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DIVERGENCE AMONG MOBILE BASIN POPULATIONS OF THE PLEUROCERID SNAIL GENUS, *LEPTOXIS*, ESTIMATED BY ALLOZYME ELECTROPHORESIS

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ABSTRACT

Although the Mobile River Basin of Alabama was historically a center of great pleurocerid diversity, populations today are small and scattered. We obtained samples of all four nominal species of Mobile Basin *Leptoxis* currently extant: *L. ampla* (3 populations), *L. picta* (1 population), *L. plicata* (2 populations), and *L. taeniata* (2 populations). Gene frequencies at nine variable enzyme loci were determined for about 30 individuals from each population using horizontal starch gel electrophoresis. Samples of about 30 individuals from three populations of the widespread *Leptoxis praerosa* were analyzed as controls. Within populations, 18 of 99 loci were polymorphic, none showing genotype frequencies significantly different from Hardy-Weinberg expectation. Between populations within species, statistically significant divergence was apparent at most loci. Comparisons among the nominal species showed *L. praerosa* and *L. plicata* to be quite distinct from each other, and from all other populations. Much lower levels of divergence among populations nominally *L. picta, L. ampla,* and *L. taeniata* seem more consistent with a hypothesis of geographic isolation than reproductive isolation. We refer to these three taxa as the "*Leptoxis picta* group." Our results compare favorably in most respects with previously published data on mitochondrial 16S rRNA gene sequence divergence among these taxa, especially in the genetic distinction of *L. plicata*. The relationships within the *L. picta* group warrant further study.

Key words: genetics, isozymes, speciation, freshwater, gastropods, Alabama, endangered species.

INTRODUCTION

The rivers and streams of Alabama's Mobile River Basin have recently attracted attention as a center of endemism for a variety of aquatic life, including turtles, fish, bivalves and prosobranch snails (Lydeard & Mayden, 1995). Based primarily on the revisions of Goodrich (1922, 1924, 1936, 1941), Burch (1989) recognized 77 species of pleurocerid snails from the region, 95% of which were unknown outside the Mobile Basin. Burch's list included 6 species of *Gyrotoma*, 5 species of *Pleurocera*, 52 species of *Elimia* (synonymizing *Goniobasis* as used by Goodrich), and 14 *Leptoxis* (lowering *Anculosa*, as used by Goodrich, to subgeneric level). During the present century, however, most of the larger rivers of the Mobile Basin have been impounded for hydroelectric power, channelized, or otherwise modified for navigation. The Mobile Basin pleurocerid fauna has also been adversely impacted by changing patterns of land use, first from siltation due to intensive agriculture, and more recently from pollution. Lydeard & Mayden (1995) presumed extinct 29 species of Mobile Basin pleurocerids, including all six species of the endemic genus *Gyrotoma*.

The *Leptoxis* species of the basin have been the object of special concern. Of the 11 species of *Leptoxis* known historically from the Coosa River, Bogan & Pierson (1993a) found only *L. taeniata* (Conrad, 1834), apparently restricted now to but a few small tributaries. Of the four *Leptoxis* species documented from the Cahaba River, only *L. ampla* (Anthony, 1855) apparently survives, inhabiting a 30 km reach of the main river and several smaller Cahaba tributaries (Bogan & Pierson, 1993b). The only *Leptoxis* population remaining in the Black Warrior drainage is *L. plicata* (Conrad, 1834), restricted to a short reach of Locust Fork. Based on these data, as well as extensive U. S. Fish & Wildlife Service field records, Hartfield (1997) identified *L. taeniata, L. ampla,* and *L. plicata* as candidates for addition to the U. S. list of endangered and threatened wildlife and plants. The status of the only other nominal *Leptoxis* species

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known with certainty to have survived in the Mobile Basin, *L. picta* (Conrad, 1834) of the main Alabama River, continues to be monitored.

But Hartfield noted that the genetic relationships among these four nominal species of *Leptoxis* are poorly understood. They are distinguished primarily by minor attributes of shell shape and size, traits long known for clinal variability (Goodrich, 1934, 1935). The non-genetic component of some aspects of pleurocerid shell morphology is well-documented (Chambers, 1982; Dillon, 1984a).

