MOLECULAR SYSTEMATICS OF SELECTED MEMBERS OF THE BLACK BASSES, GENUS *MICROPTERUS*, WITH CONCENTRATION ON THE SPOTTED BASS (*M. PUNCTULATUS*) SPECIES COMPLEX

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ABSTRACT

This study examined genetic relationships among selected populations of black basses. These centrarchid fishes, separated by both physical barriers (land formations) and distance, have shown varying degrees of differentiation, but retain many morphometric characters in common. Eight populations representing four taxa and geographical extremes in the genus *Micropterus*, with concentration on the spotted bass complex, were selected and evaluated for biochemical genetic characters. This study examined two species and two subspecies of spotted basses. The type species from Kentucky represented *Micropterus punctulatus punctulatus*; a population from Alabama represented *M. p. henshalli*. A Texas population, previously classified as conspecific with spotted bass but now listed as a distinct species, was included. One primary objective of this study was where the Louisiana populations of *M. punctulatus* align within this group, as these populations are found at a central geographic position in the distribution of these differentiated basses. Since previous studies have revealed low levels of genetic variability, a technique more sensitive to genetic differences was used, and compared to results from allozyme analysis, the more traditional method for assessing genetic differentiation. Both allozyme analysis and random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) were used to assess genetic relationships.

These two techniques resolved very different relationships. The allozyme study showed the type species, Kentucky bass, as most divergent, but supported the predicted relationships among the remaining four populations. The RAPD-PCR results were in

basic agreement with the expected taxonomy. Based on similarities at 302 polymorphic RAPD loci, the two Louisiana and Kentucky populations closely clustered, with the subspecies *M. p. henshalli* the next most divergent, and *M. treculi*, diverging next, but completing a cohesive cluster with the other spotted bass relative to the outgroups. A yet unnamed new form from Florida, the Chipola bass, was also analyzed with this technique. PCR results place this form approximately equal distances from the other two outgroup species and the *punctulatus* group. Therefore, this analysis would support species recognition for the Chipola bass, and regrouping the Texas strain of spotted bass in the *M. punctulatus* species complex.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Our knowledge and understanding of molecular differences between species, breeding populations, and the importance of genetic diversity to local adaptation and survival has increased greatly in the last half century. Central to the issue of genetic diversity is the degree of population subdivision and structuring within any species, which directly affects the amount of gene flow occurring between populations capable of interbreeding. In this regard, the exercise of systematics has new importance in that it reflects the historical record of gene exchange and is useful for identifying currently contiguous breeding populations.

As more ecosystems and fisheries are subject to human disturbance and management, the importance of understanding and maintaining genetic diversity and unique gene pools becomes increasingly critical (Soule, 1986; Ryman, 1991). Effective management is dependent, in part, on understanding the interrelatedness of the gene pools of disjunct, conspecific populations, as well as, the genetic differences in potentially hybridizing subspecies and species. Accordingly, this understanding involves first estimating ranges and distributions of populations and species, and then obtaining baseline measures of genetic parameters (Dobzhansky, 1970; Wright, 1978).

Simpson (1961) discussed the concept of the 'evolutionary species' as a group which comprised a lineage, i.e., an "ancestral-descendant sequence of populations....evolving separately and with its own evolutionary role and tendencies". Three and a half decades of research have followed, and still the application of the

concept of species in the natural world remains challenging. Whether information comes from paleontological, morphological, embryological, or molecular techniques, a single definition of species has proved difficult. Studies of the genetic structure in natural populations have shown more variation and diversity among these populations than expected. This was an unexpected finding in the late 1960's and early 1970's, as the belief at that time was that natural selection would select the most fit phenotype, and corresponding genotype, and thus weed out undesirable variation and keep allelic variation low or nonexistent (Muller, 1950; Kimura, 1968; Lewontin, 1974).

However, this extent of variation at the molecular level was not simply a discovery of interest, but a new tool to indirectly examine many questions pertaining to the effect of breeding and population structure on genetic composition of a population. Patterns in this variation could reveal a record of not only current breeding structure, but also taxonomic history of a group of populations and / or species. Many questions could be addressed, including the extent of interbreeding and movement among related groups, subspecies, and species. This type of information was applied in constructing and reconstructing networks of genetic relationships among groups, not only over physical distance, but over time as well.

To assess certain impacts to biological communities (e.g., disruption or promotion of gene flow within and among populations) over time, we must first understand the genetic structure of populations. This entails assessing genetic similarities of geographically proximate as well as disjunct populations. Maintenance of at least current levels of genetic variability is proposed to be an important factor in the

long-term survival of a population. Recognition of the significance of genetic variability (measured as the percent of loci that are polymorphic in a population and average percent of loci herterozygous per individual) is central to the field of conservation genetics (Ryman, 1991). One concern of this discipline is obtaining an accurate estimate of genetic variability (i.e., percent of loci polymorphic in a population, and average percent of loci heterozygous / individual) within and among natural populations. Such information is important as a baseline for future comparisons of measures of these parameters. Thus, genetic variability can be monitored, and programs designed to mitigate its loss before habitat disturbances occur and disrupt population structure and gene flow.

One primary concern in conservation genetics deals with habitat disruptions, and alterations in physical barriers to migration, with concomitant decrease or increase in subdivision within a population (Soule, 1986). If a natural population had shown subdivided structure, but now is becoming more homogeneous due to habitat changes or introduction of exotic, but interfertile individuals, there is a potential loss of unique local adaptive complexes. Alternately, physical or ecological barriers to migration between colonies can lead to increased reproductive isolation among subpopulations and is predicted to lead to a decrease in effective population size. Effective population size (N_e) is defined as the average number of individuals in a population that contribute genes to the next generation. Even in a populations of large numbers of individuals, effective population size is reduced when individuals tend to mate in higher frequency with nearby individuals. A consequence of a reduction in the effective population size is an increase

in genetic drift, with subsequent decrease in genetic variability, fixation of rare or less adaptive genes, and eventual genetic divergence (Nei, 1975; Wright, 1943).

An additional problem, with local consequences, is sometimes seen with species important to recreational fishing. Intentional and inadvertent introductions of exotic species into foreign habitats have set the stage for either genetic mixing, or isolation of a newly established peripheral population, followed by subsequent genetic changes (i.e., increased homogeneity or divergence of allele frequencies among subpopulations). To demonstrate decreased genetic differences between two or more groups, the magnitude of genetic differences among subpopulations (or putative taxa) must first be estimated. If populations are to be managed for maximum likelihood of survival, it is important first to have an understanding of such genetic differences and population structure. Genetic changes in subpopulations, and between potentially hybridizing taxa can be spatially and temporally monitored for loss in genetic diversity, and decrease in genetic variability. This monitoring is needed to determine if impacts on genetic structure and variability in populations or taxa appear to be correlated with natural or anthropogenic changes.

As adults, the black basses, *Micropterus* spp. (Centrarchidae, subfamily Micropterinae) are top-level carnivores, important in recreational fisheries in the southeast and south-central United States. Populations are often isolated by physical barriers or distance, which interrupt gene flow and provide conditions that lead to establishment of phenotypically distinct populations. In addition, some species and subspecies can hybridize (Morizot et al., 1991). These factors have made systematic

classification difficult. Morphological, meristic, and other phenotypic characters are not completely effective in discerning systematic relationships and levels of gene flow among populations. However, various biochemical techniques have aided classification by providing more accurate estimates of genetic differentiation. Such knowledge is also useful for management efforts designed to minimize loss of genetic diversity as manifested in these unique gene pools in nature.

As early as the 1800's, biologists recognized two different forms of North American black bass. Prior to 1926, this group was believed to comprise one genus, containing two species: *Micropterus dolomieu* Lacepede, 1802 (smallmouth bass, SMB), and *Micropterus salmoides* (Lacepede, 1802) (largemouth bass, LMB). Carl Hubbs (1926) considered the LMB to be distinct and assigned it to a monotypic genus, *Aplites*. Later, Hubbs and Bailey (1940) recognized the degree of character overlap among black basses and recommended moving the LMB back into the genus *Micropterus*.

In 1927, Hubbs reported a third species of black bass; its adult form showed several ventral rows of progressively shortened, dark spots below the lateral line. This new species was similar to SMB in several characteristics, including the pyloric caeca (few and primarily unbranched), moderate mouth size, scaleless preopercle, scaled interradial membranes on the soft dorsal and anal fins, and the dorsal fin shallowly emarginate. It differed from *M. dolomieu* in 10 features, which included larger scales, a longer jaw, fewer dorsal and pectoral fin rays, and a more elevated and rounded margin of the spinous dorsal fin. It was similar to LMB in large scale size, number of dorsal rays, and color pattern in the young. This form differed from both recognized species in

the number of pectoral rays, the presence of a tooth patch on the tongue, and color pattern in the adult. However, given the described similarities and generally conserved form, Hubbs assigned this new bass to the genus *Micropterus*, and, with regard to the similarities shared with the LMB, gave it the species name *pseudaplites*.

This new species was found in a variety of habitats, from muddy bayous to swift, rocky creeks. Specimens of similar description had been found in West Virginia, Indiana, Texas, and Alabama. The center of the range appeared to be in central Kentucky, and the Licking River in central Kentucky provided the type specimens for its description. Therefore, Hubbs (1927) gave this fish the common name Kentucky bass.

Hubbs and Bailey (1940) recognized the Kentucky bass as probably the same species described by Rafinesque in the 1800's and recommended the original name, *Micropterus punctulatus* (Rafinesque, 1819), be retained. In that 1940 revision of black basses, Hubbs and Bailey also reported evidence of two new *punctulatus* subspecies, based on meristic and morphologic differences and similarities. The recommended names for these two new subspecies were *M. p. wichitae*, for the form from south-central Oklahoma, and *M. p. henshalli* for the form found in the Alabama River system in Alabama, Mississippi, and Georgia. The subspecies designation for the remaining spotted basses would be *M. p. punctulatus*. A putative fourth species of black bass was found in the Coosa River system in Alabama, and accordingly named *M. coosae* Hubbs and Bailey, 1940. They also reported two undetermined but potentially new subspecies of *M. punctulatus* from the Colorado River in Texas, and the Chipola River in Florida. Possible hybrids were reported between *M. p. punctulatus* and *M. d. dolomieu,* in Texas

and between *M. p. henshalli* and *M. coosae* in Florida. To further confuse classification, they found specimens in two localities that displayed intermediate characters of the local taxa found there. A new subspecies of SMB was also described with the proposed name *M. d. velox.*

Micropterus punctulatus henshalli aligned with *M. p. punctulatus* for a number of characteristics: presence of a tooth patch on the tongue, the shallow emargination of the dorsal fin, number of dorsal fin soft rays, size of cheek scales, jaw size, adult color pattern and markings (both show a basal caudal spot, and an opercular spot), and a more elongate body form. However, the modal number of pectoral rays was different in the two forms (16 more often in *M. p. henshalli*; 15 in *M. p. puncutlatus*), but no unique numbers were seen in either. Scale size along the lateral line and around the caudal peduncle was smaller in *M. p. henshalli*, and the adult body form was more streamlined, i.e., more similar to *M. dolomieu*. Specimens of this subspecies were identified from the Alabama River system in Mississippi, Alabama, and Georgia, and from the Pascagoula and Pearl River systems in Mississippi. Forms of this subspecies were also represented in tributaries of Lake Ponchartrain in Mississippi and Louisiana.

