DYNAMICS OF INTROGRESSIVE HYBRIDIZATION BETWEEN PECOS PUPFISH (*CYPRINODON PECOSENSIS*) AND SHEEPSHEAD MINNOW (*C. VARIEGATUS*) IN THE PECOS RIVER, TEXAS

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INTRODUCTION

In this paper, I use allozyme and mitochondrial DNA (mtDNA) variation to describe the cytonuclear genetic structure of a hybrid swarm that developed with extreme rapidity throughout a large geographic area. Within five years after the introduction of sheepshead minnow (Cyprinodon variegatus) into the Pecos River, sometime between 1980 and 1984, locally panmictic genetic admixtures of the introduced species and the endemic Pecos pupfish (C. pecosensis) extended over more than 500 river-kilometers of the Pecos River in Texas (Echelle and Connor, 1989; Wilde and Echelle, 1992). Surveys in 1985 and 1986 revealed clines in both morphology and allozymes that suggested an initial introduction of C. variegatus into a mid-reach of the river, near Pecos, Texas, followed by dispersal of introduced genetic material into other areas (Echelle and Connor, 1989; G. R. Wilde, pers. comm.). The average frequency of introduced alleles across four diagnostic, protein-encoding loci ranged from a high of 0.84 near the town of Pecos to lows of, respectively, 0.18 and 0.40 in areas upstream and downstream from there.

Two general models can explain instances where the isolated introduction of an exotic species leads to a hybrid

swarm: 1) multilocus selection for introduced genetic elements (selection hypothesis herein), and 2) genetic replacement without selection (replacement hypothesis). The replacement hypothesis predicts that the frequencies of all diagnostic markers of the introduced genome would be approximately the same. In a closed population, the frequencies would be determined primarily by the relative abundances of the two parental species at the time of introduction. In contrast, the selection hypothesis predicts divergent frequencies of such markers, depending on selection differentials for individual genetic elements.

Echelle and Connor (1989) postulated extremely rapid multilocus selection to explain the genetic changes in the morphology and allozyme structure of pupfish populations in the Pecos River. The possibility of genetic replacement without selection was excluded because it implied an introduction of a magnitude that seemed highly unlikely for a small, nongame fish like C. variegatus. However, recent reports of extensive fish kills in the Pecos River (James and De La Cruz, 1989; Rhodes and Hubbs, 1992) have made replacement more plausible.

Predictions of the replacement hypothesis (equal frequencies of all introduced genetic markers) represent a null model against which allozyme and mtDNA data can be examined for possible effects of a variety of evolutionary forces. Comparison of mtDNA and allozymes should be

particularly informative. Mitochondrial DNA generally is a maternally inherited cytoplasmic element, and is not likely to be subjected to indirect selection (hitch-hiking) due to selection for nuclear DNA. Thus, mtDNA provides an opportunity to test the hypothesis that multilocus selection explains the high frequency of introduced allozymes in the hybrid swarm of pupfish in the Pecos River. The selection hypothesis requires large selection differentials to explain the rapid change in allozyme frequencies. This leads to the expectation that any selection differential between the alternative mtDNAs is likely to be significantly smaller than those associated with the allozymes and that, therefore, the introduced mtDNA should be rare or uncommon relative to the introduced allozymes.

There is some possibility of selection on mtDNA, for example as a result of cytonuclear coevolution (Moritz et al., 1987). However, such effects have been difficult to demonstrate (Forbes and Allendorf, 1991a,b; Nigro and Prout 1990) and there is no evidence for mtDNA selection differentials of a magnitude that would affect my results (Forbes and Allendorf, 1991a,b; MacRae and Anderson, 1988, 1990; Nigro and Prout 1990). Because mtDNA is maternally inherited, the direction of frequency differences between mtDNAs and allozymes can be examined for evidence of factors such as assortative mating, selection against specific hybrid combinations, and sexually skewed migration (Lansman

et al., 1983; Dowling et al., 1989; Dowling and Childs, 1992).

Of particular interest in my study was the question of changes in the allozyme structure of the hybrid swarm since 1985 and 1986. Most pupfishes have extended breeding seasons of several months and reproduction is initiated at such small body size that they can potentially produce more than one generation per year (e.g., Hildebrand and Schroeder, 1927; Garrett, 1981). Thus, up to the time of my sampling in 1991, the hybrid swarm had gone through a minimum of five generations and possibly as many as 10 or more since the development of the hybrid swarm. With time, and assuming no immigration, a newly established random-mating hybrid swarm should exhibit reduced linkage disequilibrium among allozymes (Brown, 1975) and a reduced amount of geographic structure in frequencies of allozymes diagnostic of the two parental species (Forbes and Allendorf, 1991a).

MATERIALS AND METHODS

Pupfish were collected from six localities in Texas (Fig. 1) that encompass the region occupied by the hybrid swarm in the Pecos River: 1) Red Bluff Reservoir in Loving Co. near the east end of the dam forming the reservoir; 2) Pecos River on the downstream side of an irrigation diversion dam ("Brush Dam"), 10 km SE of Mentone, Loving Co.; 3) Pecos River at U. S. Highway 80 bridge, 1.5 km east of Pecos; 4) Pecos River at Horsehead Crossing, 20 km SW of Crane; 5) Pecos River at State Highway 349, 8 km NW of Iraan, Pecos Co.; 6) Pecos River at Pandale Crossing, 10 km south of Pandale, Val Verde Co. Samples from sites 2-5 were made in June, 1991, while samples from sites 1 and 6 were made in August, 1992. Samples were frozen on dry ice in the field and subsequently stored in the laboratory at -70 C. Reference specimens of C. pecosensis were taken in June, 1991 from two sites on Salt Creek, one at a low-water bridge below the spillway from Red Bluff Reservoir, and the other at the U. S. Highway 285 bridge, 10 km NW of Orla, Culberson Co., Texas. The latter locality showed no evidence of introgression by C. variegatus in 1984, 1985, or 1988 (Echelle et al., 1987; Echelle and Connor, 1989; Wilde and Echelle, 1992). Reference specimens of C. variegatus were

taken in August, 1992 from an introduced population (Stevenson and Buchanan, 1973) in Lake Balmorhea (site V1), Reeves Co. Texas. Another sample of C. variegatus was taken in April, 1992 from the Edinburg Water Supply Canal, 1 km N of Edinburg (site V2), Hidalgo Co., Texas in the lower Rio Grande Valley approximately 600 km downstream from the mouth of the Pecos River.

Standard methods of horizontal starch-gel electrophoresis (Selander et al., 1971; Siciliano and Shaw, 1976) were used to examine the products of four presumptive gene loci exhibiting fixed or nearly fixed differences between C. pecosensis and C. variegatus: alcohol dehydrogenase-1 (ADH-1, EC 1.1.1.1), esterase-1 (EST-1, EC 3.1.1.1), glucose-6-phosphate-isomerase-A (GPI-A, EC 5.3.1.9), and proline-dipeptidase-1 (PEPD-1, EC 3.4.13.9). ADH-1 and PEPD-1 products were resolved from liver tissue on a continuous tris-citrate buffer system (TC-8.0., Shaw and Prasad, 1970). EST-1 products were resolved from eye/brain tissue on the continuous Tris-borate-EDTA buffer system described by Turner (1983). GPI-A products were resolved from eye/brain tissue on a discontinuous lithium hydroxide buffer system (LiOH; Selander et al., 1971). Loci were scored following Echelle and Connor (1989, see Results for an exception involving GPI-A). Sex of each specimen was determined based on the presence of a black band on the posterior edge of the caudal fin, in males.

Departures from Hardy-Weinberg expectations were assessed using the exact-probability test provided by BIOSYS-1 (Swofford and Selander, 1981). Fixation indices (F) were calculated for each locus at each site using the same program. Burrows' composite measure of gametic disequilibrium (D) was calculated from genotypic frequencies for each pairwise combination of the allozyme-encoding genes (Weir, 1979, 1990). To test the hypothesis that $D = 0$, I used the X^2-distributed statistic Q (Weir, 1990). However, I report the standardized measure of linkage disequilibrium ($D' = D/D_{max}$ where D_{max} is the maximum possible value for the sample; Hedrick, 1983).

