be interpreted as estimates of overall genetic relatedness.

Results

Spatial structuring of mtDNA haplotypes

A total of 173 individuals from 45 localities was screened for mtDNA variation. Nine of 11 enzymes produced poly-

morphic fragment profiles, revealing 65 restriction sites and 22 distinct haplotypes (Table 1). Differences among these haplotypes were attributable to changes at 30 restriction sites.

Phenetic analyses revealed two distinct clusters that differ by an estimated sequence divergence of $P = 0.038$ (Fig. 1a). This fundamental distinction in the UPGMA phenogram was also evident in the qualitative parsimony analysis, where statistical support based on the bootstrap replicates conducted was at the 100% level (Fig. 1b). Five enzymes (*Bam*HI, *Bgl*II, *Dra*II, *Hinc*II, and *Pvu*II) contributed to the fundamental genetic distinction by providing diagnostic and mutually concordant support for these two haplotype groups. These mtDNA groups also showed a strong geographic orientation, with the genetic break following a south-west-north-east orientation from the Pascagoula River in south-eastern Mississippi to the headwaters of the Savannah River in north-eastern Georgia (Fig. 2). At no locale were representatives of both mtDNA groups observed, but this may be due to the small sample sizes per locality.

Within either mtDNA assemblage, strong statistical support for additional mtDNA groupings was generally lacking (Fig. 1b). Nonetheless, geographic population structure was evident in the distributions of particular

Table 1 Polymorphisms in *Gambusia* mtDNA expressed as presence (1) or absence (0) of restriction sites for 11 endonucleases. 'Restriction fragment size data will be provided by KTS on request

	Restriction site										
Haplotype	<i>Sma</i> I	<i>Eco</i> RI	<i>Hind</i> III	<i>Dra</i> II	<i>Bgl</i> II	<i>Spe</i> I	<i>Hinc</i> II	<i>Bgl</i> III	<i>Pvu</i> II	<i>Bcl</i> I	<i>Bam</i> HI
a	11	11	11111111	1100101011001000	1110011	111011111	111111	110	11111	1010	110
b	11	11	11111111	1100101011001000	1110011	111011111	111111	100	11111	1010	110
c	11	11	11111111	1101101011001000	1110011	110111111	111111	100	11111	1100	110
d	11	11	11111111	1101101011001000	1110011	111011111	111111	100	11111	1010	110
e	11	11	11111111	1100101011001000	1110011	110111111	111111	100	11111	1111	110
f	11	11	11111111	1101101011001000	1110011	110111111	111111	100	11111	1111	110
g	11	11	11111111	1100101011001000	1110011	110011111	111111	110	11111	1010	010
h	11	11	11111111	1100101011001000	1110011	110011111	111111	110	11111	1010	110
i	11	11	11011111	1100101011011000	1110011	110011111	111111	110	11111	1010	110
j	11	11	11011111	1100101011011000	1110011	110011111	111111	100	11111	1010	110
k	11	11	11011111	1100101011001000 ¹	1110011	110011111	111111	100	11111	1010	110
l	11	11	11110111	1100101011001000	1110011	110011111	111111	100	11111	1010	110
m	11	11	11110111	1100101011001000	1110011	110011111	111111	110	11111	1010	110
n	11	11	11011111	1100101011001000	1110011	110011111	111111	110	11111	1010	110
o	11	11	11111111	1100101011001001	1110011	110011111	111111	110	11111	1010	110
p	11	11	11111111	1100101011001000	1110011	110111111	111111	100	11111	1111	010
q	11	11	11111111	1100101011001000	1110011	110111111	111111	110	11111	1111	010
r	11	11	11111111	1100101011001000	1110011	100111111	111111	100	11111	1111	010
s	11	11	11011111	1100110101101010	1101111	110101111	101111	101	10101	1111	101
t	11	11	11011111	1100100101101010	1101111	110101111	101111	101	10101	1111	101
u	11	11	11011111	1110100101101100	1101111	110111111	101111	101	10111	1111	111
v	11	11	11011111	1100110101101010	1101111	110101111	101111	101	10111	1111	101