Hubbs and Bailey (1942) recognized the distinctness of a Texas population of spotted basses from the Colorado, Guadalupe, and San Antonio River systems in south-central Texas, and recommended assigning it subspecies status, *M. p. treculi*. These central Texas populations were also distinct from the spotted bass in the East Texas Brazos and Trinity River systems, which more closely resembled *M. p. punctulatus*. The specimens examined were similar to *M. punctulatus* in the presence of a tooth patch on

the tongue, moderate mouth size, color pattern in the young, and number of dorsal soft rays. These two forms differed in that the Texas population had slightly smaller scales along the lateral line and abdomen (average counts: *M. p. treculi*, 65; *M. p. punctulatus*, 64), but larger and fewer cheek scales (average counts: *M. p. treculi*, 13.2; *M. p. punctulatus*, 14.4, per row), modal count for pectoral rays (*M. p. treculi*, 16; *M. p. punctulatus*, 15). They also differed in body form: the body was deeper, and the caudal peduncle typically shorter and wider in *M. p. treculi*. Bailey and Hubbs (1949) reported examination of a sufficient number of additional samples from both central and east Texas river systems to clarify this taxonomic question. All specimens from east Texas were identified as *M. p. punctulatus*, while those from the Colorado, Guadalupe, and San Antonio Rivers were identified as a distinct subspecies, *M. p. treculi* (Vaillant and Bocourt, 1874). Jurgens and Hubbs (1953) first suggested the Texas spotted bass be considered a separate species. Clark Hubbs (1954) observed *M. p. treculi* and *M. p. punctulatus* living sympatrically but with no evidence of hybridization. Based on numerous records of these two distinct forms being collected from the same water system, he treated this (*M. p. treculi*) as a distinct species, with an extended distribution.

Bailey and Hubbs (1949) examined six specimens from a limestone sink area on a tributary of the Sante Fe River in northern Florida. This small bass represented a "strikingly distinct species", and they recommended the name *M. notius*. The presence of interradial scales on the soft dorsal and anal fins, simple pyloric caeca, and a shallowly emarginate dorsal fin aligned it with the subgenus *Micropterus*. Also similar to the subgenus *Micropterus*, these specimens showed a basicaudal spot and three oblique lines

across the cheek from the eye. However, the body form, color, size of the scales, and the occasional branched pyloric caecum showed affinity with *M. salmoides* of the subgenus *Huro*. Based on the characters shared with both subgenera, Bailey and Hubbs (1949) postulated that *M. notius* "retains a generalized position in the genus close to the prototypic *Micropterus*". *M. notius* most probably descended directly from an ancestor that gave rise to the two independent lines of evolution leading to the two subgenera of spotted basses, *Huro* and *Micropterus*, seen at present. Bryan (1969) recorded vertebral counts as the ratio of abdominal to caudal vertebrae on specimens of *M. punctulatus* (representing a cline of subpopulations along the Ohio River) and *M. notius*; both were compared with *M. salmoides*, and *M. dolomieu*. He found the modal count for abdominal + caudal vertebrae of 14 + 18 grouped *M. notius*, *M. s. floridanus*, and *M. punctulatus*; whereas, *M. dolomieu* and *M. s. salmoides* shared a count of 15 + 17. He concluded that *M. notius* represented the base of two separate evolutionary lines leading to the subgenera *Micropterus* and *Huro*.

Several specimens from the Apalachicola River system (Chipola River, Florida), referred to as "shoal bass", were difficult to place taxonomically (Bailey and Hubbs, 1949; Ramsey and Smitherman, 1972). Bailey and Hubbs (1949) suggested this fish was a form of *M. punctulatus*, but thought it premature to assign it to the *punctulatus* group. Some structural features (e.g., skull measurements) were closely aligned with features of *M. punctulatus* (Ramsey, 1975). Another specimen discovered later appeared more similar to *M. coosae*, but was larger, grew faster, and showed differences in color pattern. These differences in growth rate and size were attributed to differences in

habitat. However, Ramsey and Smitherman (1972) conducted rearing experiments, and demonstrated a genetic basis to the differences in growth rate and color pattern between the "shoal" bass and *M. coosae*. In addition, the two forms were found together in several areas with no evidence of hybridization (Ramsey, 1975), indicating reproductive isolation, and perhaps speciation. Although the general phenotype of the "shoal" bass is similar to *M. coosae*, it may be more closely linked to the *punctulatus* lineage. Species status has been proposed for the "shoal" bass, with the suggested name *M. cataractus* (Dr. Jim Williams, personal communication).

Extant freshwater black basses of North America probably represent three main paths of evolution that were differentiated and evolving along separate lines by the end of the Pliocene (Ramsey, 1975). Among the characters distinguishing these three distinct groups, in addition to morphometric and meristic differences, are pigmentation and color pattern development in the young:

1--before scale formation, the fry have a wide, intensely-dark lateral stripe, *M. salmoides*;

2--the lateral band is almost nonexistent in scaleless fry; narrow, vertical bars appear in scaled young; the bars lighten and disappear with age, *M. dolomieu* and *M. coosae*;

3--before scale formation, the fry have a narrow, weakly developed lateral band that persists with age and becomes augmented by vertical bars, *M. p. punctulatus*, *M. p. henshalli*, *M. treculi*, *M. notius*, and the Chipola bass.

Members within each of the above groups are linked also by distribution patterns. The first group, the largemouth bass, occupies a wide variety of habitats, including habitats unsuitable to the other two groups. It is found in the brackish water of river deltas, as well as, in more inland lakes and rivers. The two species in the second group are upland forms, not normally found in coastal areas. Members of the third group are found in coastal areas and probably represent geographic isolates descended from a common ancestral coastal plain stock that was probably similar to *M. p. punctulatus* in structure and color.

The early classification of black basses was primarily based on differences in morphological and meristic characters. Ramsey (1975) suggested that the intermediate nature of certain characters in some of the black basses resulted from the role that hybridization played in black bass evolution. These characters are often influenced also by environmental factors (Bryan, 1964). Bryan (1964) demonstrated a clinal variation in some of the meristic characters used to distinguish species of black basses. He found the mean number of anal rays in *M. punctulatus* was greater in downstream fishes than those found upstream. While examining five species and subspecies of *Micropterus*, he found overlap in the total vertebral counts in the species examined. This trait also varied in downstream and upstream fishes, but the ratio of abdominal to caudal vertebrae did have taxonomic value. Bryan concluded, corroborating the hypothesis proposed by Barlow in 1962, that meristic counts were in part related to growth rate: slow growth during ontogenic differentiation resulted in an increase in the number of meristic elements, while fast growth led to a decrease in elements. Therefore, slower growth of fishes in colder

habitats is expected to result in differences for these traits from fishes found in warm habitats.

Consequently, morphological and meristic characters in bass are influenced by environmental conditions and may be similar due to convergent evolution, rather than common ancestry (Wallace, 1973). In contrast, a number of biochemical techniques facilitate a less ambiguous assessment of genetic relatedness. These include isozyme analysis, with starch gel electrophoresis, and polymerase chain reaction (PCR), using random arbitrary primers.

In spite of extensive and careful analysis by previous researchers, the classification of the black basses is still not completely resolved. In addition, no one has yet performed a complete study of the spotted bass species complex, and included southern Louisiana populations, that are currently classified as *M. p. punctulatus.* These southern populations are particularly of interest because the current taxonomy of spotted basses places the subspecies *M. p. henshalli* in streams that feed Lake Pontchartrain in southeastern Louisiana.

A number of molecular techniques are applicable to address this question, two of which I will use in this study: isozymes and RAPD-PCR (random amplified polymorphic DNA- polymerase chain reaction). Each method theoretically assays different 'types' of DNA. Isozyme analysis detects genetic mutations manifested in structural proteins and enzymes, for which changes are constrained by natural selection. RAPD-PCR theoretically detects mutations in both coding (structural) and noncoding regions of DNA. Noncoding regions of DNA do not produce a functional product and may not be

subjected to direct selection and, therefore, are predicted to evolve at higher rates (Nei, 1987; Stepien and Kocher, 1997). The RAPD-PCR technique assays mutations in both categories and is expected to be sensitive to a wide range of DNA conservation, i.e., to different levels of genetic divergence (Welsh and McClelland, 1990).

OBJECTIVES

I will use molecular techniques to assess the genetic relationships of Louisiana spotted bass populations to their geographic neighbors, and to specimens from the type locality in the Dix-Licking River system in central Kentucky. I plan to examine two populations from Louisiana, that ostensibly have restricted gene exchange due to land barriers and the Mississippi River. I will make genetic comparisons between these two Louisiana populations and populations representing each of the *M. punctulatus* subspecies: *M. p. punctulatus*, from Kentucky, and *M. p. henshalli* from Alabama. The genetic relationships among these four populations and *M. treculi* will be assessed, as well as, between all the populations and two successively more distantly related taxa, *M. salmoides* and *M. notius*. These latter two species will serve as the 'outgroups' that are used to calibrate, or to 'root', a phenetic tree constructed from the data. I will also evaluate representatives of the Chipola bass and estimate the genetic affinities of this population with these seven populations of black basses.

In this study, I will employ two different molecular techniques to obtain estimates of genetic distance between these populations: allozymes and a modification of the polymerase chain reaction (random amplified polymorphic DNA-polymerase chain reaction, RAPD-PCR). The results obtained from 'genetic fingerprinting' with RAPD-

PCR will be compared to the results from the allozyme analysis. Both techniques provide data for estimates of genetic relatedness among populations.