Genomic DNA was extracted, with minor modifications, following Hillis et al. (1990), and digested with two restriction enzymes (HindIII, XhoI) diagnostic for C. variegatus and C. pecosensis. Fragments produced by restriction endonuclease digestion were separated by horizontal electrophoresis in 1% agarose gels, denatured and transferred to nylon membranes (Southern, 1975) and hybridized to probe mtDNA labeled with [^{32}P]dCTP (random priming kit from U.S. Biochemical). Hybridization conditions, washes, and autoradiography followed Davis (1986).

Cytonuclear disequilibria were calculated as in Asmussen et al., (1987), using their estimate of gametic disequilibrium D. This parameter, like the dilocus nuclear

D, measures departures of gametic frequencies from random expectations. Following Forbes and Allendorf (1991a) I report values of the test-statistic Q (Asmussen et al, 1987) with the sign of D.

RESULTS

Frequencies of Allozymes and MtDNA Haplotypes

Populations sampled within the hybrid swarm (localities 1-6, Fig. 1) were segregating for the alleles of C. variegatus and C. pecosensis at all four diagnostic allozyme-encoding loci (Table I), just as in 1985 (Echelle and Connor, 1989). Also as in 1985, reference samples of C. pecosensis (sites P1 and P2, Fig. 1) showed low frequencies (< 0.03) of "variegatus" alleles for EST-1 and PEPD-1 (Table I), suggesting that these alleles were present in the C. pecosensis genome prior to the introduction of C. variegatus into the Pecos River. Wilde and Echelle (1992) reported evidence of genetic introgression at sites near the mouth of Salt Creek in 1988. Frequencies of introduced allozymes in a 1988 sample of 54 specimens from near site P1 were as follows: ADH-1 (0.009), EST-1 (0.019), GPI-A (the GPI-A-b allele, 0.028), and PEPD-1 (0.009). Frequencies were notably higher (0.102, 0.102, 0.065, and 0.120, respectively) in Salt Creek approximately 1.2 km downstream from my site P1 (2.4 km upstream from the convergence with the Pecos River). This site was just below a series of small waterfalls (<0.5 m high) that would retard upstream

dispersal of pupfish. Reference specimens of C. variegatus in the present study (sites V1 and V2, Fig.1) exhibited low frequencies ($\leq$ 0.05) of "pecosensis" alleles for ADH-1, EST-1, and PEPD-1 (Table I). These alleles were not present in previous reference samples of C. variegatus, possibly due to differences in collection locality.

Previous allozyme surveys of the hybrid swarm did not resolve the presence of cryptic GPI-A allele variation involving the GPI-A-d allele of C. pecosensis and the GPI-A-c allele of C. variegatus (Table I). I found GPI-A-c at a frequency of 0.07 in the reference sample of C. variegatus from Edinburg and at a frequency of 0.15 in the sample from the introduced population of C. variegatus in Lake Balmorhea. GPI-A-c was absent in reference samples of C. pecosensis and its frequency varied directly (Pearson's r = 0.999, $P < 0.001$) with the mean introduced allozyme frequency at the six sample sites from within the hybrid swarm (Fig. 2); thus, GPI-A-c apparently was introduced into the Pecos River with C. variegatus.

The introduced GPI-A-c allele forms an unusually high percentage (29-57%) of the introduced genome, at all sites in the hybrid swarm. In native populations of C. variegatus from three Texas sites, two in the Rio Grande drainage and one on the Gulf Coast, GPI-A-c occurred at a frequency of 0.00 to 0.07 (Echelle and Connor, 1989; Wilde and Echelle, 1992; this study). In a survey of C. variegatus over a wide

geographic area, Darling (1976) reported one predominant allele and two minor alleles for GPI-A. One of the minor alleles had the relative mobility appropriate for GPI-A-c; it occurred at a frequency of 0.00 to 0.13 at 27 sites from the Atlantic and Gulf coasts including five localities on the Texas coast where the frequency ranged from 0.00 to 0.07. Thus, the frequency of this allele appears unusually high in the introduced genomes in the Pecos River drainage.

Most samples collected in 1991-1992 conformed with Hardy-Weinberg expectations. An exact-probability test provided by BIOSYS-1 (Swofford and Selander, 1981) revealed three significant departures, all of which were heterozygote deficiencies: EST-1 at Pandale Crossing ($\underline{P}$ = 0.016) and PEPD-1 at Horsehead Crossing ($\underline{P}$ = 0.005) and Pandale Crossing ($\underline{P}$ = 0.029). Fixation indices ($\underline{F}$) gave no indication of an overall tendency toward heterozygote deficiencies; both positive and negative values occurred at all sites.

Geographic variation in 1991-1992 across the six localities (sites 1-6) within the hybrid swarm (Fig.3) conformed with the clinal patterns in the frequency of introduced alleles in 1985 and 1986 (Echelle and Connor, 1989; Wilde and Echelle, 1992). The clinal pattern between sites 2 and 5 appears to have flattened slightly since 1985-1986, with significant decreases in $\underline{C}$. $\underline{variegatus}$ allele frequencies at Pecos, Texas (the presumed site of initial

introduction; Echelle and Connor, 1989) and significant increases (since 1986) both up- and downstream from there (Fig. 4).

Statistical comparison of introduced allele frequencies at each of the four diagnostic loci revealed only one significant difference between 1985 and 1986 (Fig. 4); the frequency of the introduced allele for PEPD-1 at Brush Dam was significantly lower in 1986, resulting in significant overall heterogeneity across the four loci. Frequencies of introduced alleles decreased between 1985 and 1986 in 15 out of 20 cases (4 loci X 5 sites, Fig. 4; sign test, $0.01 < \underline{P} < 0.05$).

Significant interlocus, within-site heterogeneity in frequency of introduced allozymes occurred only at Horsehead Crossing (2 X 4 heterogeneity $\underline{X}^2 = 10.9$; 3 d.f., $\underline{P} = 0.012$). There was no consistent difference in interlocus abundances of the introduced allozymes across the six sites within the hybrid swarm. Correspondingly, ranked allele frequencies were not significantly concordant among sample localities (Kendall's $\underline{W} = 0.403$; $0.05 < \underline{P} < 0.10$). The near significance of the test of concordance may reflect interdependence among samples; the sampled populations are not completely isolated from one another.

The diagnostic differences in restriction fragment profiles were confirmed by the HindIII and XhoI mtDNA digests from reference samples of C. pecosensis (sites P1

and P2) and C. variegatus (sites V1 and V2). These enzymes revealed one mtDNA haplotype in the reference samples of C. pecosensis from site P2 in Salt Creek, and two mtDNA haplotypes in the reference sample of C. variegatus from Edinburg. The two haplotypes at Edinburg, here designated A and B, occurred at frequencies of 58% and 42%, respectively. Except for the Red Bluff Reservoir sample, all samples from the hybrid swarm were polymorphic for two mtDNAs: the one typical of C. pecosensis, and haplotype A of C. variegatus. The Red Bluff Reservoir sample was polymorphic for those two mtDNAs plus a third haplotype (C) resembling the type A haplotype of C. variegatus except for the presence of an apparent tandem repeat of approximately 2300 base pairs.

There was no consistent difference in frequency of introduced mtDNAs relative to introduced allozymes among the six hybrid localities (Fig. 3). The number of introduced mtDNAs expected from the frequency of introduced allozymes was equal to the observed number at site 2, greater at sites 3, 4, and 5, and lower at sites 1 and 6 (Table II). The only significant departure from the expected number occurred at Pandale Crossing ($P < 0.05$; Table II), where the expected frequency of the introduced mtDNA was 0.75 and the observed frequency was 0.93; however, this departure was not significant when adjusted for multiple tests (Rice, 1989). There were no significant sexual differences in allozyme or mtDNA frequencies in samples taken from 1991-92 (Table III).