Fig. 1 UPGMA phenogram (A) and consensus Wagner parsimony network (B) of mtDNA haplotypes in *Gambusia affinis* and *G. holbrooki*. MtDNA clones are lettered as in Table 1. Values on branches indicate levels of bootstrap support for particular groups in the Wagner network (which is unrooted).

mtDNA clones. For example, mtDNA haplotype (a) was nearly confined to the northern Atlantic coast drainages surveyed, and haplotypes (v) and (u) were confined to samples from Texas and eastern Louisiana, respectively (Fig. 2). Beyond these cases, only two mtDNA haplotype distributions warrant mention. In the UPGMA analysis, two genotypic clusters within the *G. holbrooki* group differed by

an estimated $P = 0.01$, and this distinction also received moderate (67%) bootstrap support in the parsimony analysis (Fig. 1). One of these putative lineages (clones c, e, f, p, q and r) was confined to Gulf coast locales, whereas the other was widespread throughout the *G. holbrooki* range and was fixed in the samples from Atlantic coast drainages (Fig. 2).

Fig. 2 Geographic distribution of the mtDNA haplotypes described in Table 1. Closed and open circles represent the two major clonal assemblages (Fig. 1) usually characteristic of *Gambusia affinis* and *G. holbrooki*.

Fig. 3 UPGMA phenogram of *Gambusia* populations based upon allozyme analyses.

Regional differences in measures of mtDNA diversity were also observed. Lowest genetic diversity occurred in populations from northern Atlantic coast drainages of Georgia and South Carolina where only one mtDNA haplotype (a) was observed (Fig. 2). Highest mtDNA diversities were found in *G. holbrooki* populations collected from southern regions of drainages flowing into the Gulf of Mexico in Mississippi, Alabama, and Florida (due partly to the occurrence there of haplotypes representing both of the two major *holbrooki* mtDNA haplotype groups). This latter region was also characterized by a number of unique and localized mtDNA haplotypes that differ at one or two restriction sites from the common *holbrooki* genotype (h).

Spatial variation in allozyme frequencies

A total of 1259 individuals from the same 45 localities was screened for allozyme variation. At the five polymorphic nuclear loci monitored, allelic distributions revealed a macrogeographic population structure similar to that observed among mtDNA haplotypes (Figs 3 and 4), with a major genetic discontinuity in western Georgia and eastern Alabama registered in allele frequency differences and species index scores (Table 2). At most locations, relatively few *holbrooki* alleles were observed in fish that on the basis of mtDNA genotype would be characterized as *G. affinis*. Thus the western locales 1–5 exhibited no *holbrooki* alleles, whereas locales 9–15 and 27 had *holbrooki* alleles in frequencies less than 0.25. However, an exception involved

Fig. 4 Geographic distribution of the mean frequencies of 'affinis' and 'holbrooki' alleles over the five allozyme loci assayed.

Table 2 Characteristics of 45 mosquitofish populations sampled across the south-eastern United States