In light of these concerns, Lydeard et al. (1997) surveyed 15 pleurocerid populations from the Mobile Basin: seven *Elimia* and four *Pleurocera*, in addition to the four nominal *Leptoxis* species. A molecular phylogeny constructed from mitochondrial 16S rRNA gene sequences suggested that Alabama *Elimia* and *Pleurocera* are sister taxa. The four *Leptoxis* species were quite different from the *Elimia/Pleurocera* group, and depicted as paraphyletic. Levels of sequence divergence were low between *L. taeniata* and *L. ampla*, with *L. picta* and *L. plicata* appearing more distinct.

Allozyme electrophoresis is an older and more established technique for evaluating the specific status of pleurocerid populations, especially the large genus *Goniobasis*. Extensive surveys of variation at allozyme-encoding loci, involving at least 11 species and 58 populations, have established that *Goniobasis* shows unusually low levels of heterozygosity, high levels of divergence between populations within species, and very few shared alleles at any locus when compared among species (Chambers, 1978, 1980; Dillon, 1984b, 1988a; Dillon & Davis, 1980; Bianchi et al., 1994; Stiven & Kreiser, 1994). Recent evidence suggests similar trends in *Leptoxis*, although intrapopulation variation may be somewhat greater, and interpopulation variation less (Dillon & Ahlstedt, 1997).

The purposes of the present work are twofold. We survey the allozyme divergence displayed by populations representing the four nominal species of Mobile River basin *Leptoxis* to gather further evidence regarding their genetic distinction. We also compare the levels of allozyme divergence estimated here to the DNA sequence divergence estimates of Lydeard et al. (1997), as a possible guide to the future application of the newer technology.

METHODS

We analyzed eight populations of Alabama *Leptoxis* assigned to four species (Appendix). Our *L. taeniata* populations were sampled from Buxahatchee and Choccolocco creeks, two tributaries of the Coosa River. As no *Leptoxis* inhabit the 50 km reach of the Coosa River separating these two creeks, gene flow between the populations we designated Taebux and Taechc, respectively, would seem to be negligible at present. We obtained samples of *L. ampla* from three shoals of the Cahaba River separated about 20 river km from each other, labeled Ampcah1, Ampcah2, and Ampcah3 from upstream down. Our two samples of *L. plicata* are from Locust Fork, Pliloc1 about 15 river km upstream from Pliloc2. Our single sample of *L. picta* (Picala) was collected by boat from limestone walls and outcrops in the lower Alabama River.

We selected three populations of the well-characterized *Leptoxis praerosa* (Say, 1821) to provide calibration for our analysis. Populations of this species are common and widespread throughout the Ohio, Cumberland, and Tennessee river drainages. Our *L. praerosa* came from three tributaries of the Tennessee River, the Elk River (Praelk), the Duck River (Praduk), and the Sequatchie River (Praseq). *Leptoxis* from the Sequatchie and Duck rivers have been previously analyzed by Dillon & Ahlstedt (1997). Analyzing all 11 populations together, we were able to evaluate observed levels of genetic divergence among nominal Alabama species by comparison to divergence among *Leptoxis* populations known to be conspecific, isolated at approximately equivalent distances.

The geographic relationships among the 11 populations analyzed in this work are mapped in Figure 1, and locality data and sample sizes are given in the Appendix. Although our sample sizes were in most cases greater than 30, only 21 individual *L. picta* were available. The Appendix also provides catalog numbers for voucher specimens deposited in the Academy of Natural Sciences of Philadelphia.