Associations Between Loci

Nuclear Gametic Disequilibria

Standardized linkage disequilibrium values (D') are presented in Table IV. None exhibited table-wide significance (Rice, 1989) at the 0.05 level. There is, however, some evidence for residual linkage disequilibrium between GPI-A and EST-1, two loci that may be physically linked on the same chromosome (Echelle and Connor, 1989). Only two D-values were statistically significant in the individual tests, and both involved GPI-A/EST-1 from the two localities farthest downstream (sites 5 and 6); in both instances, the sign of D' was positive, indicating an excess of coupling gametes involving GPI-A and EST-1. As previously mentioned, GPI-A was incorrectly scored in the 1985 and 1986 samples due to the presence of a cryptic allele in the hybrid swarm. The D' values reported for GPI-A/EST-1 in the 1985-1986 samples were markedly higher than those for all other pairs of loci, including GPI-A/ADH-1 and GPI-A/PEPD-1. Thus, linkage between GPI-A and EST-1 is indicated for those samples, despite the scoring difficulty.

My sample sizes are too small and allele frequencies are too extreme (at sites 2 and 3) to accurately assess significance of individual disequilibrium values (Brown, 1975). However, the distribution of signs for D' indicates that disequilibrium has declined since 1985 and 1986. In a

population at equilibrium, the distribution of D' values should be symmetrical around zero (Forbes and Allendorf, 1991a). In my 1991-1992 collections, there were five D' values of 0.000 and 15 positive and 16 negative values. In contrast, ignoring comparisons involving the incorrectly scored GPI-A, there were significant excesses of positive values in ten samples from 1985 (24 positive, 6 negative values; $X^2 = 10.4$, $P < 0.005$) and 16 samples from 1986 (34 positive and 14 negative, $X^2 = 8.3$, $P < 0.005$) in samples from Red Bluff Reservoir and the Pecos River (Echelle and Connor, 1989; Wilde and Echelle, 1992).

Cytonuclear Disequilibria

Pairwise cytonuclear (mtDNA/allozyme) disequilibrium values for all 1991-1992 samples within the hybrid swarm are provided in Table V. None of the pairwise tests are significant, nor are the sums of the chi-squares (Qs) for single tests over all localities (column sums in Table V). Like the nuclear gametic disequilibria, there is no significant positive or negative trend in cytonuclear gametic disequilibria in the 1991-1992 samples (12 positive and 12 negative values, Table V). Ignoring lack of independence due to linkage, the allelic counts in the individual 2 X 2 cytonuclear disequilibrium tables can be pooled into a single contingency test for each locality (Table VI; Forbes and Allendorf, 1991a). Although not a

conservative test, none of the samples exhibited significant cytonuclear association.

DISCUSSION

Data from 1991-1992 provide no support for multilocus selection as an explanation for the rapid genetic changes that followed the introduction of C. variegatus into the Pecos River in the early 1980s. Equivalent within-sample frequencies between introduced mtDNA and allozymes at different sites within the hybrid swarm are explained most parsimoniously by the hypothesis that portions of the genome of C. pecosensis have been replaced, without selection, by the introduced genome.

The multilocus selection hypothesis seems untenable because it would require an mtDNA selection differential that is both very large and equivalent to those associated with the rapid change in allozyme frequencies. This in turn implies a level of constraint or epistatic selection that might be detectable in assessments of cytonuclear disequilibrium. There is no evidence of such disequilibrium in the hybrid swarm (Table V). Similarly, Forbes and Allendorf (1991a) found no evidence of cytonuclear disequilibrium in another study of hybrid swarms between divergent subspecies of cutthroat trout (mtDNA sequence divergence = 2%; Nei's genetic identity from allozymes = 0.74).

The alternative mtDNAs of hybridizing species generally seem to be effectively equivalent against a wide range of nuclear backgrounds. For example, Forbes and Allendorf (1991b) found no effects of parental mtDNAs on morphology or developmental stability in their study of cutthroat trout hybrid swarms. Also, in a study of genetic introgression between two cyprinid fishes, Dowling and Hoeh (1991) found the mtDNA of one species (Notropis cornutus) to be nearly fixed in the nuclear background of the other species (N. chrysocephalus) in a population geographically removed from the present zone of contact and exhibiting no nuclear evidence of introgression. In that situation, the parental mtDNAs are highly divergent (7-8% mtDNA sequence divergence, Dowling and Hoeh, 1991) and hybrids are selectively inferior (Dowling and Moore, 1985). Given such observations, selection due to cytonuclear co-evolution is not likely to explain the high, parallel frequencies of introduced allozymes and mtDNAs in Pecos River pupfish.

The lack of any dramatic change in the genetic structure of C. pecosensis in Salt Creek further indicates that selection does not explain the rapid development of the hybrid swarm in the Pecos River. Sites P1 and P2 are upstream from a series of small waterfalls (<0.5 m high) near the mouth of Salt Creek that would retard upstream gene flow from the Pecos River. However, in 1987 and 1988, high stream flows resulted in frequent spillway discharge from

Red Bluff Reservoir into Salt Creek just upstream from site P1. Correspondingly, collections from near P1 in 1988 revealed low frequencies (0.02-0.04) of ADH-1 and GPI-A alleles diagnostic of C. variegatus (Wilde and Echelle, 1992). My sample revealed no evidence of those alleles in 1991. There has been no report of fish kills in Salt Creek upstream from the spillway. Thus, it appears that, in the presence of an abundant population of C. pecosensis, the hybrids introduced from Red Bluff Reservoir had little effect on the genetic structure of the Salt Creek population.

The only potential support for the multilocus selection hypothesis is that, at all except one site in the hybrid swarm (site #3), the frequencies of introduced allozymes increased between 1985-1986 and 1991-1992 at each of the four diagnostic nuclear loci (Fig. 4, but see ADH-1 at Pandale Crossing for an exception). At site 3, however, the trend was reversed, with a decline in introduced allozymes at all four loci. These parallel interlocus changes may reflect the decline in between-site heterogeneity that is expected as a result of gene flow in neutral clines (Endler, 1977). Regarding sites in the Pecos River proper (sites 2-6), the declines in introduced allozymes occurred at the site (3) having the highest frequencies in 1985 and 1986, while increases occurred at sites with lower frequencies. Similar adjustments might explain the change in frequency in

Red Bluff Reservoir. Red Bluff Dam precludes upstream gene flow from downstream populations in the Pecos River and the pupfish population (C. pecosensis) is sparse upstream from the reservoir (J. E. Brooks, pers. comm.). Thus, the increased frequencies of introduced allozymes at site 1 might reflect gene flow among genetically heterogeneous subpopulations within the lake.

The replacement hypothesis requires that the introduction of C. variegatus into the Pecos River coincided with a drastic decline in abundance of the endemic species, C. pecosensis. It seems unlikely that there would have been massive introductions of a small, nongame species like C. variegatus. For example, the relative abundance of introduced allozymes in 1985 (Echelle and Connor, 1989) indicates that, at the inception of the hybrid swarm, introduced genetic elements outnumbered those of C. pecosensis by a factor of about 9 to 1 in the vicinity of Pecos. Explaining these observations under the assumption that the endemic species was present at the usual density of pupfish in the area would require the introduction of perhaps hundreds of thousands of C. variegatus. It seems more likely that the introduction occurred at a time when the abundance of the native species was extremely low.

The potential for dramatic declines in the abundance of C. pecosensis is indicated by reports of extensive fish kills resulting from toxins released during blooms of the

alga, Prymnesium parvum, in the lower Pecos River. The first recorded kills occurred in the fall seasons of 1985 and 1986 and apparently extended from Iraan (site 5, Fig 1) downstream to the backwaters of Lake Amistad, an impoundment of the Rio Grande (James and De La Cruz, 1989). Most of this area lies outside the historical range of C. pecosensis, except for the collection of a single specimen approximately 75 km downstream from Iraan (Echelle and Echelle, 1978). A geographically more extensive kill in the fall of 1988 extended from southeastern New Mexico downstream at least as far as Pandale (my site 6; James and De La Cruz, 1989; Rhodes and Hubbs, 1992). I am aware of no reports of fish kills between 1980 and 1984 when C. variegatus was introduced into the Pecos River. However, unreported fish kills are not difficult to imagine for this remote, primarily rural area. The river in the study area flows through no centers of human habitation and supports a limited amount of human recreation.