Location*	mtDNA N	allozyme N	Frequency of <i>affinis</i> alleles†	Gametic disequilibrium‡	Species scores§										
					0	1	2	3	4	5	6	7	8	9	10
1 (A)	4	30	1.000												30
2 (A)	4	30	1.000												30
3 (A)	4	30	1.000												30
4 (A)	4	30	1.000												30
5 (A)	4	30	1.000												30
6 (A)	4	29	0.541							1	4	12	8	4	
7 (H)	4	29	0.114	-0.025 - 0.062*	11	9	8				1				
8 (H)	4	30	0.743	-0.092 - 0.056*					1	4	4	7	7	5	2
9 (A)	4	30	0.900	-0.049 - 0.003*								1	7	16	6
10 (A)	4	30	0.887	-0.033 - 0.013								3	9	10	8
11 (A)	4	30	0.884	0.000 - 0.016								2	7	15	6
12 (A)	4	30	0.873	-0.019 - 0.035*							1	2	8	11	8
13 (A)	4	30	0.930	-0.017 - 0.028							1	2	14	13	
14 (A)	4	30	0.817	-0.023 - 0.054*						1	2	3	11	11	2
15 (A)	4	30	0.767	-0.067 - 0.020							4	10	8	8	
16 (H)	4	4	0.500					2		2					
17 (H)	4	29	0.438	-0.013 - 0.023	1	4	10	9	5						
18 (H)	4	30	0.560	-0.013 - 0.003		1	6	3	15	5					
19 (H)	4	30	0.140	0.000 - 0.010	18	7	5								
20 (H)	4	30	0.060	-0.012 - 0.023*	16	10	4								
21 (A)	3	30	0.356	-0.026 - 0.133*	1	2	7	5	7	2	4	2			
22 (H)	4	30	0.000		30										
23 (H)	4	30	0.232	-0.017 - 0.070*	14	12	3		1						
24 (H)	4	30	0.053		29	1									
25 (H)	4	30	0.236			20	6	4							
26 (H)	4	30	0.013		26	4									
27 (A)	4	30	0.953	-0.011 - 0.010									2	10	18
28 (H)	4	30	0.083	-0.012 - 0.053*	12	16	1	1							
29 (H)	4	30	0.067	-0.020 - 0.000	13	14	3								
30 (H)	4	30	0.000		30										
31 (H)	4	30	0.047		28	2									
32 (H)	4	30	0.057		17	9	4								
33 (H)	4	30	0.040		19	10	1								
34 (H)	4	7	0.286				3	2	2						
35 (A)	2	5	1.000												5
36 (H)	4	30	0.023		24	5	1								
37 (H)	3	30	0.097		5	21	4								
38 (H)	4	30	0.000		30										
39 (H)	4	30	0.080		11	14	5								
40 (H)	2	16	0.081	0.000 - 0.031	8	5	1	2							
41 (H)	4	30	0.120	0.000 - 0.025	7	14	6	2	1						
42 (H)	3	30	0.063	-0.019 - 0.000	14	13	3								
43 (H)	4	30	0.147	0.000 - 0.007	4	13	8	5							
44 (H)	4	30	0.147		3	15	7	5							
45 (H)	4	30	0.096	-0.004 - 0.000	18	9	3								

*Letters in parentheses indicate the mtDNA type (H, *holbrooki*; A, *affinis*) present at that location.

†Mean *affinis* allele frequency over five loci. Allele frequency data for all loci and populations are available on request from the senior author.

‡Distribution values of composite measures of gametic disequilibrium (Weir 1979; Weir & Cockerham 1989) for all pair-wise comparisons of five allozyme loci (nonzero values only shown). Asterisks represent one or more values significantly greater than zero ($P \leq 0.05$).

§Number of individuals which had the given number of *affinis* alleles.

Fig. 5 Three-dimensional representation of nuclear disequilibria across geographic samples of *Gambusia*. Latitude and longitude positions for each sample are represented in Universal Transverse Mercator metrics (km).

locale 6 in eastern Louisiana, where 'affinis' nuclear alleles predominated on a 'holbrooki' mtDNA background. Conversely, along the Gulf coast several locales that exhibited *holbrooki*-type mtDNA (8, 16–18) also had in high frequency ($q > 0.4$) allozyme alleles normally characteristic of *G. affinis* (Table 2).

At the nuclear loci assayed, many populations along the mtDNA contact area between *G. holbrooki* and *G. affinis* were characterized by significantly higher non-zero gametic disequilibria than described for most other localities (Fig. 5). Estimates of nuclear gametic disequilibrium were significant in populations 7, 8, 20, 23 and 28, which exhibited *holbrooki* mtDNA, and populations 9, 12, 14 and 21, which exhibited *affinis* mtDNA (Table 2). All of these populations occur in the general region of contact between *G. affinis* and *G. holbrooki* in Alabama and western Georgia.