CHAPTER 2

ISOZYME ANALYSIS

INTRODUCTION

Protein electrophoresis, a standard biochemical, taxonomic tool, is useful for estimating genetic divergence based on observed differences at structural gene loci, i.e., loci coding for enzymes and other proteins. The technique is also referred to as isozyme, or allozyme, analysis. Genetic distance estimates obtained with this technique have proved useful for separating closely related taxa, even in cases where phenotypic discrimination, either morphometric, meristic, or both, have failed (Avise, 1974). Application of this technique has been useful in some areas of black bass systematics. For example, although individuals from two populations of largemouth bass are difficult to distinguish with phenotypic criteria, allozyme analysis showed the Florida population to be distinct from the northern largemouth bass. These two populations have fixed allelic differences for isocitrate dehydrogenase (Idh-B) and glutamate-oxaloacetate transaminase (Got-B) (Philipp et al., 1983; Carmichael et al., 1986). Williamson et al. (1986) demonstrated allelic frequency differences between *M. s. salmoides* and *M. s. floridanus* at an additional nine loci. Whitmore and Butler (1982), first used an index derived from eleven meristic characters to separate two species of black basses from Texas into three groups: *M. treculi*, *M. dolomieu* and hybrids. Isozyme analysis showed that three loci (Ldh-C, Mdh-B, Idh-B) were diagnostic for these two species and hybrids. Only two of 24 specimens were misidentified with use of the meristic index, supporting

an overall congruence of meristic and biochemical data. Morizot et al. (1991) found discrete isozyme markers that identified each of four species, *M. s. salmoides*, *M. s. floridanus*, *M. dolomieu*, and *M. treculi*. They demonstrated the existence of hybrids between *M. treculi* and *M. dolomieu*, and between *M. s. salmoides* and *M. treculi*. They also described a cross containing alleles from all three species. Dave Philipp (personal communication) performed electrophoresis on 10 populations of spotted bass from Texas to Georgia. He found the Alabama subspecies (*M. p. henshalli*) to be distinct from the northern form (*M. p. punctulatus*), and *M. treculi* to also be distinct from the northern subspecies (*M. p. punctulatus*). Rex Dunham (personal communication) also found individuals from the northern range of the *M. p. henshalli* distribution in Alabama to be electrophoretically distinct from individuals in the southern part of the distribution.

Objectives

With the extent of population subdivision and genetic differentiation observed over such small geographic ranges as discussed above, I am interested in comparing populations from Louisiana with representatives of each of the above taxa. I also am interested in exploring the role of the Mississippi River as a barrier to east-west migration for coastal stream fishes (Chernoff et al., 1981). Therefore, I will attempt to estimate the level of genetic differentiation between two coastal populations of spotted bass in south Louisiana, separated by the Mississippi River, and compare that with estimates of differentiation between these populations and conspecific populations from more distant locales.

I will estimate the genetic distances between two Louisiana populations representing *M. p. punctulatus*, one population from Alabama, representing *M. p. henshalli*, a population from Kentucky representing *M. p. punctulatus*, and a representative population from Texas for *M. treculi*. One Louisiana collection will be sampled from the Tickfaw River, east of the Mississippi River. The second Louisiana collection will be from the Atchafalaya Basin, a site west of the Mississippi River. Polymorphic and repeatable enzyme systems will be determined for this group of five basses. Allele frequencies at the polymorphic loci will be used to estimate genetic distances between each pair of populations. A phenogram displaying the relationships among these five bass populations and species will be constructed to illustrate genetic distances.

MATERIALS AND METHODS

Study sites

Five populations of spotted basses were collected. These populations were chosen because they either represented a type locality for a taxonomic group (Kentucky, Texas, Alabama), or represented a population with questionable genetic affinities (the two Louisiana populations). Samples for the allozyme study were collected from the following localities (Figure 1):

Tickfaw River, Louisiana (LE),

Atchafalaya Basin, Louisiana (LW),

Lake Herrington, Kentucky (KY),

Lake Jordan Reservoir, Alabama (AL),

Heart of the Hills Hatchery, Guadalupe River, Texas (TX).

The Atchafalaya Basin in Louisiana is a bottomland, hardwood swamp, initially resulting from the shifting of channels of the Mississippi River and fed today by the Atchafalaya River. There is little water flow outside the main channel after spring floodwaters recede, and rich vegetation occurs both in open water and along the shores. The Tickfaw River in east Louisiana is a free-flowing river with rocky, sandy bottom and with large communities of vegetation along the shores. Lake Herrington in Kentucky is part of the Dix River. The Dix River was dammed in 1929 by Kentucky Utilities to create a cooling lake for a power plant; this lake contains the original native spotted black bass. The bottom and shores of the lake are rich in limestone, with some rocks and submerged vegetation near shores. Lake Jordan Reservoir is in central Alabama and part of the Mobile drainage. The lake is the most downstream impoundment on the Coosa River and was created in 1928 by damming the river. The habitat above the dam has moderate water flow, and rocky banks with a few trees. The water below the dam is more typical of lake habitat, with a rocky bottom and little or no water flow.

Sample collection

Fish were obtained from two different sources: a state hatchery, and field collections. Specimens of the Guadalupe bass (*M. treculi*) were obtained from Heart of the Hills Hatchery in Kerrville, TX., operated by Texas Parks and Wildlife, and certified pure. The remaining specimens were collected from natural habitats. Wild caught fishes were captured by either electrofishing (boat and backpack) or angling (Table 1).

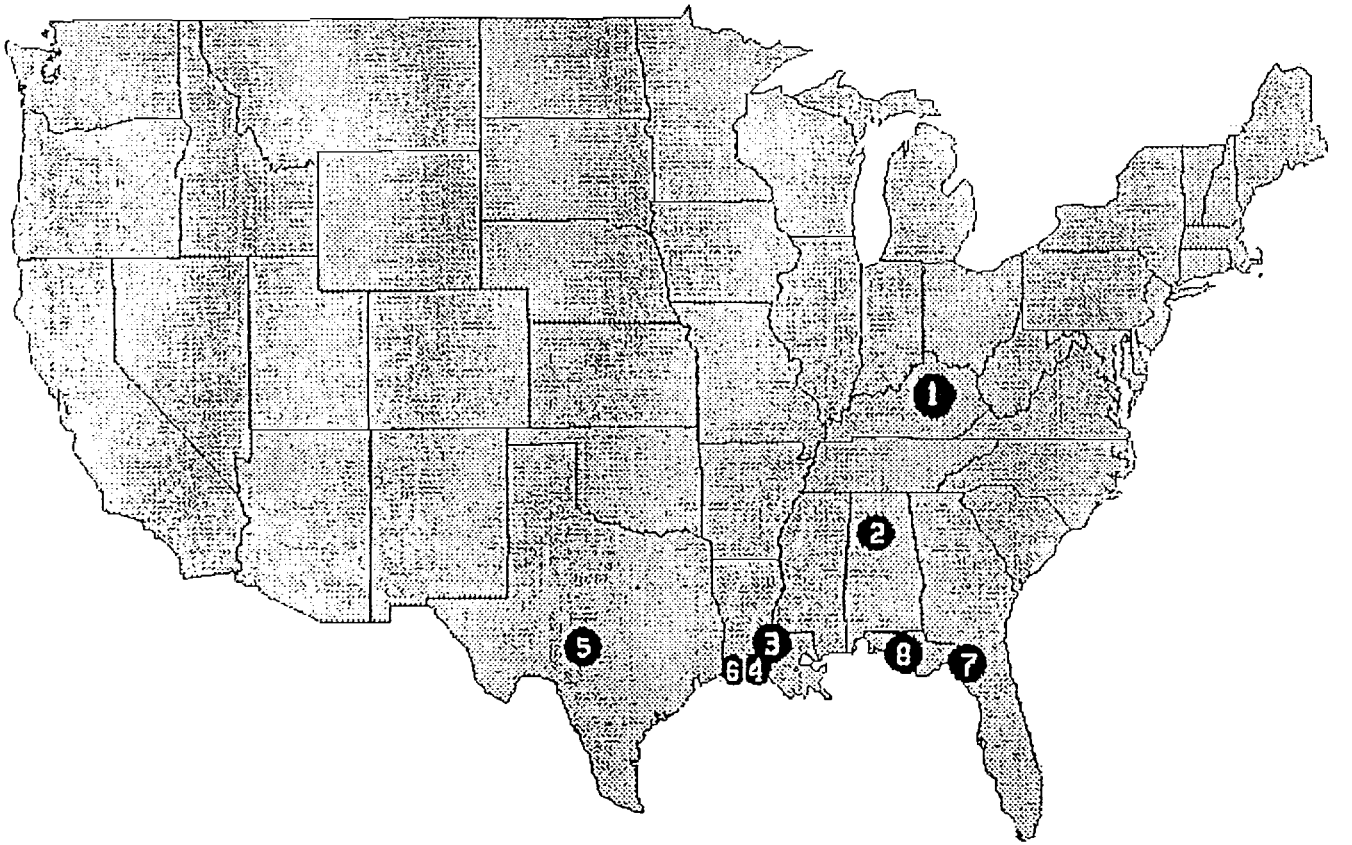


Fig. 1. Collection sites for *Micropterus* populations. Locations are as follows: 1 = Lake Herrington, KY.; 2 = Lake Jordan Reservoir, AL.; 3 = Tickfaw River, LA; 4 = Atchafalaya Basin, LA; 5 = Guadalupe River, TX; 6 = Sante Fe River, FL; 7 = Chipola River, FL.

Fish immobilized with boat electrofishing were netted out of the water and placed in a live-well of water with aeration. Fish remained in the live-well until the end of the sampling period, at which time blood was taken. However, if collecting was slow (collection time at a site exceeded 30 to 45 minutes), blood was drawn from captive fish at points during the period of fishing, so that the fish did not become severely stressed. Fish collected with the backpack electroshocker were placed in a 5-gallon bucket of water. When the bucket became crowded with fish (or if the fish had been in the bucket more than about 30 minutes), the collecting team returned to the starting station and bled fish there.

The whole fish was placed in a pre-labeled plastic, freezer-storage bag (Ziploc) and placed on ice. Fish were transported back to the laboratory and stored at -80° C, until the removal of tissue for isozyme analysis. For the Louisiana collections, fish were immediately placed on ice for transport back to the laboratory. Fish collected at two sites (Kerrville, TX, and Lake Jordan Reservoir, AL) were frozen at -20°C, and transported to Baton Rouge on ice while still frozen. Additional fresh samples for isozyme analysis were obtained immediately prior to use from Lake Herrington, KY, and Heart of the Hills Hatchery, Kerrville, TX. Samples from these two sites were collected by state personnel, frozen, and shipped on dry ice overnight to the University of Houston (UH), where I did the isozyme analysis.

Processing tissues

Whole fish to be used in isozyme analysis were frozen at -80° C. Just prior to isozyme analysis, fish were partially thawed, and tissues removed. Fish were placed in a

Table 1. Collection locality, date, and collection method for specimens of the *Micropterus punctulatus* species group.

Collection Locality (Designation)	Sample Number	Collection Date Year	Collection Method
Lake Herrington, Kentucky (KY)	20	5 Oct 94	Electrofishing
Lake Jordan Reservoir, Alabama (AL)	39	9 Nov 94	Electrofishing
Guadalupe River, Texas (TX)	42	2 Nov 92	Hatchery fingerlings
Tickfaw River, Louisiana (LE)	28	8 Jul 94 10 Aug 94 29 Aug 94	Angling
Atchafalaya Basin, Louisiana (LW)	20	1 Nov 93 4 Nov 93 29 June 94	Electrofishing

tray on a container of ice to keep samples cold while being dissected. Heart, liver, and a section of tail muscle were each removed and placed in a 2.0-ml Eppendorf tube. *M. treculi* specimens from Heart-of-the Hills Hatchery were all juveniles, ranging in length from 4 to 7 cm. A posterior section of the fish, which included the tail muscle and liver (approximately one third) was removed; the combined tissues were stored in a single tube. Tissue samples were refrozen to -80° C. Tissues from individuals of three populations (LE, LW, AL) were removed in Baton Rouge and transported on dry ice to UH for processing and electrophoresis. Individuals from the other two populations (TX and KY) were shipped to UH frozen, overnight, and dissected there, according to the protocol described above.