There was no quantification of the effect of the reported fish kills on pupfish abundance in the Pecos River. James and De La Cruz (1989) stated that "all fish inhabiting the fish kill areas were affected," although apparently there was no attempt to sample for live fish. Rhodes and Hubbs (1992), on the other hand, suggested that the pupfish was less affected by the algal toxins than were other fishes. Rhodes and Hubbs (1992) noted that, during the 1988

kill, the pupfish in the field "seemed lethargic", but did not exhibit any other signs of ichthyotoxin poisoning. The intensity of algal-induced fish kills probably varies from locality to locality and among years, depending on environmental conditions. The introduction of C. variegatus might have coincided with a particularly severe kill.

The allozyme and mtDNA data together indicate that the hybrid populations I examined probably originated from at least two separate introductions, one in Red Bluff Reservoir and one downstream in the Pecos River proper. A single introduction in the vicinity of Pecos would explain the clinal patterns of allozyme variation in the Pecos River proper (Echelle and Connor, 1989), but it does not explain the presence of two introduced mtDNA haplotypes in Red Bluff Reservoir, one of which (haplotype C) was not detected elsewhere in the hybrid swarm. Dispersal into the Pecos River from a single introduction in Red Bluff Reservoir appears unlikely to explain the genetic structure of the hybrid swarm because the average 1991 introduced allozyme frequencies at some sites in the Pecos River (0.87 and 0.85 at Brush Dam and Pecos, respectively) are much higher than in the reservoir (0.39-0.50; $\bar{X} = 0.46$) and frequencies at intervening sites in 1985 were even lower (<0.30, Echelle and Connor, 1989).

Thus, the data indicate two separate introductions, both of which coincided with extremely low population

densities for the native species, C. pecosensis. This, in turn, suggests that transport and release of C. variegatus, possibly as a result of sport- and/or baitfishing activities, is a persistent human activity in the Pecos River area of Texas, and there is some evidence from historical collections that this is true. In addition to the introductions in Red Bluff Reservoir and the Pecos River proper, there have been at least two other introductions of C. variegatus into isolated waters of the Pecos River drainage, one in Lake Balmorhea (Stevenson and Buchanan, 1972) and another in a springfed section of Leon Creek (Hubbs, 1980). The latter population was eliminated in the late 1970s (Hubbs, 1980), but the former persists as a dense population and is a possible source of stock for the introductions in Red Bluff Reservoir and the Pecos River proper.

Lake Balmorhea has supported an introduced population of C. variegatus since the 1960s (Stevenson and Buchanan, 1972). Although isolated from the Pecos River proper, Lake Balmorhea is only about 60 km from the river and incidental transport of fishes between the two bodies of water is easy to envision. Correspondingly, my sample from this population had a moderately high frequency (15%) of GPI-A-c, an allele that is normally rare in natural populations of C. variegatus on the Texas Coast (0-7%), but represents an unusually high proportion (29-57%) of the introduced GPI-A

alleles in Red Bluff Reservoir and the Pecos River populations. Possibly due to sampling error, the introduced mtDNA known only from Red Bluff Reservoir (haplotype C) was absent from the 40 specimens examined from Lake Balmorhea; about 13 out of 100 such samples would not include a haplotype present at a frequency of 0.05.

The following hypothesis may explain the rapid spread, sometime between 1980 and 1984, of introduced genetic elements over more than 400 river-kilometers (Echelle and Connor, 1989) of the Pecos River proper. In this scenario, the introduction of C. variegatus would have occurred in the vicinity of Brush Dam or Pecos, at a time when C. pecosensis was extremely uncommon, perhaps due to an algal-induced fish kill. The two species then would have formed a population of intergrades (hybrid swarm) which expanded in numbers and began to disperse. Dispersal of intergrades would have occurred more rapidly than would have been possible at times when pupfish and other fishes occurred in greater densities. A similar effect may explain the present common occurrence of intergrades in downstream areas (e.g., Pandale) at least 55 km downstream from the recorded range of C. pecosensis (Wilde and Echelle, 1992). Historically, C. pecosensis was either absent or extremely rare in the Pandale area, possibly as a result of community constraints imposed by the relatively diverse, freshwater fauna that occupied the area (Echelle and Echelle, 1978). An attempt in 1986 to collect

pupfish downstream from Pandale, in the Pecos River arm of Lake Amistad, was unsuccessful (A. A. Echelle, pers. comm.).

In summary, the pattern of geographic variation in frequencies of introduced genetic elements in the Pecos River proper and in Red Bluff Reservoir can be explained on the basis of replacement (without selection) of a portion of the genome of C. pecosensis, following separate introductions of C. variegatus in Red Bluff Reservoir and in the Pecos River proper. All measures of within-sample genetic structure are compatible with local panmixia in an admixture of the two species. Between-locality differences in frequencies of individual elements in the Pecos River proper are attributable to dispersal from a single site of introduction, together with genetic drift, either at the time of dispersal of intergrades, or subsequently, perhaps during population bottlenecks due to fish kills. Genetic drift would explain the striking lack of between-site concordance in ranked frequencies of introduced genetic elements. Mitochondrial DNA is more susceptible to genetic drift than are nuclear genes. Correspondingly, the between-site differences in frequency of mtDNA were greater than that for any of the four nuclear genes (Fig. 3). With time, the between-site differences should diminish in magnitude, and there is some evidence that this is occurring.

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APPENDICES

APPENDIX A

TABLES

Table I. Genotypic arrays for four loci in reference samples of C. pecosensis (samples P1 and P2) and C. variegatus (samples V1 and V2) and for pupfish from the Pecos River drainage of Texas (samples 1-6). Allele assignments are shown at the bottom of the table (P = C. pecosensis; V = C. variegatus). Locality numbers are in parentheses. Alleles are given letters in alphabetic order of decreasing anodal mobility.

	Locus			
Locality (number)	ADH-1	EST-1	GPI-A	PEPD-1
C. pecosensis:				
Salt Creek (P1)	40 aa	39 aa	39 dd	2 bc
		1 ab	1 de	38 cc
Salt Creek, Hwy 285				
Crossing (P2)	24 aa	24 aa	24 dd	1 bc
				23 cc
Pecos River mainstem:				
Red Bluff				
Reservoir (1)	16 aa	8 aa	4 bb	8 bb
	17 ac	24 ab	1 bc	21 bc
	7 cc	8 bb	12 bd	11 cc

TABLE I. Continued.

Locality (number)	Locus			
	ADH-1	EST-1	GPI-A	PEPD-1
			1 be	
			5 cc	
			4 cd	
			4 ce	
			9 dd	
Brush Dam (2)	1 aa	1 aa	9 bb	28 bb
	9 ac	5 ab	14 bc	10 bc
	30 cc	34 bb	7 bd	2 cc
			1 be	
			6 cc	
			3 cd	
Pecos (3)	8 ac	1 aa	11 bb	26 bb
	32 cc	8 ab	7 bc	12 bc
		31 bb	11 bd	2 cc
			7 cc	
			4 cd	

TABLE I. Continued.

Locality (number)	Locus			
	ADH-1	EST-1	GPI-A	PEPD-1
Horsehead (4)	13 aa	5 aa	9 bb	1 ab
	14 ac	17 ab	9 bc	13 bb
	13 cc	18 bb	12 bd	11 bc
			1 cc	15 cc
			5 cd	
			4 dd	
Iraan (5)	6 aa	4 aa	7 bb	14 bb
	20 ac	23 ab	14 bc	17 bc
	14 cc	13 bb	8 bd	9 cc
			1 cc	
			5 cd	
			5 dd	
Pandale (6)	1 aa	4 aa	4 bb	28 bb
	17 ac	7 ab	13 bc	8 bc
	22 cc	29 bb	7 bd	4 cc
			5 cc	
			3 cd	
			8 dd	

TABLE I. Continued.