Our equipment and techniques for horizontal starch gel electrophoresis of whole animal homogenates have been previously described (Dillon, 1985, 1992). Samples were initially run on gels of five different buffer systems and stained to visualize 13 different enzymes. We simultaneously screened these gels and stains by requiring that clearly inter-

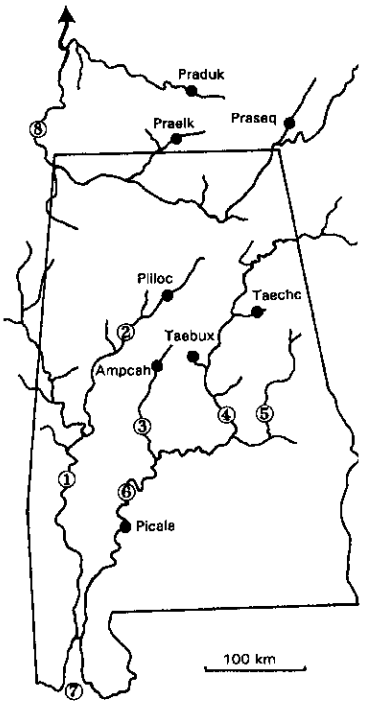


FIG. 1. The state of Alabama, showing major drainages and sample sites. (1) Tombigbee River, (2) Black Warrior River, (3) Cahaba River, (4) Coosa River, (5) Tallapoosa River, (6) Alabama River, (7) Mobile Bay, (8) Tennessee River.

pretable polymorphism be present in an initial comparison of Praduk and Ampcah1, selected as the most different pair of populations in our study. Ultimately we identified the products of nine putative gene loci for detailed analysis over all 11 populations.

The Poulik buffer (Poulik, 1957) was used to resolve glucose phosphate isomerase (GPI, EC 5.3.1.9) and octopine dehydrogenase (ODH, EC 1.5.1.11). The AP6 buffer (Clayton & Tretiak, 1972) was used to resolve mannose phosphate isomerase (MPI, EC 5.3.1.8), 6-phosphogluconate dehydrogenase (6PGD, EC 1.1.1.44), and isocitrate dehydrogenase (IDH, EC 1.1.1.42). The products of two putative loci were apparent on the IDH gel, one migrating cathodally ("IDHF") and the other anodally ("IDHS"). The TEB8 buffer (buffer III of Shaw & Prasad, 1970) was also employed for IDHF, xanthine dehydrogenase (XDH, EC 1.2.1.37), and esterase (EST1, EC 3.1.1.2). Superoxide dismutase (SOD, EC 1.15.1.1) activity was visualized as light bands on TEB8 gels darkly stained for XDH or IDH.

Allozyme phenotype has been shown to result from simple Mendelian inheritance of codominant alleles at the 6PGD locus by Chambers (1980), working with *Goniobasis floridana*. Dillon (1986) reported similar findings for GPI, ODH, and EST1, based on a mother-offspring analysis in *Goniobasis proxima*. Although the esterase stain employed here (α-napthyl acetate as substrate) yields a complex, multi-banded phenotype for each individual, only the slowly-migrating, strongly staining products of the single locus designated EST1 by Dillon (1986) were accorded a genetic interpretation in the present work.

Population Praduk served as the standard for allelic designations. We adopted here the same designations used for this population by Dillon & Ahlstedt (1997) for the four shared loci (EST1, GPI, MPI, and ODH), and labeled all new alleles accordingly. For the five loci not reported by Dillon & Ahlstedt (IDHS, IDHF, XDH, 6PGD, and SOD), the most common allele in Praduk was considered to migrate 100 mm and all other alleles labeled by their relative electrophoretic mobilities in millimeters faster or slower.

Gene frequencies, tests to Hardy-Weinberg expectation (by chi-square, with pooling for rare genotypes), and Nei's (1978) unbiased genetic identities and distances were calculated using BIOSYS version 1.7 (Swofford & Selander, 1981). Tests for homogeneity between populations within nominal species were by Fisher's exact method in 2 × 2 cases, otherwise by chi-square contingency tests, pooling the rarest rows or columns as necessary. We analyzed the matrix of genetic distances using the multidimensional scaling module of STATISTICA (Release 5.0, StatSoft, Inc.), with a standard Guttman-Lingoes (principal component) starting configuration. The distances between any pair of populations sharing no alleles at any locus (i.e., similarity = 0.0) were set to 5.0, a figure greater than any value actually observed.