Tissues were homogenized with a Kinematic GMBH polytron tissue homogenizer in a 50-ml polyallomer centrifuge tube, in 1:1 (volume:volume) homogenizing buffer (0.2 M Tris-HCl, pH 7.4-0 .0002 M polyvinyl pyrolodine-0.015 M EDTA, 1.0 ml 1% NAD, 1.0 ml 1% NADP, total volume of 100 ml). Each tube with tissue sample and buffer was held in a beaker of ice during processing; liver was homogenized for 15 seconds, muscle, for 30 seconds. Samples were centrifuged at 12,000 rpm for 30 minutes in a superspeed refrigerated centrifuge (model IEC B-20A). The supernatant was pipetted into clear, 2.0-ml Eppendorf tubes and stored at -80° C until use.

Electrophoresis

Allozymes were evaluated on 12% starch gels, containing a mixture of two starches. The optimum concentration of the two different starches was 24-g

Electrostarch plus 24-g Sigma starch. Forty-eight grams of starch was dissolved in 400-ml buffer, and heated to boiling with constant stirring over a Bunsen burner. Gel solution was aspirated by vacuum for a maximum of 45 seconds (or until solution had gone from vigorous bubbling to slow bubbling). The gel solution was immediately poured into a 400-ml gel mold (17.25 cm x 19.1 cm x 1.0 cm); bubbles and particulates were removed with a Pasteur pipet. The gel was cooled to room temperature (i.e., to a temperature that felt cool to touch), and then wrapped with plastic wrap (Saran Wrap); any air bubbles between gel and plastic were removed by pulling a straight edge (e.g., a credit card) over the plastic surface.

A horizontal cut was made the width of the gel, 7.6-cm from one end, with a plexiglass guide and straight edge spatula. Each sample was absorbed onto a paper wick (6 mm x 10 mm, Whatman 3-mm chromatography paper) held with fine tipped forceps; excess moisture was absorbed on a clean sheet of filter paper, and wicks were placed against the face of the 'cathodal' section of the gel. Sixteen samples were placed in each gel, with two intra-gel repeats, and one or more inter-gel repeats. A bromophenol blue marker was included at the end of the line of samples. The two sections of the gel were pressed firmly together, removing any air bubbles between gel and wicks. The gel was again covered with plastic wrap; 3.8 cm of gel were left exposed at the cathodal end, and 7.6 cm exposed at the anodal end. The gel was placed horizontally across buffer trays, with Handiwipe sponge cloths serving as conduits between buffer trays and gels. A sheet of glass, and a 22.9 cm. x 33.0 cm tray of ice were placed on the the gel for cooling.

Electrophoresis and histochemical staining followed the methods of Selander et al. (1971), Philipp et al. (1983), Siciliano and Shaw (1976), and Harris and Hopkinson (1976). Power supplies were Heathkit, regulated high voltage (0-400 V DC). The maximum voltage applied was specific for each buffer type (Table 2), but never greater than voltages that generated current exceeding 50 ma. The electrophoretic run was stopped when the bromophenol blue marker reached within 1 cm of the anodal sponge cloth. Seven gel and tray buffers (Table 2) were tested with thirty-eight protein and enzyme stains (Table 3).

Four to six 0.15-mm slices were obtained from each gel, the slicing apparatus made with the thinnest guitar string (key 'E') stabilized in an acrylic frame. Each slice was placed in a labeled, plastic stain box, the appropriate stain poured over the gel slice; and gently shaken to insure even distribution of stain. The gel slice and stain were incubated according to stain protocol, either at 37° C, or room temperature. When dark zones appeared, enzyme reactions were arrested by pouring off the stain, rinsing the gel in tap water, and adding 3:1 methanol:acetic acid fixative. Gels were scored and photographed.

For zymograms representing gene products of multiple loci, the loci were numbered in ascending order corresponding to decreasing migration distance from the origin (i.e., fastest migrating isozyme was labeled '1'). In scoring polymorphic loci, the most common allele in the Kentucky population was designated 'M'. Alleles migrating faster (more anodally) were designated 'F', with a numerical subscript identifying the faster migrating alleles by lower numbers. Alleles migrating slower than the common

allele (more cathodally) were designated 'S', with the same rule for subscripts. Alleles possessing nearly identical electrophoretic mobility, such that multiple bands were not consistently distinguishable in the heterozygote, were lumped into one electrophoretic mobility category.

Data were analyzed with the computer package NTSys (Numerical Taxonomy and Multivariate Analysis, Version 1.80, Rohlf, 1993). Nei's genetic distance (Nei, 1972) was calculated between each pair of populations. The UPGMA (unweighted pair-group method using an arithmetic average) clustering method (Sokal and Sneath, 1963; Sneath, 1973; Nei, 1987; Rohlf, 1970) was used to construct a dendrogram for these five populations based on Nei's genetic distances. I used the computer package POPGENE, Version 1.2 (Yeh and Boyle, 1997) to calculate Fst values between these populations.

RESULTS

Allozyme polymorphisms and allele frequencies

Thirty-eight commonly used enzyme systems that have been observed to be polymorphic in other species (Selander et al., 1971; Siciliano and Shaw, 1976; Harris and Hopkinson, 1976), and for which chemicals were available were screened for reliable banding patterns (Table 3). Only enzymes producing discrete, unambiguously definable banding zones in at least 90% of the individuals were used in this study. Several enzyme systems were variable and scorable in certain populations, but were not consistently scorable across all individuals and runs. Therefore, these were not included in the population analyses due to lack of confidence in genotype designations. Problems for

Table 2. Buffers tested for use in allozyme analysis. The pH of both the gel and tray buffers, and maximum voltage applied for each gel type are specified.

	gel pH	tray pH	maximum voltage
Tris-citrate	6.7	6.3	130
Tris-citrate	7.5	7.5	150
Tris-citrate	8.0	8.0	250
Poulik, discontinuous	8.2	8.7	250
Tris-versene-borate	8.0	8.0	250
Tris-borate-EDTA	8.2	8.2	250
Lithium hydroxide	8.3	8.1	300

Table 3. Enzymes screened for polymorphisms in populations of spotted basses. Primary tissue for expression of each enzyme, and the buffer system(s) determined best for visualizing the system are listed. Polymorphic systems are marked with an asterisk.

Enzyme	Tissue	Buffer
Adenylate kinase*	Liver	LiOH
Isocitrate dehydrogenase*	Liver	TC 7.5 (+NADP)
Catalase*	Liver	Poulik
Creatine kinase	Muscle	TBE
Lactate dehydrogenase	Liver / muscle	Poulik, TVB
Sorbitol dehydrogenase	Liver (2 sys: 1 in liver only)	Poulik (liv), TC 7.5 + NADP (mus)
∝Glycerol-3-phosphate dehydrogenase	A/B: muscle A/B: liver(two systems)	TBE (mus) TC 7.5 (liv)
Glutamate-oxaloacetate transaminase	A: muscle (bottom slice), B: liver (primarily)	TC 7.5, TBE, TVB
Malate dehydrogenase	A: all; B: muscle (primarily)	TVB
Catalase	Liver only	TC 7.0

(table cont.)

Enzyme	Tissue	Buffer
Phosphoglucomutase	3 sys: 1: liver only, 2: muscle only, 3: present in both tissues	TC 7.5
6-Phosphogluconate dehydrogenase	Liver (primarily)	TC 7.0, 7.5
Super oxide dismutase	Liver (primarily)	Poulik (on Sdh)
NADA esterase	Liver (2 sys)	Poulik/LiOH
∝β-Naphthyl proprionate* esterase	Liver	TC 7.5
∝β-Naphthyl acetate esterae	Liver, muscle	TC 7.5
Malate dehydrogenase	Liver (best), muscle	TBE/TC
Phosphoglucose isomerase	Liver, muscle (2 Sys)	TC 7.0, Poulik
Glyceraldehyde-3-phosphate dehydrogenase	Liver	TVB (+NAD), LiOH

(table cont.)

Enzyme	Tissue	Buffer
Alcohol dehydrogenase	Liver	TVB
Peptidase (leu-tyr)*	1: liver; 1: muscle	Poulik
Peptidase (phe-ala-leu)	Liver	Poulik
Aldolase*	Liver	TVB(var), Poulik
Phosphoglycerate kinase	Liver	TVB
Adenosine deaminase*	Liver	TVB
Mannose-6-phosphate isomerase*	Liver	T.C. 7.5
Glucose-6-phosphate dehydrogenase*	Liver, muscle (2 sys)	TVB (liv), T.C. 7.5 (+NAD, NADP)
Aconitase	All tissues, (mitochondrial)	T.C. 7.0, TVB
Creatine kinase	Muscle (2 systems)	TVB
Hexokinase	Liver	Poulik
Alkaline phosphatase*	Liver, muscle	T.C. 7.5

(table cont.)

Enzyme	Tissue	Buffer
Glycerate-2-dehydrogenase	Liver	T.C. 7.5
Acid phosphatase	Liver	T.C. 7.0
∝Hydroxybutyrate dehydrogenase*	Liver, muscle	T.C. 7.0 (liv)
Nucleoside phosphorylase*	Muscle	TVB

*Enzyme systems that showed polymorphisms; the enzymes for which allele frequencies are not reported showed problems with reproducibility, or differential denaturation of alleles.

scoring included differential denaturation of alleles, blurred or non-discrete banding such that heterozygotes were difficult to differentiate from homozygotes in some individuals, or low reproducibility across individuals. In spite of attempted manipulations in tissue processing, buffer systems, and staining, consistent results could not be achieved with confidence for these systems. However, these systems appeared informative. These enzyme systems included nucleoside phosphorylase, glyceraldehyde-3-phosphate dehydrogenase, adenylate kinase, 6-phosphogluconate dehydrogenase, ∝-hydroxybuterate dehydrogenase, one peptidase, glucose-6-phosphate dehydrogenase, and catalase.