Locality (number)	Locus			
	ADH-1	EST-1	GPI-A	PEPD-1
C. variegatus:				
Balmorhea (V1)	1 ac	20 bb	14 bb	19 bb
	19 cc		6 bc	1 bc
Edinburg (V2)	29 cc	3 ab	1 ab	1 ab
	1 ee	27 bb	1 ac	29 bb
			25 bb	
			3 bc	
Allele assignments:	P = a	P = a	P = d, e	P = c
	V = c,e	V = b	V = a,b,c	V = a,b

Table II. Contingency tests (Zar 1974) for mtDNA and allozyme markers. Values are observed (OBS) and expected (EXP) numbers of mtDNA haplotypes for C. pecosensis (P) and C. variegatus (V) and the test statistic G (using Williams' correction). Expected values are based on the average frequency of diagnostic nuclear genes (ADH-1, EST-1, GPI-A, PEPD-1) at each locality. No tests were significant at the 0.05 level when corrected for multiple tests (Rice, 1989). A = the common haplotype for C. variegatus, and C = the haplotype detected only at Red Bluff Reservoir.

Locality	mtDNA	OBS	EXP	G
Red Bluff Reservoir	P	18	21	0.453
	V (A,C)	15,6	18	
Brush Dam	P	5	5	0.000
	V	35	35	
Pecos	P	11	6	1.833
	V	29	34	
Horsehead Crossing	P	22	17	1.231
	V	18	23	
Iraan	P	21	15	1.791
	V	19	25	
Pandale	P	3	10	4.529*
	V	37	30	

* $0.025 < P < 0.05$.

Table III. Gene frequency differences by sex: 2 x 2 G-test values for counts of C. pecosensis and C. variegatus mtDNA and nuclear alleles by sex. (+) = males have excess pecosensis alleles. (-) = females have excess pecosensis alleles.

Locality	Genetic Element						
number	MtDNA	ADH-1	EST-1	GPI-A	PEPD-1	SUM	df
1	0.823	0.011	0.106	0.037	0.161	1.138	5
	(+)	(-)	(+)	(-)	(+)		
2	1.660	2.295	0.028	1.173	0.087	5.243	5
	(+)	(-)	(-)	(+)	(+)		
3	0.025	0.180	1.181	0.757	0.307	2.450	5
	(+)	(+)	(-)	(+)	(-)		
4	1.650	0.000	0.159	0.844	0.280	2.933	5
	(+)	--	(+)	(+)	(+)		
5	0.044	0.005	0.000	0.896	0.183	1.128	5
	(-)	(+)	--	(+)	(-)		
6	0.366	0.870	0.041	0.457	0.000	1.734	5
	(+)	(-)	(+)	(+)	--		

No tests were significant ($P > 0.10$ for all tests).

Table IV. Standardized linkage disequilibrium values (D') in pupfish from the Pecos River in Texas. No tests were significant at the 5% level when corrected for multiple tests (Rice, 1989), although uncorrected individual tests were significant for samples 5 and 6.

	Locality number					
Paired loci	1	2	3	4	5	6
ADH-1, EST-1	0.000	-0.986	0.146	0.076	-0.232	0.022
ADH-1, GPI-A	-0.131	-1.373	0.000	-0.328	-0.156	-0.825
ADH-1, PEPD-1	-0.119	0.016	0.287	-0.053	0.000	0.066
EST-1, GPI-A	-0.102	0.183	0.032	0.254	0.449*	1.038**
EST-1, PEPD-1	0.111	0.275	-1.026	0.025	0.168	-0.340
GPI-A, PEPD-1	0.000	-0.453	0.000	-0.291	-0.217	-0.275

* $P < 0.025$; ** $P < 0.001$.

Table V. Pairwise cytonuclear disequilibria for hybrid pupfish populations in the Pecos River, Texas. Values are the chi-square distributed statistic Q with sign of D (Forbes and Allendorf, 1991). Chi-square values are summed over all sites (df = number of sites) for each pairwise comparison. No tests were significant at the 5% level, uncorrected for multiple tests.

		Paired Loci			
		MtDNA Versus:			
Locality number	N	ADH-1	EST-1	GPI-A	PEPD-1
1	39	0.371	-0.413	-0.626	-0.015
2	40	0.188	-0.548	-0.068	0.025
3	40	-0.502	0.018	-0.003	0.070
4	40	-0.101	0.522	0.595	-0.404
5	40	-0.067	0.314	-3.151	0.040
6	40	0.164	2.079	1.726	-0.811
Total X^2		1.393	3.894	6.169	1.365
df = 6					

Table VI. Combined test for cytonuclear disequilibria across all diagnosic nuclear loci and all hybrid localities within the Pecos River, Texas. Values are total C. pecosensis (P) and C. variegatus (V) nuclear alleles at loci listed in Table V vs. mtDNA haplotypes. G values are listed for each pooled 2 x 2 test (df = 1), along with the sign of the disequilibrium association. Significance (SIG) of one-tailed probability tests for positive association are also shown (see Forbes and Allendorf, 1991).

Locality number	Nuclear alleles	MtDNA Haplotype P	MtDNA Haplotype V	G	SIG
1	P	75	94	0.468 (-)	NS
	V	69	74		
2	P	5	38	0.035 (-)	NS
	V	35	242		
3	P	13	36	0.027 (-)	NS
	V	75	196		
4	P	74	58	0.102 (+)	NS
	V	102	86		
5	P	60	61	0.662 (-)	NS
	V	108	91		
6	P	9	67	2.461 (+)	NS
	V	15	229		

APPENDIX B

FIGURES

Figure 1. Collection sites for reference samples of C. pecosensis (sites P1 and P2) and C. variegatus (sites V1 and V2) and samples from the hybrid swarm in the Pecos River drainage of Texas (sites 1-6). The study area extends from the New Mexico-Texas border (Red Bluff Reservoir), southeast through Texas.