RESULTS

Example shells from each of the four nominal Alabama *Leptoxis* species are shown in Figure 2. Their differences were not striking. The shells of *L. picta* tended to be heavier, with a higher spire, while those of *L. ampla* were lower and more rounded, and *L. taeniata* intermediate. Apical erosion made spire height difficult to evaluate, however, especially in the *L. ampla* population. The shells of *L. plicata* were less eroded, with more shouldered whorls. They were characterized by low folding (or plication) on the whorl periphery, barely visible in Figure 2. Although such plications have been reported to occur in *L. ampla*, we saw no evidence of them in our samples.

Gene frequencies are given in Table 1. Levels of intrapopulation variation were low, although perhaps not quite as low as in the better-studied *Goniobasis*. Over all 9 × 11 = 99 loci, we found 18 polymorphic as judged by the 95% criterion. Genotype frequencies at none of these 18 loci differed significantly from Hardy-Weinberg expectation.

All nominal species for which more than one population was sampled are listed in Table 2, along with the loci at which any intraspecific polymorphism was observed. Every nominal species showed significant interpopulation allelic frequency difference in at least one locus. This was especially striking at the ODH locus in *L. ampla* , and at both the ODH and EST1 loci in *L. plicata*, where the most common allele changed over distances as short as 15 river km. Not only did the three *L. praerosa* populations differ significantly from each other, the present Praseq and Praduk populations differed from the Sequatchie and Duck samples of Dillon & Ahlstedt (1997) located 20–30 km downstream.

Figure 3 shows Nei's unbiased genetic identities among all pairs of *Leptoxis* populations. The three *L. praerosa* populations were strikingly different from all others, as were the two *L. plicata* populations. The levels of genetic identity among *L. ampla, L. picta,* and *L. taeniata* populations were much higher. Figure 3 also depicts the Nei's genetic distances in two dimensions, from multidimensional scaling. After 100 iterations, the stress for this solution was 0.0015. The six populations comprising *L. ampla, L. picta,* and *L. taeniata* occupy one end of the long axis of the scale, the three *L. praerosa* the other end, and *L. plicata* appears intermediate.

DISCUSSION

The species concept under which the pleurocerid fauna of the Mobile Basin has been described and revised differs substantially from the biological concept in currency today. In his (1922) monograph on the "Anculosae" (*Leptoxis*) of Alabama, Goodrich wrote, "That collection of individuals in the Pleuroceridae may be called a species whose predominant characters are not the predominant characters of another collection of individuals. If we see only a few specimens of a single species its own peculiar characters may often seem to be submerged by characters linking it with another species. But in a long series the individual characters stand out, and we are compelled then to recognize the existence of definable differences and to proceed to describe them and provide the label of a name."