Seventeen zones of banding were resolved consistently in all five populations and scored as putative loci. The monomorphic and polymorphic enzymes scored in this study are listed by status in Table 4. Four of these putative loci were polymorphic and scorable across all five populations: two phosphoglucose isomerase loci, Pgi-1 and Pgi-3, mannose-6-phosphate isomerase, Mpi, and a naphthol AS-D acetate (NADA) substrate esterase locus (Tables 4 and 5). Two more enzyme systems, adenosine deaminase (Ada) and alkaline phosphatase (Alp), were polymorphic, well resolved, and informative, but were found in high concentrations in liver tissue, and not present in appreciable amounts in muscle tissue (Richardson, Baverstock, and Adams, 1986). Therefore, these two enzymes were not consistently detectable in the samples representing the Guadalupe bass, due to lack of sufficient liver tissue. Although an attempt was made to include liver tissue in this processing, apparently adding the appropriate volume of buffer for the amount of muscle tissue present, left the liver

enzymes too dilute to consistently visualize. Therefore, allele frequencies were available for only the four populations for which these two enzymes could be scored (Table 6).

Two alleles were observed at Pgi-1 in the five populations, three at each Pgi-3 and NADA-esterase, and four at Mpi. The M allele for the Pgi-1, Pgi-3, and NADA-esterase loci was found in highest frequency in all five populations. Alabama (AL) and Kentucky (KY) were both fixed for the M allele at Pgi-1; i.e., the M allele was the only allele present in either of these populations, and therefore, the frequency of M was 1.0. The rare allele (S_1) at this locus was present in the other three populations at a frequency of 0.15, or less. All five populations had the Pgi-3 M allele in the highest frequency (> 0.89); this allele was fixed in the *M. treculi* population from the Guadalupe River. At the esterase locus, all five populations showed the M allele at a frequency of 0.62 or greater; however, the Alabama collection differed from the other populations in the rank order of the remaining alleles (Table 5).

The Mpi locus presented an interesting, but more complex picture. The Kentucky population was fixed for the M allele. This allele was not found in a frequency greater than 0.2 in the other four populations, which were the southern U.S. populations These four southern populations showed the same allele (F_2) in highest frequency. This allele (F_2) was fixed in the population from the Tickfaw River (LE).

The distribution of alleles at the Ada locus showed a trend across the four populations evaluated similar to that seen at the Mpi locus for the same groups (Table 6). Three alleles were seen at this locus; all four populations shared the same

Table 4. Monomorphic and polymorphic enzymes scored in five spotted bass populations. Data are from isozyme analysis for *M. punctulatus* ssp. and *M. treculi*.

Monomorphic enzymes:	
isocitrate dehydrogenase (Idh)	
lactate dehydrogenase (Ldh)	
malate dehydrogenase (Mdh)	
glycerol-3-phosphate dehydrogenase (Gpdh)	
phosphoglucomutase (Pgm)	
glutamate-oxaloacetate transaminase (Got)	
6-phosphogluconate dehydrogenase (6-Pgd)	
superoxide dismutase (Sod)	
creatine kinase (Ck)	
aldolase (Ald)	
alcohol dehydrogenase (Adh)	
Polymorphic enzymes	**Number of alleles**
phosphoglucose isomerase-1	2
phosphoglucose isomerase-3	3
esterase, NADA substrate	3
mannose-6-phosphate isomerase	4
adenosine deaminase	3
alkaline phosphatase	3

Table 5. Allele frequencies at four isozyme loci in *M. punctulatus* ssp. and *M. treculi*. Allozyme designations are F (fast), S (slow), based on migration relative to M (the most common allele in the Kentucky population). Alleles are numbered within a mobility class with increasing numbers for greater migration distance from the origin; n = sample size. KY = Lake Herrington, KY; AL = Lake Jordan Reservoir, AL; LE = Tickfaw River, LA; LW = Atchafalaya Basin, LA; TX = Guadalupe River, TX.

		Locality				
Locus	Allele	KY	AL	LE	LW	TX
Pgi-1	M	1.00	1.00	0.89	0.94	0.85
	S_1	0.00	0.00	0.11	0.06	0.15
	n	20	35	23	18	41
Pgi-3	M	0.95	0.91	0.94	0.89	1.00
	S_1	0.00	0.09	0.00	0.03	0.00
	S_2	0.05	0.00	0.06	0.08	0.00
	n	20	37	24	18	42
NADA	F_1	0.28	0.07	0.26	0.17	0.06
	F_2	0.10	0.23	0.11	0.03	0.00
	M	0.62	0.70	0.63	0.80	0.94
	n	17	30	23	18	41

(table cont.)

		Locality				
Locus	Allele	KY	AL	LE	LW	TX
Mpi	F_1	0.00	0.18	0.00	0.00	0.06
	F_2	0.00	0.76	1.00	0.96	0.73
	M	1.00	0.04	0.00	0.04	0.19
	S	0.00	0.02	0.00	0.00	0.02
	n	20	23	20	13	40

Table 6. Allele frequencies at two isozyme loci analyzed from liver tissue. These were not obtained from the *M. treculi* population. Allozyme designations are F (fast), S (slow), based on migration relative to M (the most common allele in the Kentucky population). n = sample size. KY = Lake Herrington, KY; AL = Lake Jordan Reservoir, AL; LE = Tickfaw River, LA; LW = Atchafalaya Basin, LA.

Locus	Allele	KY	AL	LE	LW
Ada	F	0.00	0.13	0.12	0.05
	M	0.58	0.74	0.75	0.86
	S	0.42	0.13	0.13	0.09
	n	19	23	26	21
Alp	F	0.00	0.00	0.04	0.00
	M	0.86	0.92	0.92	0.63
	S	0.14	0.08	0.04	0.37
	n	18	19	23	19

common allele. The two Louisiana and Alabama populations had two other alleles in low, but similar, frequencies in each population ($\leq$ 0.13, Table 6). Again, the Kentucky population differed in having the rarer S allele in a relatively high frequency (0.42), the absence of the F allele, and the M allele was at a comparatively low frequency (0.58), relative to frequencies of 0.74 to 0.86 in the other three populations. Three alleles were also seen at the Alp locus. All four populations had the same common allele in frequencies ranging from 0.63 to 0.92; however, the frequencies seen in the Atchafalaya (LW) population for the M and S alleles diverged from the other three populations at this locus. The Tickfaw River (LE) and the Alabama populations showed nearly identical allele frequencies at both Ada and Alp loci. One notable difference was that the LE population contained a rare allele at the Alp locus (F, frequency = 0.04), not seen in any other population (Table 6).

Wright's Fst

Heterogeneity in the allele frequencies observed among a group of populations, and thus population subdivision, was quantified with a single statistic, Fst (Wright, 1951; Nei, 1973) (Table 7). An Fst of 0.291 was calculated for the five bass populations and four polymorphic loci (Pgi-1, Pgi-3, NADA, and Mpi). By eliminating the Mpi locus for the five populations, an Fst = 0.074 was obtained. For the four populations with inclusion of the Ada and Ap loci and with Mpi, the Fst was 0.236. With both Ada and Ap included, but eliminating Mpi, the Fst for the four populations was 0.080.

Table 7. Wright's Fst values for isozyme data in spotted basses. Values are calculated from isozyme data for different combinations of allozyme loci and the five populations.

Populations	# of Polymorphic Loci	Fst
5 (KY, LE, LW, AL, TX)	4 (Pgi-1, Pgi-3, Mpi, NADA)	0.291
5 (KY, LE, LW, AL, TX)	3 (Pgi-1, Pgi-3, NADA)	0.074
4 (KY, LE, LW, AL)	4 (Pgi-1, Pgi-3, Mpi, NADA)	0.318
4 (KY, LE, LW, AL)	6 (Pgi-1, Pgi-3, Mpi. NADA, Ada, Ap)	0.236
4 (KY, LE, LW, AL)	5 (Pgi-1, Pgi-3, NADA, Ada, Ap)	0.080

Genetic distances

Nei's genetic distances and genetic identities were calculated between each pair of the five populations based on the four polymorphic loci present in all five populations (Table 8). The genetic distance between the two Louisiana populations suggests a close genetic relationship. This pair of Louisiana populations also shows close genetic relationships with the samples collected from Alabama (AL) and Texas (TX), as indicated by genetic distances of 0.023 to 0.044, respectively. The population collected from Lake Herrington, Kentucky was the genetically most distinct among the collections, with distances ranging from 0.237 (TX) to 0.369 (LE).

Three additional analyses were carried out (Tables 9-11). One analysis excluded the discordant data from the Mpi locus for all five populations (Table 9). Therefore, results were based on eight alleles from three variable loci. The other two analyses incorporated the two additional polymorphic loci, Ada and Alp, that were not detectable in the Texas population, *M. treculi.* Consequently, the Texas population was not included in either of the latter analyses. These data including allele frequencies from Ada and Alp were also analyzed with, and without, the Mpi locus. A total of eighteen alleles was present in the analysis with the inclusion of the Mpi data, and a total of 14 alleles without the Mpi data. Tables 10 and 11 list genetic distances and identities for the four populations (TX not included) analyzed with Ada and Alp, both with, and without Mpi, respectively.

Table 8. Nei's genetic distances and genetic identities based on four polymorphic allozyme loci in five populations of spotted basses. Population designations are by collection site: KY = Lake Herrington, Ky., LE = Tickfaw River, La., LW = Atchafalaya Basin, La., AL = Lake Jordan Reservoir, Al., TX = Guadalupe River, Tx. The first four populations represent *M. punctulatus* spp. according to current classification; the Texas population represents *M. treculi*. Nei's genetic distances are below the diagonal; genetic identities are above the diagonal.

	Locality				
	KY	LE	LW	AL	TX
KY	0.0000	0.6911	0.7142	0.7493	0.7886
LE	0.3694	0.0000	0.9912	0.9703	0.9571
LW	0.3366	0.0089	0.0000	0.9770	0.9773
AL	0.2886	0.0302	0.0233	0.0000	0.9672
TX	0.2374	0.0439	0.0230	0.0333	0.0000

Table 9. Nei's genetic distances and genetic identities based on three polymorphic allozyme loci, excluding the Mpi locus, in five populations of spotted basses. Population designations are by collection site: KY = Lake Herrington, Ky., LE = Tickfaw River, La., LW = Atchafalaya Basin, La., AL = Lake Jordan Reservoir, Al., TX = Guadalupe River, Tx. The first four populations represent current classification as *M. punctulatus* ssp.; the Texas population represents *M. treculi*. Nei's genetic distances are below the diagonal; genetic identities are above the diagonal.

	Locality				
	KY	LE	LW	AL	TX
KY	0.0000	0.9958	0.9871	0.9832	0.9610
LE	0.0043	0.0000	0.9888	0.9800	0.9718
LW	0.0130	0.0112	0.0000	0.9829	0.9882
AL	0.0170	0.0202	0.0172	0.0000	0.9670
TX	0.03981	0.0286	0.0119	0.0336	0.0000

Table 10. Nei's genetic distances and genetic identities based on six polymorphic allozyme loci, including Ada and Alp, in four populations of spotted bass. Population designations are by collection site: KY = Lake Herrington, Ky., LE = Tickfaw River, La., LW = Atchafalaya Basin, La., AL = Lake Jordan Reservoir, Al. All populations represent current classification as *M. punctulatus* ssp.; the Texas population, *M. treculi*, is not included due to lack of sufficient liver tissue. Nei's genetic distances are below the diagonal; genetic identities are above the diagonal.