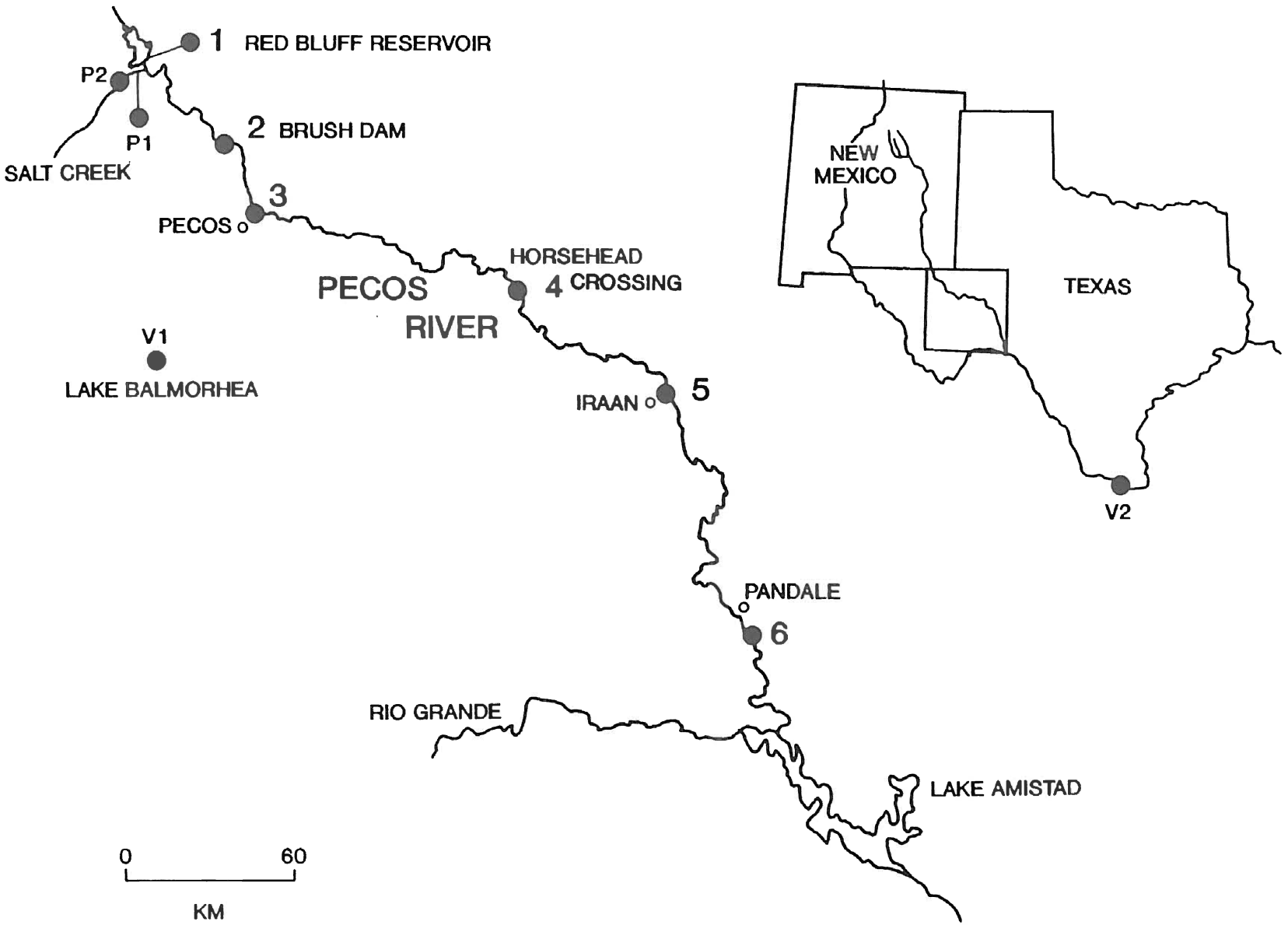

1 RED BLUFF RESERVOIR
P2
P1
SALT CREEK
2 BRUSH DAM
3
PECOS
HORSEHEAD
4 CROSSING
PECOS
RIVER
V1
LAKE BALMORHEA
IRAAN
5
PANDALE
6
RIO GRANDE
LAKE AMISTAD
NEW
MEXICO
TEXAS
V2
0
60
KM

Figure 2. Mean frequency of diagnostic C. variegatus alleles at four loci and the frequency of a cryptic and typically rare C. variegatus allele (GPI-A-c) at six localities in the hybrid swarm (sites 1-6, Fig. 1). Distance = river-kilometers.

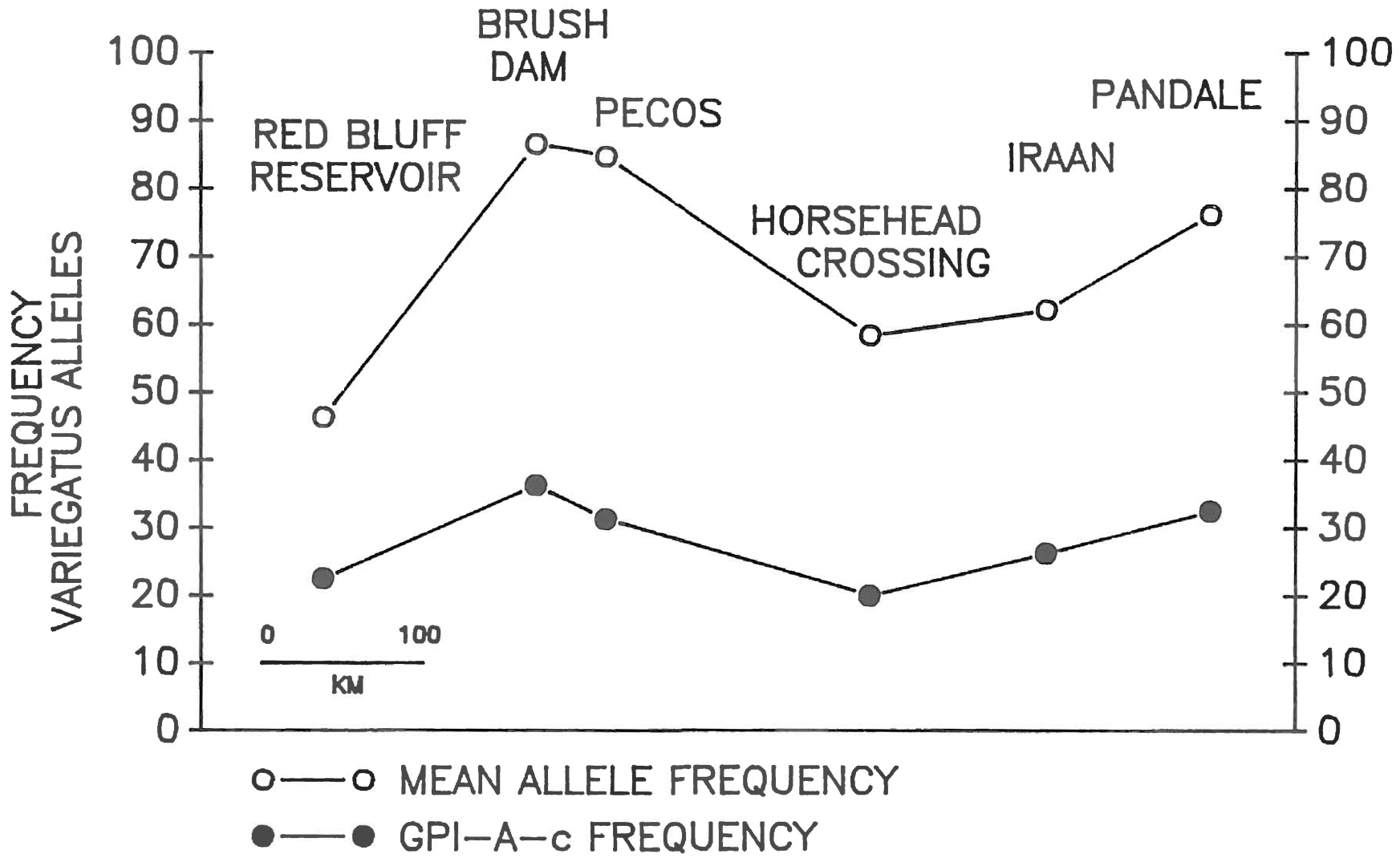

BRUSH
DAM
PECOS
PANDALE
RED BLUFF
RESERVOIR
IRAAN
HORSEHEAD
CROSSING
FREQUENCY
VARIEGATUS ALLELES
100
90
80
70
60
50
40
30
20
10
0
0
100
KM
MEAN ALLELE FREQUENCY
GPI–A–c FREQUENCY

Figure 3. Frequency of diagnostic C. variegatus alleles and mtDNA haplotypes in samples of pupfish from the mainstream of the Pecos River in Texas (sites 1-6, Fig. 1). Distance = river-kilometers.