Discussion

Whereas a spatial genetic partitioning of *G. affinis* and *G. holbrooki* would appear straightforward based on mtDNA designation alone (Figs 1 and 2), lack of perfect concordance with the allozyme allele frequencies, and the presence of nonparental genotypic combinations and intermediate species index scores for nuclear loci in the general contact region, raise the likelihood that historical introgression has occurred over a broad central area in the south-eastern USA. On the other hand, observations of nonrandom associations among nuclear alleles in this same area suggests that there also has been a strong tendency for species-specific assortative mating (and/or selection against specific nuclear genotypes). The picture is further complicated because nuclear alleles of both species also segregate in low frequency in some populations outside the current geographic region of the major genetic discontinuity.

Spatial genetic structure within and among species

The spatial distributions of distinct mtDNA haplotypes and nuclear allozymes documented here are generally consistent with expectations based on the recently described ranges of *G. holbrooki* and *G. affinis* (Wooten *et al.* 1988). [This outcome was not a foregone conclusion, because *Gambusia* relationships and taxonomy have been highly controversial, and all populations in the south-eastern USA are often treated as belonging to a single species with geographic and genetic affinities uncertain (e.g. Lee *et al.* 1980)]. Furthermore, the boundary region between the *holbrooki* and *affinis* genetic types generally is concordant with mtDNA phylogeographic discontinuities reported within several other fish species in the region (Birmingham *et al.* 1986), as well as with an area of concentration for species' distributional limits within numerous taxonomic assemblages of freshwater fishes (Swift *et al.* 1985; Wiley & Mayden 1985). These similarities in distinction between closely related eastern and western fish faunas of the south-eastern USA argue strongly for an overriding effect of historical biogeographic phenomena in shaping macrogeographic population genetic structure (Avice 1992).

A second genetic subdivision of lesser magnitude, recognized in mtDNA analyses (Figs 1 and 2) as well as by overall allozyme frequency differences (Table 2), distinguishes many (but not all) populations of *G. holbrooki* from the Gulf coastal plain from those to the east in peninsular Florida, Georgia, and the Carolinas. Thus, for the south-eastern *Gambusia* complex as a whole two important historical separations are implicated: an early, well-supported genetic separation leading to the current differences between *G. affinis* and *G. holbrooki*, and a later, less well-supported genetic separation leading to eastern vs. Gulf coast forms of *G. holbrooki*.

One possibility is that ancestors of *G. holbrooki* may have been isolated in an eastern refugium, perhaps the Florida peninsula, which appears to be the stronghold for the eastern genetic form of many freshwater fish species (Bermingham & Avise 1986). A second dispersal or vicariant event may then have initiated the separation between eastern and western *holbrooki* types. If so, there may also have been recent gene flow between these latter areas, because the mtDNA genotype (h) (as well as others within the same haplotype group; Fig. 1) are shared by Gulf coast and Atlantic coast populations (Fig. 2). Alternatively, such inter-regional sharing of genotypes might be attributable to the retention of unmodified ancestral haplotypes.

Additional population substructure within *G. affinis* and *G. holbrooki* provides further clues to historical patterns of gene flow and population separation. For example, *G. holbrooki* in the northern Atlantic drainages is depauperate in mtDNA variation and carries in high frequency an otherwise uncommon mtDNA haplotype, (a), which is only one mutation step removed from (h) which predominates to the south (Fig. 2). These northern drainages were probably colonized fairly recently from a larger and more variable source population in Florida or southern Georgia. Within *G. affinis*, a distinctive mtDNA genotype (u) (Fig. 1) was confined to and fixed in the sample from one locale in eastern Louisiana (Fig. 2), indicating that geographic structure occurs within this species as well.