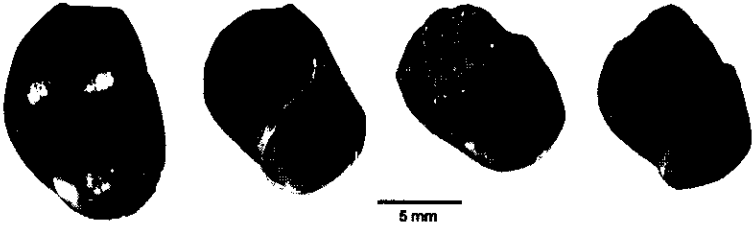


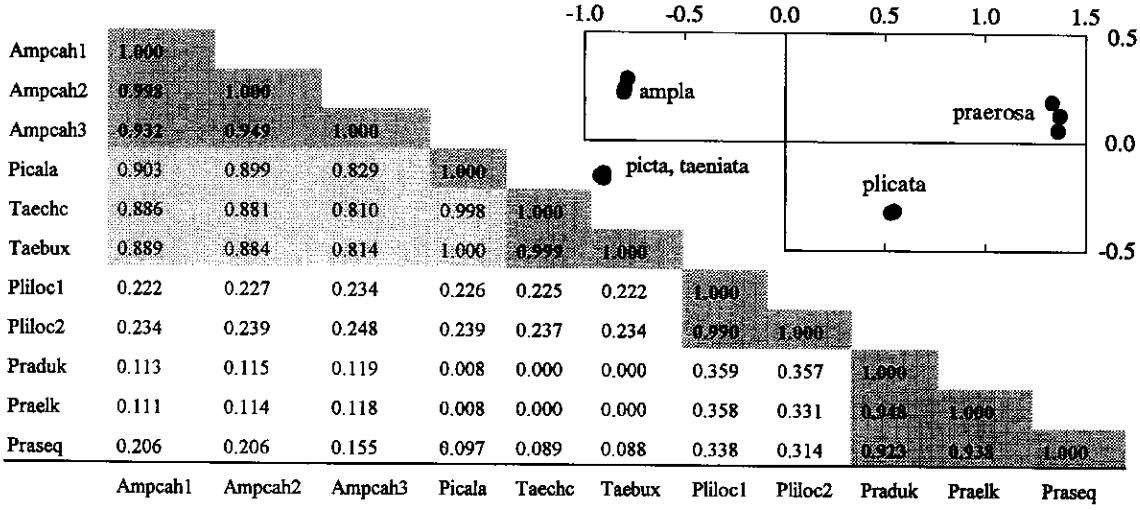
FIG. 2. Example shells of four Mobile Basin *Leptoxis* species. From left, *L. picta* (Picala), *L. taeniata* (Taechc), *L. ampla* (Ampcah1), and *L. plicata* (Pliloc2).

TABLE 1. Gene frequencies at nine allozyme loci for 11 populations of *Leptoxis*

Locus	Allele	Amp-cah1	Amp-cah2	Amp-cah3	Picala	Taechc	Taebux	Pliloc1	Pliloc2	Praduk	Praelk	Praseq
GPI	108				0.024			0.726	0.726			
	104	1.000	0.855	0.625	0.976	1.000	1.000					
	97							0.274	0.274	1.000	1.000	0.838
	94		0.145	0.375								
	90											0.162
MPI	100							1.000	1.000		0.016	
	98				0.929	1.000	1.000					
	95	1.000	1.000	1.000	0.071					1.000	0.984	1.000
EST1	106							0.613	0.387			
	105									1.000	1.000	1.000
	104	1.000	1.000	1.000	1.000	1.000	1.000					
	99							0.387	0.613			
6PGD	106	1.000	1.000	1.000	1.000	1.000	1.000	0.767	0.900			
	100							0.233	0.100	1.000	1.000	0.952
	94											0.048
ODH	121							0.355	0.242			
	118							0.065	0.048			
	115							0.323	0.532	0.855	0.177	0.054
	113							0.258	0.177	0.145	0.823	0.203
	110		0.057	0.682								
	107	1.000	0.943	0.318	1.000	1.000	1.000					0.743
IDHF	103							1.000	1.000			
	100									1.000	1.000	1.000
	98	1.000	1.000	1.000	1.000	0.875	1.000					
	95					0.125						
IDHS	103	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000			
	102											0.177
	100									1.000	1.000	0.823
XDH	103	1.000	1.000	1.000	1.000	1.000	1.000					
	100							1.000	1.000	1.000	1.000	1.000
SOD	110	1.000	1.000	1.000	1.000	1.000	1.000					
	100							1.000	1.000	1.000	1.000	1.000

Goodrich often reported that Mobile Basin species overlapped not just in character, but in geographic range as well. Populations identified by Goodrich (1922) as *L. picta* historically inhabited the lower Coosa River, the lower Cahaba River, and the Alabama River downstream to Claiborne, Monroe County. Goodrich reported the range of *L. taeniata* as substantially identical to that of *L. picta*, except that *L. taeniata* extended further up the Coosa River and its tributaries. Goodrich listed *L. ampla* from both the Coosa and Cahaba rivers and their tributaries, although not from the main stem of the Alabama River. Goodrich did not consider that the geographic range of *L. plicata* overlapped with those of *L. picta*, *L. taeniata*, or *L. ampla*. He recorded *L. plicata* as occurring in the Black Warrior River, the Tombigbee River, and their tributaries only.