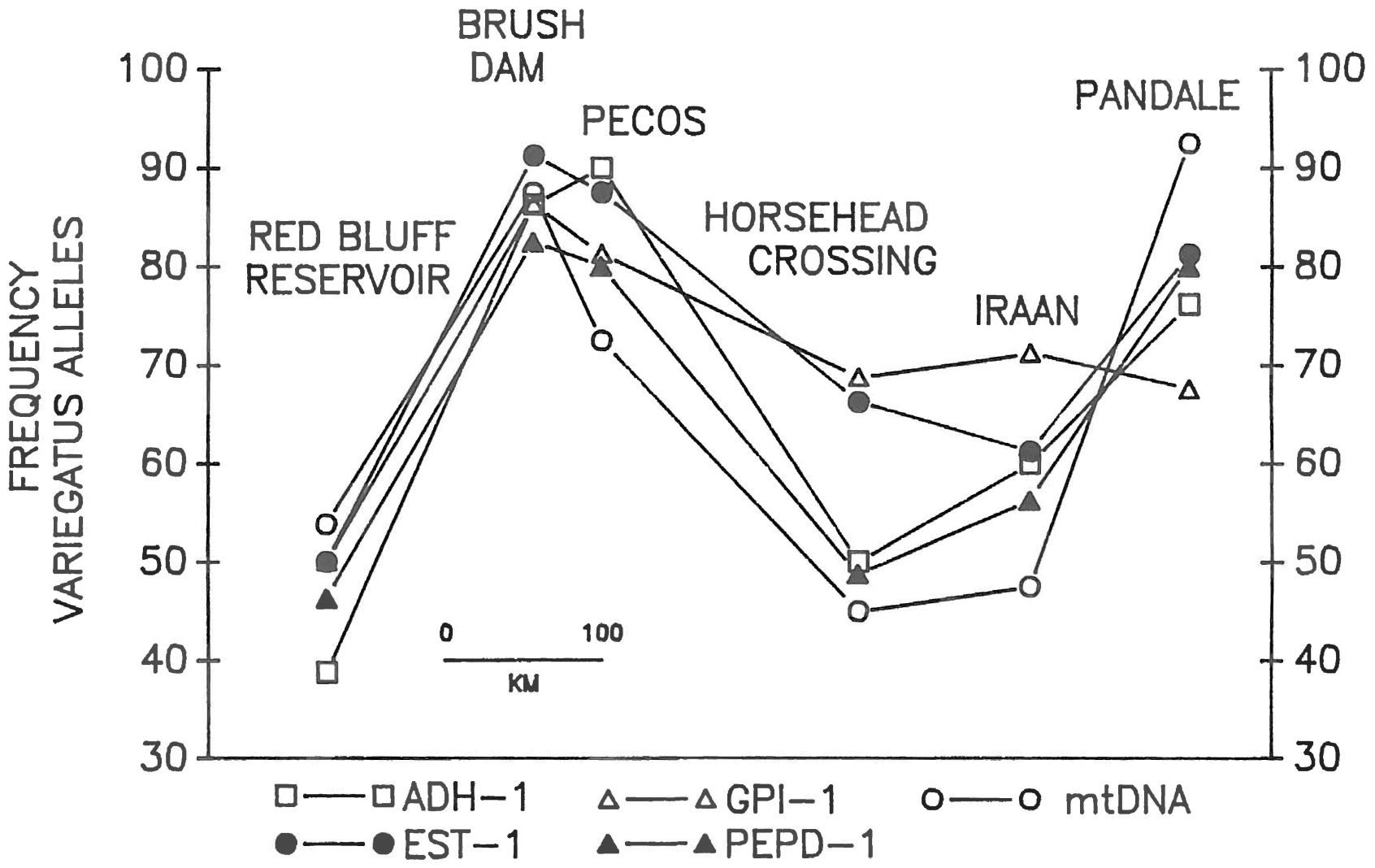
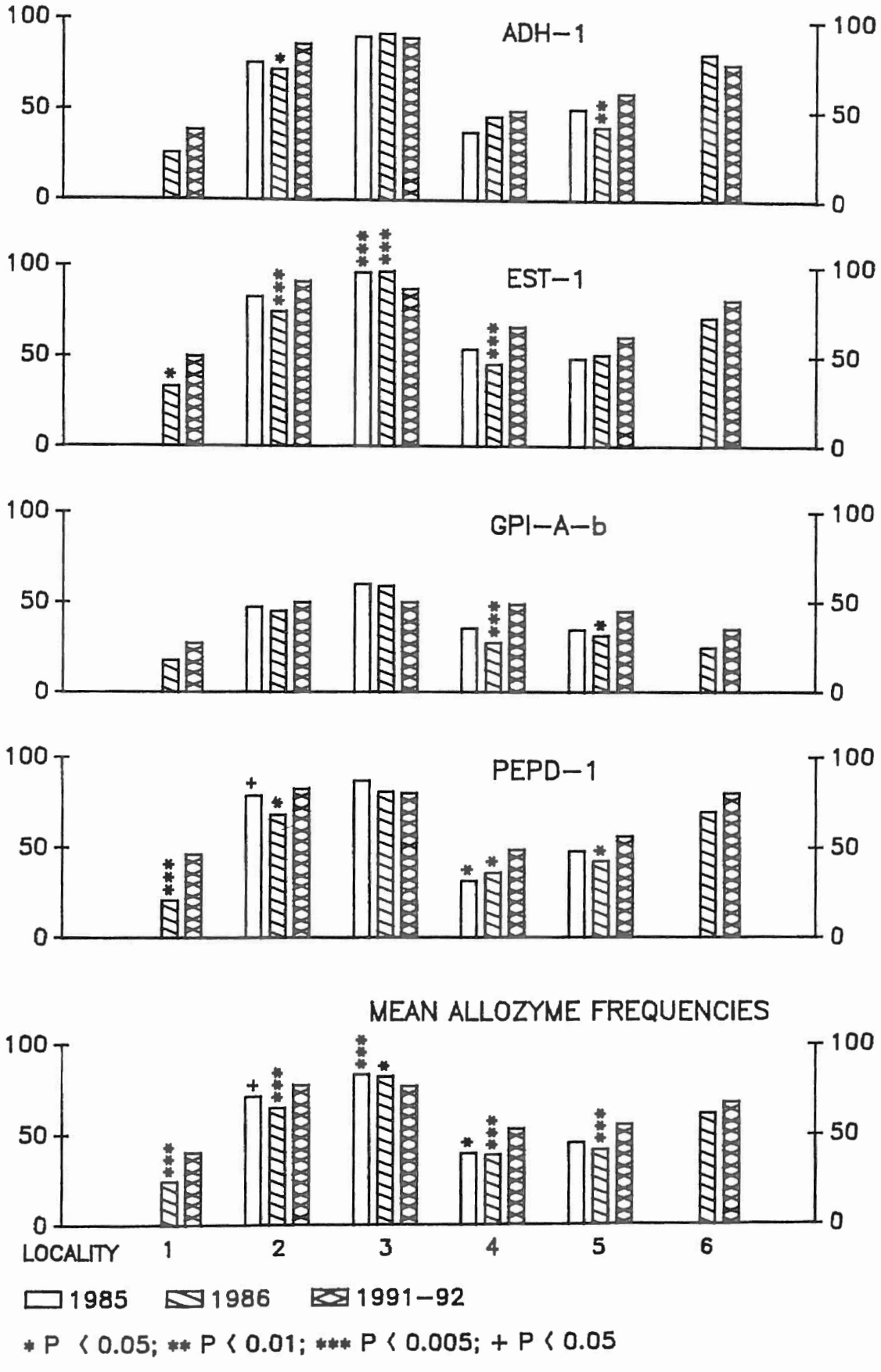

BRUSH
DAM
PECOS
PANDALE
RED BLUFF
RESERVOIR
HORSEHEAD
CROSSING
IRAAN
FREQUENCY
VARIEGATUS ALLELES
100
90
80
70
60
50
40
30
0
100
KM
ADH–1
GPI–1
mtDNA
EST–1
PEPD–1

Figure 4. Frequency histogram of diagnostic C. variegatus alleles at individual nuclear loci in samples of pupfish from the Pecos River drainage in Texas (localities 1-6) in 1985 (Echelle and Connor, 1989), 1986 (Wilde and Echelle, 1992), and 1991-1992 (present study). Asterisks (*) indicate significant differences between 1991-92 data and either 1985 or 1986 data, depending upon the location of the symbol. Likewise, (+)s indicate significant differences between 1985 and 1986 data. Statistical significance is based upon heterogeneity chi-squares, provided by BIOSYS-1 (Swofford and Selander, 1981).


ADH–1
EST–1
GPI–A–b
PEPD–1
MEAN ALLOZYME FREQUENCIES
100
50
0
LOCALITY 1 2 3 4 5 6
1985
1986
1991–92
* P < 0.05; ** P < 0.01; *** P < 0.005; + P < 0.05

APPENDIX C

GENOTYPIC ARRAYS

Genotypic scores of specimens surveyed across four loci by gene pairs for Red Bluff Reservoir (site 1) in 1992.