	Locality			
	KY	LE	LW	AL
KY	0.0000	0.7680	0.7613	0.8087
LE	0.2640	0.0000	0.9704	0.9777
LW	0.2727	0.0300	0.0000	0.9625
AL	0.2124	0.0225	0.0382	0.0000

Table 11. Nei's genetic distances and genetic identities based on five polymorphic allozyme loci in four populations of spotted bass, including Ada and Alp, but omitting Mpi. Population designations are by collection site: KY = Lake Herrington, Ky., LE = Tickfaw River, La., LW = Atchafalaya Basin, La., AL = Lake Jordan Reservoir, Al. All populations represent current classification as *M. punctulatus* ssp.; the Texas population, *M. treculi,* is not included due to lack of sufficient liver tissue. Nei's genetic distances are below the diagonal; genetic identities are above the diagonal.

	Locality			
	KY	LE	LW	AL
KY	0.0000	0.9791	0.9508	0.9715
LE	0.0212	0.0000	0.9631	0.9850
LW	0.0505	0.0376	0.0000	0.9642
AL	0.0289	0.0151	0.0364	0.0000

Phenogram

Nei's genetic distances calculated from these data were used to construct phenograms based on the UPGMA method (unweighted pair-group method with arithmetic mean). In the phenogram constructed from the data inclusive of Mpi and all five populations, the two Louisiana populations clustered most closely (genetic distance, D = 0.009) (Fig. 2). The Alabama population was the next most closely aligned population to this pair (average D = 0.027); the Guadalupe bass (*M. treculi*) diverged next (average D = 0.033). The Kentucky population was the most divergent population within the *punctulatus* group. This was primarily due to the fixation of the relatively rare M allele at the Mpi locus. This allele was not present in a frequency higher than 0.19 in any of the other four populations, and was completely absent in the LE population. The F_2 allele was the most common in the other four populations, and was fixed in the LE population (see Table 5).

A phenogram was constructed for each modified data set (Figs. 3 - 5). For the five populations with the Mpi locus omitted (Fig. 3), KY now aligned with LE. LW and TX were closely associated, and the most distinct of the five populations. Only the Louisiana, Kentucky, and Alabama populations could be included in the analyses including allele frequencies at the Ada and Alp loci. When the Mpi locus was included in the analysis with these two additional loci (Fig. 4), the LE and AL populations showed the closest genetic relationship (D = 0.023), and the LW population was the most divergent of these four (D = 0.273, Table 10). If the Mpi locus was eliminated (Fig. 5),

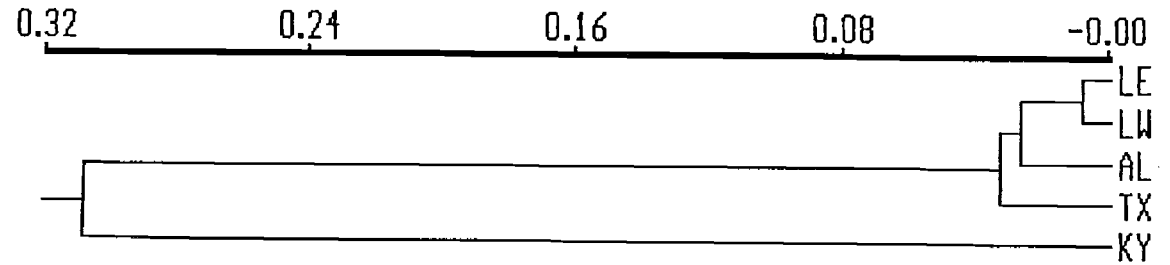


Fig. 2. Phenogram of five spotted bass populations, based on isozyme data at four polymorphic loci. Scale is Nei's unbiased genetic distance. KY = Lake Herrington, KY; AL = Lake Jordan Reservoir, AL; LE = Tickfaw River, LA; LW = Atchafalaya Basin, LA; TX = the Guadalupe River, TX.

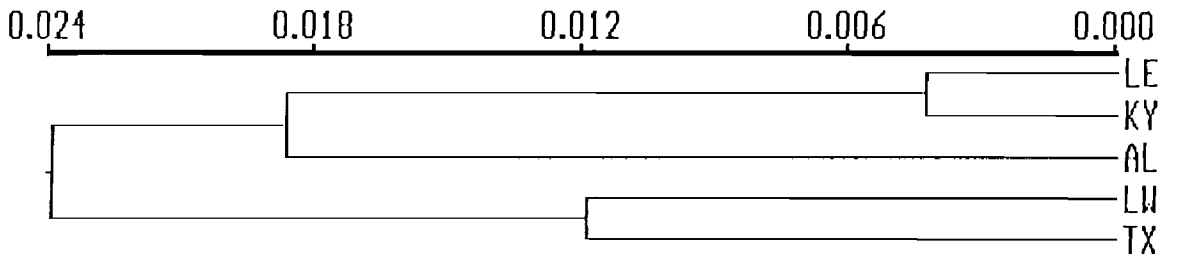


Fig. 3. Phenogram of five spotted bass populations, based on isozyme data at three polymorphic loci. Scale is Nei's unbiased genetic distance. KY = Lake Herrington, KY; AL = Lake Jordan Reservoir, AL; LE = Tickfaw River, LA; LW = Atchafalaya Basin, LA; TX = the Guadalupe River, TX.

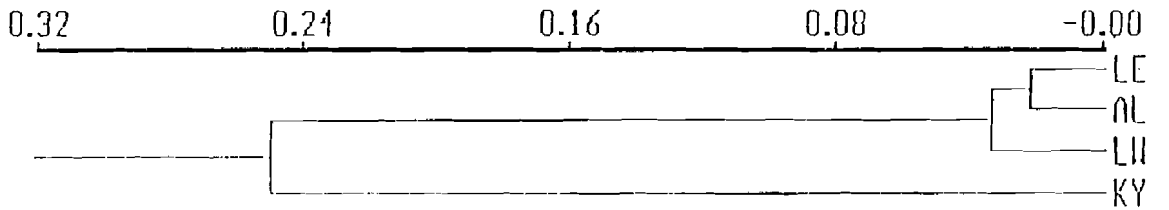


Fig. 4 Phenogram of four spotted bass populations, based on isozyme data at six polymorphic loci. Scale is Nei's unbiased genetic distance. KY = Lake Herrington, KY; AL = Lake Jordan Reservoir, AL; LE = Tickfaw River, LA; LW = Atchafalaya Basin, LA.

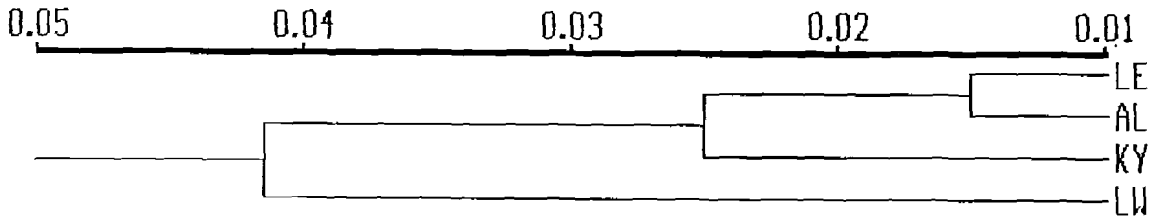


Fig. 5. Phenogram of four spotted bass populations, based on isozyme data at five polymorphic loci. Scale is Nei's unbiased genetic distance. KY = Lake Herrington, KY; AL = Lake Jordan Reservoir, AL; LE = Tickfaw River, LA; LW = Atchafalaya Basin, LA.

the LE population aligned most closely with the KY population. The AL population branched within this cluster, at an average distance of ~ 0.02. The LW and TX populations were the most distant from these three, and aligned more closely with each other, than with any of the other three.

DISCUSSION

In this study, I examined allozymes in spotted bass populations and focused on the polymorphic loci which were clearly reproducible, with heterozygotes that were unambiguously scorable, and allelic differences that provided information on differentiation among these populations. No estimates of genetic variability are made because loci showing low variability (allele frequency < 0.05 over all five populations) and problems in reproducibility were not analyzed for the complete data set (as additional samples could not be obtained). There also were several loci which showed scorable polymorphisms in some populations, but were not repeatable and consistent in all populations. In addition, the samples representing the Guadalupe bass were all small juveniles, and adequate liver tissue was not obtained from all individuals. Thus, the two polymorphic loci found only in liver tissue were not scorable in these samples.

The degree of genetic heterogeneity seen among these five bass populations was estimated with Wright's Fst (Table 7). Heterogeneity in the allele frequencies observed among a group of populations, and thus population subdivision, can be quantified with this single statistic, Fst (Wright, 1951; Nei, 1973). Fst is theoretically the correlation between two gametes drawn at random from each subpopulation relative to the correlation between two gametes drawn at random from the total population (Nei,

1973). In practice, Fst is calculated as a standardized variance of allele frequency within a group of populations:

$$Fst = s^2 / (\bar{p} (1 - \bar{p})),$$

where s^2 is the sample variance of the allele frequencies over populations being compared, and $\bar{p}$ is the average frequency of allele 'p' over all populations (Weir, 1996). An Fst of 0.10 - 0.15 has been observed in natural populations known to be subdivided (Wright, 1978).

The Fst values were calculated with the POPGENE computer package (Yeh and Boyle, 1997) for different combinations of allozyme loci and populations (Table 7). These analyses include all monomorphic loci in this study, and differing numbers of polymorphic loci. An Fst value of 0.291 for these five bass populations in the analysis with the Mpi locus included supports the hypothesis that these populations show signs of subdivision. Similarly, with Ada, Ap, and Mpi included in the analysis with four populations, an Fst value of 0.236 also indicates subdivision. However, when the Mpi locus is eliminated from the analysis, Fst values of 0.074 without Ada and Ap, and 0.080 with Ada and Ap, were obtained. These values are well below that predicted to indicate population subdivision. Therefore, the single Mpi locus appears to be driving the conclusion of population subdivision among these basses. If this locus is not considered, the Fst values are well under values proposed to indicate population subdivision, and these five spotted bass populations appear to be relatively genetically homogeneous (Nei and Chakraborty, 1973).

The relationships among these basses are visually presented in phenograms (Figs. 2 - 5) constructed with the UPGMA clustering algorithm (Sneath and Sokal, 1973). This method assumes constant and equal (or nearly equal) mutation rates along all branches in the tree. If the distance measure is linearly related to time since divergence among taxa, UPGMA produces 'correct' trees. Since the true shape and length of a tree can never be known, the reliability of this method can only be evaluated in computer simulations and with expected values. In simulated comparisons with other methodologies, UPGMA performs well and has often better represented the expected 'true' species tree (Huelensenbeck and Hillis, 1993; Swofford and Olsen, 1990).