The concept of the species differs today, as does the distribution of *Leptoxis* in the Mobile Basin. Under the biological species concept, local variation in gene frequencies (and by extension, external appearances) is a not-unexpected consequence of limited gene flow in



	Ampcah1	Ampcah2	Ampcah3	Picala	Taechc	Taebux	Pliloc1	Pliloc2	Praduk	Praelk	Praseq
Ampcah1	1.000										
Ampcah2	0.998	1.000									
Ampcah3	0.932	0.949	1.000								
Picala	0.903	0.899	0.829	1.000							
Taechc	0.886	0.881	0.810	0.998	1.000						
Taebux	0.889	0.884	0.814	1.000	0.999	1.000					
Pliloc1	0.222	0.227	0.234	0.226	0.225	0.222	1.000				
Pliloc2	0.234	0.239	0.248	0.239	0.237	0.234	0.990	1.000			
Praduk	0.113	0.115	0.119	0.008	0.000	0.000	0.359	0.357	1.000		
Praelk	0.111	0.114	0.118	0.008	0.000	0.000	0.358	0.331	0.948	1.000	
Praseq	0.206	0.206	0.155	0.097	0.089	0.088	0.338	0.314	0.923	0.938	1.000

FIG. 3. Below the diagonal are Nei (1978) unbiased genetic identities among *Leptoxis* populations. The identities of nominally conspecific populations are shaded darkly. Identities among the members of the *L. picta* group are lightly shaded. Above the diagonal is the result of a multidimensional scaling based on Nei (1978) genetic distances.

TABLE 2. The probability of homogeneity among nominally conspecific populations of *Leptoxis* (p_x from χ^2 tests, p_f from Fisher's exact tests). A subscript "c" indicates that rows or columns were combined for the test. The table is blank for loci where conspecific populations were not polymorphic.

	L. ampla	*L. taeniata*	*L. plicata*	*L. praerosa*
GPI	$p_{xc} < 0.001$		$p_f = 1.0$	$p_{fc} < 0.001$
EST1			$p_f = 0.019$	
6PGD			$p_f = 0.085$	
ODH	$p_x < 0.001$		$p_{xc} = 0.062$	$p_x < 0.001$
IDHF		$p_f = 0.006$		
IDHS				$p_{fc} < 0.001$

populations of organisms with dispersal capabilities as low as freshwater snails. For example, a culvert placed in a small North Carolina stream in the 1950s caused significant divergence at the ODH locus between upstream and downstream populations of *G. proxima* over a distance of just 10 meters (Dillon, 1988b). That this did not comprise a speciation event became clear when the barrier was removed, and the genetic difference disappeared. Indeed, geographically isolated populations of *G. proxima* sharing no alleles at as many as six allozyme loci have nevertheless demonstrated no evidence of reproductive isolation when transplanted (Dillon, 1986, 1988a). Evidence of similar interpopulation divergence is clear in our three samples of *L. ampla*, our two samples of *L. taeniata*, and our two samples of *L. plicata*. Whether the significant differences highlighted in Table 2 are due to some unrecognized barriers to dispersal, or whether they may be due to isolation by distance alone, cannot be told at present. But it is clear that our three populations of *L. ampla*, for example, do not constitute different species. It is also clear that, extending the levels of divergence illustrated within *L. ampla* down the 120 km length of the Cahaba River as was the situation earlier in this century, the *Leptoxis* of the Alabama River would be expected to show striking genetic differences with the *Leptoxis* of the headwaters, through isolation by distance. There is little expectation, however, that reproductive isolation will evolve in such a circumstance, or that headwaters populations and populations from the main river will speciate parapatrically.

The divergence among *L. taeniata*, *L. picta*, and *L. ampla* is negligible, given their geographic distance. *Leptoxis picta* has uncommon alleles at the GPI and MPI loci not detected in *L. taeniata*, and one *L. taeniata* population has an allele at IDHF not seen in *L. picta*. The levels of divergence appeared somewhat greater between *L. ampla* and *L. picta/taeniata*, due to the results at the MPI locus. But although *L. ampla* is fixed for an allele not seen in *L. taeniata*, Table 1 shows that both MPI alleles are found in the *L. picta* population that may have connected them in the main Alabama River. Such small and clinal differences are not comparable to those normally displayed by species of pleurocerid snails presumed distinct, as illustrated by *L. praerosa* and *L. plicata*. We therefore refer to all three of these taxa, *L. picta*, *L. taeniata*, and *L. ampla*, as the "*Leptoxis picta* group."