Historical and contemporary hybridization

Recent attention has been focused on concordance and discordance of different molecular markers in hybrid zones. Use of multiple markers is particularly important for the study of introgression because the complexity of hybridization processes precludes a universal marker for understanding these phenomena (Baker *et al.* 1989; Avise & Ball 1990). Previous genetic studies of hybridizing taxa have documented that geographic patterns based on allozyme loci are not always consistent with one another, or with those based on mtDNA (Hunt & Selander 1973; Ferris *et al.* 1983; Powell 1983; Spolsky & Uzzell 1984; Harrison *et al.* 1987; Arntzen & Wallis 1991; Dowling & Hoeh 1991). Such discrepancies are believed to arise through stochastic historical effects including founder events or other idiosyncratic demographic factors (Gyllenstein & Wilson 1987), ecological and behavioral factors (Harrison 1986; Lamb & Avise 1986), and locus-specific selection intensities on alleles in heterospecific genetic backgrounds (Hunt & Selander 1973). Other studies have provided examples of concordant distributional patterns for uni- and biparentally inherited markers in hybrid settings (e.g. Avise *et al.* 1984; Szymura *et al.* 1985; Baker *et al.* 1989; Dowling *et al.* 1989).

The availability of several allozyme and mtDNA markers in the current study allows examination of the degree of geographic concordance across independent loci (Figs 2 and 4). Populations within the general contact region between *G. affinis* and *G. holbrooki* were not characterized solely by parental and F_1 individuals (species index scores, Table 2), but also included recombinant nuclear and cytonuclear genotypic associations strongly suggestive of historical introgression. Observations of significant gametic disequilibria among nuclear genotypes indicate that opposing forces are also at work in the hybrid zone that have prevented a randomization of genotypes that otherwise might be expected under extensive hybridization alone. High disequilibrium could be maintained either by gene flow of parental types into the hybrid zone or by positive assortative mating and/or selection against certain recombinant genotypes.

Additional evidence from controlled laboratory crosses (Scribner 1992, 1993), and temporal genetic studies of 'artificial' hybrid populations between *G. affinis* and *G. holbrooki* (Scribner & Avise, in press), argue strongly for the possibility of rapid genetic changes within natural *Gambusia* hybrid zones and for movements in hybrid zone position. A lower population carrying capacity for *G. affinis*, and lower fitness of juveniles of *affinis* maternal parentage in mixed populations with *G. holbrooki* (due to differences in size at birth, growth rates, and size and age at sexual maturity; Scribner 1992, 1993) suggest a directionality to changes in genotypic composition. In addition, mixed-species experimental populations exhibited a rapid and directional change in genotypic composition toward elimination of *affinis* genotypes over a period of 2 years (about four generations). These experimental studies were conducted in the absence of gene flow, and under relatively uniform environmental conditions, but the results clearly showed that genotypic composition in hybrid zones might be quite dynamic in nature as well. Existing theory predicts that hybrid zones will tend to move from areas of high fitness and population density to regions where these properties are low (Nagylaki 1976; Barton & Hewitt 1985).

Nuclear alleles normally characteristic of *G. holbrooki* were observed in low or moderate frequency particularly west and north of the zone of major mtDNA discontinuity (Figs 2 and 4). The presence of occasional electromorphs 'in advance' of the mtDNA break is suggestive of differential introgression of nuclear and mtDNA markers. This interpretation is consistent with behavioral observations of species-specific differences in degree of assortative mating: in laboratory and field experiments, female *G. affinis* mate randomly with regard to species whereas *G. holbrooki* females mate almost exclusively with males of their own species (Scribner, in press). Thus, the presence of nuclear *holbrooki* alleles in hybridized populations that carry *affinis*

mtDNA might be attributable to male-mediated introgression. It has further been reported that F_1 females from crosses between *G. holbrooki* females and *G. affinis* males are infertile, whereas no decline in fertility was observed among F_1 females from the reciprocal cross (Reznick 1981). This result too is consistent with retardation of *holbrooki* cytoplasmic penetrance through the hybrid zone. Although the direction of both pre- and post-zygotic isolating mechanisms suggests that *holbrooki* nuclear genes may precede maternally inherited mitochondrial genes through a hybrid region via male-biased gene flow, the possibility that these electromorphs represent retention of ancestral states (or the products of convergent evolution) cannot be eliminated entirely.