	EST-1				GPI-A		
ADH-1	PP	PV	VV		PP	PV	VV
PP	3	9	4		4	8	4
PV	4	11	2		3	9	5
VV	1	4	2		3	3	1

	EST-1				PEPD-1		
GPI-A	PP	PV	VV		PP	PV	VV
PP	4	4	2		4	3	3
PV	1	12	7		5	11	4
VV	1	8	1		2	7	1

	EST-1				ADH-A		
PEPD-1	PP	PV	VV		PP	PV	VV
PP	3	7	1		4	4	3
PV	4	11	6		8	11	2
VV	1	6	1		4	2	2

Genotypic scores of specimens surveyed across four loci by gene pairs for Brush Dam (site 2) in 1991.

	EST-1			GPI-A		
ADH-1	PP	PV	VV	PP	PV	VV
PP	0	0	1	0	0	1
PV	0	1	8	0	0	8
VV	1	4	25	0	10	20

	EST-1			PEPD-1		
GPI-A	PP	PV	VV	PP	PV	VV
PP	0	0	0	0	0	0
PV	0	3	8	0	3	8
VV	1	2	26	2	7	20

	EST-1			ADH-A		
PEPD-1	PP	PV	VV	PP	PV	VV
PP	0	1	1	0	0	2
PV	0	2	8	1	2	7
VV	1	2	25	0	7	21

Genotypic scores of specimens surveyed across four loci by gene pairs for Pecos (site 3) in 1991.

	EST-1				GPI-A		
ADH-1	PP	PV	VV		PP	PV	VV
PP	0	0	0		0	0	0
PV	0	3	5		0	3	5
VV	1	5	26		0	12	20

	EST-1				PEPD-1		
GPI-A	PP	PV	VV		PP	PV	VV
PP	0	0	0		0	0	0
PV	1	2	12		1	4	10
VV	0	6	19		1	8	16

	EST-1				ADH-A		
PEPD-1	PP	PV	VV		PP	PV	VV
PP	0	1	1		0	1	1
PV	0	0	12		0	3	9
VV	1	7	18		0	4	22

Genotypic scores of specimens surveyed across four loci by gene pairs for Horsehead Crossing (site 4) in 1991.

	EST-1				GPI-A		
ADH-1	PP	PV	VV		PP	PV	VV
PP	1	7	5		1	4	8
PV	2	6	6		0	9	5
VV	2	4	7		3	4	6

	EST-1				PEPD-1		
GPI-A	PP	PV	VV		PP	PV	VV
PP	1	1	2		1	0	3
PV	3	9	5		6	6	5
VV	1	7	11		8	5	6

	EST-1				ADH-A		
PEPD-1	PP	PV	VV		PP	PV	VV
PP	3	6	6		4	5	6
PV	0	4	7		4	6	1
VV	2	7	5		5	3	6

Genotypic scores of specimens surveyed across four loci by gene pairs for Iraan (site 5) in 1991.

	EST-1				**GPI-A**		
ADH-1	PP	PV	VV		PP	PV	VV
PP	0	4	2		0	3	3
PV	1	12	7		3	5	12
VV	3	7	4		2	5	7

	EST-1				**PEPD-1**		
GPI-A	PP	PV	VV		PP	PV	VV
PP	2	3	0		2	2	1
PV	1	8	4		0	6	7
VV	1	12	9		7	9	6

	EST-1				**ADH-A**		
PEPD-1	PP	PV	VV		PP	PV	VV
PP	1	6	2		1	6	2
PV	2	10	5		3	6	8
VV	1	7	6		2	8	4

Genotypic scores of specimens surveyed across four loci by gene pairs for Pandale Crossing (site 6) in 1992.

	EST-1				GPI-A		
ADH-1	PP	PV	VV		PP	PV	VV
PP	0	0	1		0	0	1
PV	2	3	12		2	3	12
VV	2	4	16		6	7	9

	EST-1				PEPD-1		
GPI-A	PP	PV	VV		PP	PV	VV
PP	3	1	4		1	1	6
PV	1	4	5		1	1	8
VV	0	2	20		2	6	14

	EST-1				ADH-A		
PEPD-1	PP	PV	VV		PP	PV	VV
PP	0	1	3		0	2	2
PV	1	1	6		0	4	4
VV	3	5	20		1	11	16

Homozygous C. pecosensis (PP) and C. variegatus (VV) genotypes, as well as heterozygotes (PV) are shown.

APPENDIX D

CYTONUCLEAR ARRAYS

Cytonuclear scores of specimens surveyed across four loci by gene pairs for Red Bluff Reservoir (site 1) in 1992.

ADH-1

MtDNA	PP	PV	VV
P	10	4	4
V	6	12	3

EST-1

MtDNA	PP	PV	VV
P	2	12	4
V	6	11	4

GPI-A

MtDNA	PP	PV	VV
P	5	6	7
V	5	14	2

PEPD-1

MtDNA	PP	PV	VV
P	5	9	4
V	6	11	4

Cytonuclear scores of specimens surveyed across four loci by gene pairs for Brush Dam (site 2) in 1991.

ADH-1

MtDNA	PP	PV	VV
P	1	0	4
V	0	9	26

EST-1

MtDNA	PP	PV	VV
P	0	0	5
V	1	5	29

GPI-A

MtDNA	PP	PV	VV
P	0	1	4
V	0	10	25

PEPD-1

MtDNA	PP	PV	VV
P	0	2	3
V	2	8	25

Cytonuclear scores of specimens surveyed across four loci by gene pairs for Pecos (site 3) in 1991.

ADH-1

MtDNA	PP	PV	VV
P	0	1	10
V	0	6	22

EST-1

MtDNA	PP	PV	VV
P	1	1	9
V	0	5	23

GPI-A

MtDNA	PP	PV	VV
P	0	4	7
V	0	11	17

PEPD-1

MtDNA	PP	PV	VV
P	1	3	7
V	1	9	18

Cytonuclear scores of specimens surveyed across four loci by gene pairs for Horsehead Crossing (site 4) in 1991.

ADH-1

MtDNA	PP	PV	VV
P	6	8	7
V	7	5	6

EST-1

MtDNA	PP	PV	VV
P	3	9	9
V	2	3	13

GPI-A

MtDNA	PP	PV	VV
P	3	10	8
V	1	7	10

PEPD-1

MtDNA	PP	PV	VV
P	7	6	8
V	7	6	5

Cytonuclear scores of specimens surveyed across four loci by gene pairs for Iraan (site 5) in 1991.

ADH-1

MtDNA	PP	PV	VV
P	2	12	7
V	4	8	7

EST-1

MtDNA	PP	PV	VV
P	2	14	5
V	2	9	8

GPI-A

MtDNA	PP	PV	VV
P	2	3	16
V	3	10	6

PEPD-1

MtDNA	PP	PV	VV
P	5	9	7
V	4	8	7

Cytonuclear scores of specimens surveyed across four loci by gene pairs for Pandale Crossing (site 6) in 1992.

ADH-1

MtDNA	PP	PV	VV
P	0	2	1
V	1	15	21

EST-1

MtDNA	PP	PV	VV
P	1	1	1
V	3	6	28

GPI-A

MtDNA	PP	PV	VV
P	2	0	1
V	6	10	21

PEPD-1

MtDNA	PP	PV	VV
P	0	0	3
V	4	8	25

P = C. pecosensis; V = C. variegatus.

VITA

Michael Ray Childs

Candidate for the Degree of

Master of Science

Thesis: DYNAMICS OF INTROGRESSIVE HYBRIDIZATION BETWEEN PECOS PUPFISH (CYPRINODON PECOSENSIS) AND SHEEPSHEAD MINNOW (C. VARIEGATUS) IN THE PECOS RIVER, TEXAS

Major Field: Zoology

Biographical:

Personal Data: Born in Phoenix, Arizona, 15 July, 1967, the son of Malcolm R. and Marie Childs.

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