In most respects, our findings coincide with those based on 16S rRNA gene sequence divergence. Lydeard et al. (1997) also found *L. plicata* to be quite distinct from all other Mobile Basin *Leptoxis*, unique at about 20% of its nucleotide bases. Lydeard's mtDNA phylogeny depicted the three members of the *L. picta* group as a single clade when transversions were weighted more than transitions. But while very little sequence divergence was apparent between *L. taeniata* and *L. ampla* (only about 2%), Lydeard reported about 20% sequence divergence between *L. taeniata/ampla* and *L. picta*. So our finding that *L. picta* and *L. taeniata* are indistinguishable in their allozyme frequencies at nine loci was quite unexpected.

A similar discrepancy between allozyme and mtDNA divergence in oysters was attributed to balancing selection at multiple enzyme loci by Karl & Avise (1992), although much more data would be required before such a suggestion could be made in our case. Lydeard et al. (1997) only analyzed a single individual for each nominal *Leptoxis* species. There is a clear need for additional surveys of

16S rRNA sequence divergence focused below the species level.

A complete understanding of the genetic relationships among *L. picta, L. taeniata,* and *L. ampla* would have required samples from populations inhabiting the lower regions of the Cahaba and Coosa rivers, where the three nominal species were once reported to co-occur. All such populations are long extinct. Regardless of their specific status, the levels of genetic diversity displayed by the small populations of the *L. picta* group that remain today, as evidenced by both mtDNA and allozyme studies, argue strongly for conservation measures. The *L. plicata* population restricted now to just 20 km of Locust Fork (Hartfield, 1997) is clearly a unique species by all measures, and deserves immediate protection.

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APPENDIX

Locality data, sample sizes, and (where applicable) catalog numbers for voucher specimens deposited in the Academy of Natural Sciences of Philadelphia

Ampcah1—Cahaba River at Co. 52, 1.5 km W of Helen, Shelby County, Alabama. Same station as CA-61 of Bogan & Pierson (1993b). N = 25 *Leptoxis ampla.*

Ampcah2—Cahaba River at River Road, 3 km S of intersection of Co. 1 and Co. 13. Shelby County, Alabama. N = 36 *Leptoxis ampla.*

Ampcah3—Cahaba River at Co. 24, Bibb County, Alabama. Just downstream from station CA-64 of Bogan & Pierson (1993b), and 10 km downstream from the *L. ampla* site of Lydeard et al. (1997). N = 24 *Leptoxis ampla.* ANSP 400111.

Picala—Alabama River about 2 km south of U.S. 84 crossing, Monroe County, Alabama. Same site as the *L. picta* site of Lydeard et al. (1997). N = 21 *Leptoxis picta.* ANSP 400112.

Pliloc1—Locust Fork, 0.4 km upstream from the Mount Olive Road boat ramp, Jefferson County, Alabama. About 20 km south of *L. plicata* site of Lydeard et al. (1997). N = 31 *Leptoxis plicata.* ANSP 400113.

Pliloc2—Locust Fork, at shoal 10 km downstream from the Mount Olive Road boat ramp, Jefferson County, Alabama. N = 31 *Leptoxis plicata.*

Taebux—Buxahatchee Creek, Shelby County, Alabama. N = 34 *Leptoxis taeniata.*

Taecho—Choccolocco Creek, Talladega County, Alabama. Same site as the *L. taeniata* site of Lydeard et al. (1997). N = 32 *Leptoxis taeniata.* ANSP 400115.

Praduk—Duck River at Shelbyville, Bedford County, Tennessee. About 30 km upstream from Duck River site of Dillon & Ahlstedt (1997). N = 31 *Leptoxis praerosa.*

Praelk—Elk River at Stump Shoals Public Access near US 64 bridge, 8 km E of Fayetteville, Lincoln County, Tennessee. N = 31 *Leptoxis praerosa.* ANSP 400114.

Praseq—Sequatchie River at Tn 28 bridge, Whitwell, Marion County, Tennessee. About 20 km upstream from Sequatchie site of Dillon & Ahlstedt (1997). N = 37 *Leptoxis praerosa.*