Based on data for the four polymorphic loci present in all five populations, both Louisiana populations appeared to be more closely aligned with *M. p. henshalli* (AL) than with *M. p. punctulatus* (KY). The Tickfaw River population from eastern Louisiana was slightly more distant than the western Louisiana population (Atchafalaya Basin) from the other three spotted bass populations. The Alabama population, representing *M. p. henshalli*, was closely related to *M. treculi* and both Louisiana populations. The small genetic distance between the two Louisiana populations (D = 0.009) was indicative of local populations connected by gene flow (Slatkin and Maruyama, 1975), and supports the contention that the Mississippi River has not acted as a substantial barrier to genetic exchanges throughout the distribution of spotted bass in southern Louisiana.

The distinctness of the Kentucky *M. punctulatus* population in this analysis was not surprising given the documented history of this population. This Kentucky

population inhabits a reservoir that was impounded in 1929 by damming the Dix River. At that time, the *M. punctulatus* population most likely experienced an initial genetic bottleneck. The new barriers to migration most likely restricted gene flow, led to reproductive isolation, and thus allowed this population to experience subsequent genetic drift. One of the proposed consequences of genetic bottlenecks and genetic drift in small, closed populations is fixation of a relatively rare allele (or alleles) (Wright, 1931; Kimura, 1955, 1962; Nei, 1975). Therefore, the proportionately larger genetic distance estimated between the Kentucky population and the other four could be due to stochastic processes in one population, and not signify overall genetic divergence among these populations of fishes. As this study measured no other parameters, regarding either population structure or environmental variables, it was impossible to ascertain whether the allele frequencies at a few loci were a reflection of selection or a stochastic event, relating to possible bottlenecks experienced at reproduction (such as founder effect, or generational bottlenecks).

The pattern of relationships among these populations at the Mpi locus was distinct from that observed at the other loci. To ascertain the effect of this single locus on the results, the analysis was run without Mpi and with just three polymorphic loci. With the Mpi locus eliminated, the Kentucky population more closely aligned with the Tickfaw River (LE) population, and the Texas population and the Atchafalaya Basin population (LW) were closely related (Figure 3). The Alabama population completed a cluster of the three most eastern populations; whereas, the more western populations (LW and TX) were distinct from these other three.

With inclusion of the two liver encoded polymorphic isozymes, adenosine deaminase and alkaline phosphatase, which were not obtained in the Texas samples, and with mannose-6-phosphate isomerase left in the analysis, the Kentucky population was again the most distant. If Mpi was omitted, with inclusion of these two liver enzymes, the Atchafalaya (LW) population was the most distinct of the four remaining populations. However, the genetic distances calculated among the four populations with these five loci were small. The largest genetic distance in this latter analysis was between the Atchafalaya (LW) population and Kentucky (D = 0.0505).

Subspecies status was supported for *M. s. salmoides* and *M. s. floridanus* when a Nei's genetic identity of 0.911 was estimated from isozyme data (Philipp et al., 1983). Genetic identity is related to the genetic distance discussed above by the equation:

$$D = -\ln_e I,$$

where "D" is Nei's genetic distance, and "I" is Nei's genetic identity (Nei, 1987). This is a measure of the genetic differences between two populations which theoretically estimates the number of gene substitutions per locus. One assumption of this estimate is that the ancestral population was in equilibrium. It is an appropriate measure of differences when populations diverge due to drift and mutation (Nei, 1972). With the Mpi locus excluded from this present analysis, the lowest genetic identity, (i.e., the two populations showing the least genetic relatedness in this group) among these five populations was between the Texas and Kentucky populations (I = 0.9610). The genetic identities calculated between each pair of populations within this group for this analysis ranged from 0.9610 - 0.9958. Therefore, with the single 'aberrant' locus not included,

these five spotted bass populations showed closer genetic affinities than estimates of genetic distance between the two subspecies of *M. salmoides*. In the analysis including the Mpi locus, but not considering the Kentucky population, the four remaining populations had genetic identities ranging from 0.9571 (TX-LE) to 0.9912 (LW-LE). Again, these identity estimates were higher than the genetic identity calculated between the two *M. salmoides* subspecies.

The estimates of genetic distance parameters in fish species vary widely depending on species and population structure. Imsiridou et al. (1997) examined the genetic structure of fifteen populations of a species of river chub (*Leuciscus cephalus*) in Greece and France by evaluating patterns of variation at 20 enzyme loci. Genetic distances ranged from 0.002 - 0.063, and gave no evidence of speciation. In a similar allozyme study of variation in populations of the Red River pupfish, *Cyprinodon rubrofluviatilis*, Ashbaugh et al. (1994) examined seventeen populations from two river drainages in Texas and Oklahoma. Roger's genetic distance between the population clusters from the two different river drainages was 0.25. Within each drainage, the distances ranged from 0.008 - 0.009 for three samples from the Brazos River, and 0.012 - 0.059 for fourteen populations in the Red River drainage system. Diagnostic alleles found in each of the two drainages, in addition to the relatively large interdrainage genetic distance compared with the intradrainage distance, support the hypothesis that these two populations represented cryptic species. Van der Bank et al. (1989) examined allozyme variation in fifteen African cichlid species. Nei's genetic distances ranging

from 0.095 to 0.565 were calculated for comparisons fifteen species within three genera, each containing two or more species.

Comparing my results to the above values, I conclude my data showed little evidence of reproductive isolation among four of these spotted bass populations and species, and among all five populations, there was no evidence of diagnostic alleles at any locus examined. The genetic distances and identities were within the range of other species, known or suspected to have recent gene exchange. The Kentucky population and Mpi locus however, presented an interesting case. The analysis based on allozymes was dependent on such a small number of polymorphic systems and genetic markers, that a single aberrant genetic locus greatly affected the results. As discussed above, a number of hypotheses can be proposed to explain this discordant locus, but in an aquatic species located in a small enclosed habitat, the occurrence of a past, as well as recurrent, genetic bottleneck is a compelling hypothesis. However, the apparent fixation of a rare allele does indicate that there is very little, or no gene exchange currently occurring between the Kentucky population and other populations.

CHAPTER 3

SYSTEMATIC ANALYSIS: RAPD POLYMERASE CHAIN REACTION

INTRODUCTION

Population genetics has seen the development and application of a number of techniques, the primary ones until recently involving isozymes and mtDNA (evaluated primarily from restriction fragment length polymorphisms, RFLPs). Isozyme analysis, a technique useful for assaying differences in functional and structural proteins, is commonly used in systematic and population studies. However, given the relative conservation of functional enzymes, as well as other protein gene products, genetic changes are slow to accumulate, and therefore, isozyme analysis may not be sensitive enough to detect genetic differences observed in the incipient stages of population subdivision (Ferguson et al., 1995; Bielawski and Pumo, 1997; Seyoum and Kornfield, 1992). Isozyme analysis has failed to detect differences between and among a number of populations or taxa that demonstrate apparent reproductive isolation (Bardakci and Skibinski, 1994). An alternative method utilizes the mtDNA molecule. The mitochondrial genome has a relatively high mutation rate and is useful for detecting very recent genetic changes (Wilson et al., 1985). However, one drawback to this technique is that it samples only the mitochondrial genome.

A technique more sensitive to genetic changes at the population level, which also assays genetic differences at the intra- and interspecific level, is needed to cover the postulated range of relationships among these basses. The polymerase chain reaction, using short, arbitrary oligonucleotides of random sequence is expected to yield such

suitable resolution. This technique theoretically surveys DNA from moderately repetitive to highly conserved areas of the genome. The use of genetic data to study population structure and genetic relationships is based on the assumption "that population structure will affect all loci in a similar fashion, while locus-specific effects will differ from locus to locus" (Richardson, Baverstock, and Adams, 1986). Therefore, the utility and reliability of molecular markers in revealing population relationships and evaluating systematics are dependent, in large part, on random and complete sampling of the genome. While it is difficult to know the extent to which these criteria are met, they can be best optimized by choosing a technique which, at least, theoretically maximizes random and complete sampling of the genome. The application of both these methods (allozymes and RAPD-PCR) should give a range of resolution sufficient to elucidate relationships among these black basses.

RAPD-PCR analysis has revealed genetic differences in several studies where isozyme and mitochondrial DNA analyses have failed to detect significant differentiation, e.g., the Atlantic coast striped bass, *Morone saxatilis* (Bielawski and Pumo, 1997), and among three species (*Oreochromis mossambicus, O. aureus, O. niloticus*), and four subspecies (*O. niloticus* ssp.)of tilapia (Bardakci and Skibinski, 1994). Atlantic coast striped bass are an extremely conserved species genetically; isozymes and isoelectric focussing show virtually no variation in that group. However, Bielawski and Pumo (1997) found they could measure nuclear DNA variation with RAPDs, and detected genetic subdivision between two river systems, that was not detected with mtDNA. Bardakci and Skibinski (1994) found isozymes were capable of discriminating among

three species of tilapia, but were not sensitive enough to detect differences among four subspecies of *O. niloticus*. Hybrids between tilapia species were identified using mtDNA, but this technique could not reveal intrapopulation variation in these species. However, RAPDs were sensitive enough to detect not only subspecies differences, but intrapopulation variation, also.

The RAPD technique was developed in the early 1990's, independently, by two different research groups (Williams et al., 1990; Welsh and McClelland, 1990). With this technique, short, arbitrary sequence DNA primers are added to a reaction mixture containing target DNA (from the organism to be analyzed), and all cofactors necessary for DNA replication. Through repeated cycles of heating and cooling, DNA is denatured (92°-95° C), annealed (37° C), and replicated (72° C). If two sites on the target genome are complementary to the arbitrary primer sequence and separated by a distance no greater than 2000-3000 base pairs, many blunt-ended products, complementary to the DNA between these sites will be replicated. Through repeated cycling of this process, these target DNA sequences will be produced in exponential numbers. The end result of this procedure is a mixture containing a nested sample of discrete DNA molecules, replicated from, and thus complementary to, specific sites in the target genome. A sufficient quantity of DNA is produced such that these fragments can be separated and visualized with agarose, or polyacrylamide, gel electrophoresis. The molecular size of DNA fragments can be estimated by comparing the migration distance with that of corresponding bands from molecular size standards included on the same gel (Tingey et al., 1992; Caetano-Anolles et al., 1991).

Each discrete band visualized on the gel represents a unique DNA molecule of a specific molecular weight, and is assumed to be the product of a single locus, with two character states, 'presence' or 'absence'. Band 'presence' reflects the presence of two complementary primer binding sites flanking a segment of DNA (generally less than about 3000 kilobases, kb, in length). Band 'absence' reflects loss of one or both of the primer binding sites, or a deletion or insertion between these two sites, such that the molecular size of the amplified fragment is modified. A data matrix can be constructed with the character states of each specific band for each primer for all individuals. A 'fingerprint' is generated, which is specific for each primer and DNA template combination. The frequency of the 'absent' (i.e., null) allele at each locus can be calculated by taking the square root of the frequency of the 'null' phenotype in each population. The frequency of the 'present' allele is then calculated by subtracting the frequency of the 'null' allele from '1'.