Conversely, nuclear alleles that normally predominate in *G. affinis* were observed occasionally in *G. holbrooki* populations from Florida and Atlantic and Gulf coastal populations (Fig. 3). The presence of *affinis* alleles could again reflect simply the retention of ancestral polymorphisms predating the species separation. Alternatively, *affinis* alleles in at least some *G. holbrooki* populations might represent evolutionary footprints from an earlier broadly distributed *G. affinis* population that historically was assimilated by ancestral *G. holbrooki* dispersing out of Florida. However, arguing rather strongly against the footprint scenario are the facts that: (a) *affinis* mtDNA genotypes were not similarly retained in *G. holbrooki* populations, despite the previously mentioned asymmetries in mating preference and hybrid fertility that would appear to favour such maintenance; and (b) *affinis*-type electromorphs were observed in moderate frequency even in Florida locations where *G. affinis* had presumably never been present.

Previous studies of introgressive hybridization in *Gambusia* species have stressed additional ecological factors as important in maintaining species integrity in sympatry, such as habitat preferences (Hubbs 1959; Hubbs & Delco 1962; Hubbs & Peden 1969). These studies involved *G. affinis* and several localized endemics in Texas (*G. geiseri*, *G. georgei* and *G. heterochin*). The authors suggested that ecological separation based on water chemistry, temperature, and distribution of vegetation contributed to positive assortative mating and prezygotic isolation. Similar factors may also be at work in the *holbrooki*-*affinis* contact region. For example, in Atlantic and Gulf coast drainages that contain both species, *G. affinis* predominates in the headwaters whereas *G. holbrooki* generally predominates along the coastal plain. This suggests that even in the absence of firm geographic impediments to dispersal, ecological preferences can have an important influence on species distributions. Whether ecological conditions partially segregate *G. affinis* and *G. holbrooki* on finer, syntopic spatial scales remains to be determined.

Synopsis

There are two general but somewhat opposing messages to be derived from these analyses of cytonuclear population structure in mosquitofish. First is the impressive amount of historical information apparently retained in a relatively small number of nuclear and cytoplasmic loci. Not only is a major distinction between two otherwise cryptic *Gambusia* forms in the south-eastern USA blatantly obvious from the genetic data, but also several finer details about geographic relationships within these groups, and probable patterns of gene flow and introgressive hybridization between them have been revealed. However, a second and competing message is that there are also definite limits to inferences that can be drawn from purely descriptive surveys of contemporary population structure. As is evident from the discussion above, alternative scenarios can often be advanced to account for observed genetic architectures (in *Gambusia* or elsewhere), such that historical reconstructions based solely on static descriptions of contemporary population structure will inevitably have elements of uncertainty that may never be overcome entirely. However, for hybridizing species that can be manipulated experimentally, additional insight into the forces governing population genetic architecture may be gained through detailed behavioral observations on ecological and mating preferences, as well as from 'population cage' experiments designed to monitor temporal genetic changes following secondary contact and introgressive hybridization in controlled settings. Results of such experiments with *Gambusia* will be presented elsewhere.

Acknowledgements

This work was supported by NSF grants to J.C.A., by the Savannah River Ecology Laboratory under contract no. DE-AC09-76SR00819 between the US Department of Energy and the University of Georgia's Institute of Ecology, and by an Oak Ridge Associated Universities Participation agreement to K.T.S. We thank M. Wooten for insightful discussions and assistance in data collection during the initial stages of this project.

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This paper is the third in a series resulting from K. T. Scribner's doctoral studies which examines the effects of population-level processes on microevolutionary changes within populations of hybridizing *Gambusia* species. Earlier experimental studies characterized differences in genotype-specific life-history traits and how these differences translated into differential rates of recruitment, mortality, and concomitantly to population changes in gene frequency. With this paper the story comes full circle as hypotheses concerning the rate and direction of evolution within *Gambusia* hybrid zones which were formulated based on experimental data are examined in natural populations.