One reason RAPDs are effective for detecting variation between such closely related groups is that the random sequence primer does not discriminate between coding and noncoding regions of DNA, and thus will amplify DNA both in moderately repetitive DNA and in structural, or coding, DNA. Thus, this technique, theoretically, assays genes ranging from highly variable to phylogenetically conserved (Welsh and McClelland, 1990; Caetano-Anolles et al., 1991). The number of fragments generated by a single primer can be quite large. Therefore, it is a reasonable assumption that this technique is better at randomly, and more extensively, sampling the genome than the more standard techniques (Lynch and Milligan, 1994). An additional advantage of

surveying a large number of loci randomly distributed throughout the genome, is that this reduces the likelihood of problems associated with inclusion of linked loci in population studies, and improves the probability of unbiased sampling of DNA variation (Nei, 1978). However, the genetic basis of the genes amplified to produce fragments is unknown without breeding and heritability studies. Additionally, the process and thus results are sensitive to cycling conditions, as well as the concentration of components in the reaction mixture.

Objectives

I will use RAPD-PCR to assess genetic divergence among eight populations of black basses. These populations include the five populations of spotted basses used in the isozyme analysis described in Chapter 2: the two Louisiana populations (*M. p. punctulatus*), one Alabama population (*M. p. henshalli*), one Kentucky population (*M. p. punctulatus*), and one Texas population (*M. treculi*). I will estimate the genetic relationships (i.e., distances) between these five spotted basses and two congeneric species (*M. salmoides*, the largemouth bass, and *M. notius*, the Suwanee bass). These two species also serve as the 'outgroups' in this study. The distances between these 'outgroups' will be used to calibrate the distances observed among the five spotted bass, so that a representative phenogram can be constructed with all populations. I will also obtain samples of the newly described black bass from the Chipola River in Florida, and determine the genetic affinities, based on the RAPD marker profile, of this distinct micropterine bass.

MATERIALS AND METHODS

Field and tissue collection

Study sites and collection methods for the five spotted bass populations in this study are described in Chapter 2. Samples were obtained from two additional species and from the recently described Chipola bass (Figure 1):

Micropterus salmoides--Atchafalaya Basin, La.

Micropterus notius--Sante Fe River, Fla.

Chipola bass--Chipola River, Fla.

The Florida specimens were all collected with boat electrofishing by Florida State Wildlife and Fisheries personnel. The Louisiana specimens of *M. salmoides* were collected while electrofishing for other samples.

Fish were kept in aerated water in a live well, ice chest, or bucket until time for bleeding. Blood was drawn either from the heart (for fish over 10 cm, approximately 90% of fish sampled), or from the dorsal artery (for fish under 6 cm, approximately 5% of fish sampled). To draw blood from the dorsal artery, the needle was inserted at a point immediately behind the anal fin. One-cc, 3-cc, or 5-cc syringes were used: needle size was appropriate to fish size (26G5/8, 25G5/8, 23G1, 22G1, or 20G1). Acid citrate dextrose (ACD), Solution B was used as an anticoagulant at a ratio of 0.1 ml of ACD for approximately 1.5 ml of blood. A volume of 0.5 to 3 ml of blood was drawn from each fish. After insuring mixing of the blood with ACD, the blood was then expelled into a sterile Eppendorf tube or left in the syringe, and then placed on ice for transport

back to the laboratory. Blood samples were stored at -20° C for 1 to 7 days, until DNA was extracted.

DNA extraction: phenol / chloroform method

For the following steps, all tubes, pipets, pipet tips, and any other supplies used with DNA were purchased sterile or sterilized in an autoclave. All pipet tips used for stock solutions of DNA had aerosol barriers, and were used only once. DNA was extracted from whole blood with a modification of the phenol-chloroform method (Ausubel, et al., 1987). DNA was extracted from blood of 5 or fewer individuals, two tubes per individual, at one time. Forty-five *u*l of whole blood were aliquoted into clear 1.5-ml Eppendorf tubes. Fifty *u*l of Tris-EDTA (0.1 mM EDTA, TE_{low}) buffer were added to this solution. This increased the volume of the aqueous phase, determined by earlier experiments to maximize the quantity of clean DNA (with reduced lipid, protein, and other contaminants) recovered from blood samples. To this mixture, 500 *u*l of 10X SSC (1.5 M sodium chloride, NaCl; 0.15 M sodium citrate, pH 7.0) were added, and mixed with a Pasteur pipet by gently pipetting the contents up and down five times. Sixty *u*l of 10% sodium dodecyl sulfate (SDS) were added. This again was gently mixed with a Pasteur pipet until the sample appeared viscous (about 30 seconds), and then 500 *u*l of phenol:chloroform:isoamyl alcohol (24:1:1) were added. This solution was mixed with a Pasteur pipet for 1 to 3 min, or until the mixture was light brown.

The microtubes with the blood/phenol/chloroform mixture were centrifuged in a counter-top centrifuge (HBI MicroCentrifuge) at maximum speed (13,000 RPM) for 5 minutes. The upper (aqueous) layer from each tube was transferred to a clean

Eppendorf tube, and 500 *u*l of phenol:chloroform:isoamyl alcohol were added to each tube. The solution was mixed vigorously, with a new Pasteur pipet for approximately 1 minute. This solution was centrifuged for 5 minutes at 13,000 RPM. The upper (aqueous) layer was transferred to a clean Eppendorf tube. This procedure was repeated a minimum of two times, or until a clear aqueous layer was obtained.

After the final spin, the aqueous layer was transferred to a clean Eppendorf tube, and 2.5 volumes of 100% cold ethanol (stored at -20° C) were added. The tube was inverted several times to ensure thorough mixing and was placed at - 20° C for at least 4 hours. The DNA/ethanol solution was then centrifuged for 5 minutes at maximum speed. Most of the supernatant was pipetted off, except a thin layer of fluid was left on top of the DNA pellet (about 10 - 20 *u*l). The pellet was washed with 70% cold ethanol: 500 *u*l of 70% ethanol were added, and the tube was centrifuged for 5 minutes. The supernatant was decanted, and the pellet was dissolved in 200 *u*l of sterile deionized water. The DNA was precipitated by adding 500 *u*l of cold 100% ethanol, mixing, and placing at -20° C for at least 20 minutes. This solution was centrifuged for 5 minutes, the supernatant decanted, and the pellet allowed to air-dry by inverting the tube on a clean sheet of filter paper (20 to 45 minutes). The pellet was resuspended in approximately 10 times the dry volume of the pellet with sterile TE_{low} buffer (50 to 150 *u*l). This solution was placed at 4° C for 4 hours, or overnight. Any remaining pellet was resuspended by gentle pipetting with a large bore pipet, until the pellet was dissociated and the solution appeared homogeneous. The DNA concentration of this

solution was immediately quantified on an agarose gel (see below), or the solution was stored at -20° C until quantification of DNA (maximum of 3 days).

DNA extraction: guanidine-HCl method

DNA was also extracted from whole blood by using the Guanidine-HCL method (see Appendix D). The quality of this DNA was later compared to that obtained with the phenol-chloroform method for quality in amplification. Blood samples from 30 fish were extracted by following this protocol. PCR amplification was carried out with DNA obtained by both the guanidine-HCl method and the phenol-chloroform-isoamyl alcohol procedure, and results from these two methods were compared.

Evaluation and quantification of DNA

Concentration and quality (measured by amount of degradation) of DNA solutions were evaluated with electrophoresis on 1% agarose gels, for both tubes of DNA from each individual. For a 1% gel, 2.5 gm of agarose (Gibco UltraPure) were dissolved in 250 ml of 1X TBE buffer and heated to boiling in a microwave oven. To this mixture, 0.5 *u*l of ethidium bromide (10 mg / ml) were added, and the solution was cooled to 45° C in a iced water bath. The agarose solution was poured into a 21.6-cm x 35.6-cm plexiglass gel mold containing two combs, each comb forming 20-wells (14 *u*l / well). After solidifying, the gel was placed into a buffer tray containing 1X TBE buffer with 2 *u*l of ethidium bromide (10 mg / ml) / 100 ml of buffer.

Five *u*l of each DNA stock were added to 0.9 *u*l of 6X loading buffer on a piece of clean parafilm. Five samples were prepared at a time to minimize evaporation while

on the parafilm. Each sample was mixed with the loading buffer by pipetting with a 10-*u*l Eppendorf pipet, and loaded directly into the wells.

Five DNA standards were included on each gel: 0.01 *u*g, 0.05 *u*g, 0.1 *u*g, 0.5 *u*g, and 1.0 *u*g. Electrophoresis was conducted at 100 V (2.8-V / cm) for 5 to 6 hours. Gels were photographed over ultra-violet light (the GD57500 system). Concentration of each DNA sample was estimated from the picture of the gel by comparing size and intensity of the sample band with that of the DNA standards.

Particular attention was given this step of quantifying the concentration of the final working stock DNA solution, because the products and banding patterns discerned with RAPD-PCR are very dependent on DNA concentration in the amplification process. Only high quality DNA that showed little or no denaturation was used for PCR. Quality was estimated by the degree of degradation of the DNA seen on the agarose gel, reflected in the tightness of banding, and lack of any streaking or subbanding. One tube of DNA solution was chosen to make the stock dilution. If the qualities of two stocks were roughly equal, the tube with the highest DNA concentration was used; if the quality appeared different between the two tubes, the tube with the highest quality DNA was used regardless of differences in concentration.

Stock solutions with an estimated DNA concentration of 5 ng / *u*l in TE_{low} buffer were made for each sample. The volumes necessary for this dilution were calculated using the equation:

*(x u*l of stock DNA) (*y* ng / *u*l) = (100 *u*l) (5 ng / *u*l),

where y = the estimated concentration of the stock DNA in ng, and x = volume of the stock DNA to use in 100 *u*l (of TE_{low}), for a final concentration of 5 ng / *u*l. Ten *u*l from each dilution were prepared as above and run on a 1% agarose / TBE gel at 35.6 cm / 100 V. Three DNA standards were included on each gel: 0.01 *u*g, 0.05 *u*g, and 0.1 *u*g DNA. The concentration of DNA in each solution was estimated, and used to make a working stock dilution of 1 ng DNA / *u*l. Concentration of this working stock solution was confirmed as 1 ng / *u*l on a 1% agarose / TBE gel, with DNA standards of 0.01 *u*g / *u*l, 0.02 *u*g / *u*l, and 0.05 *u*g / *u*l. Necessary adjustments were made by adding the appropriate volume of DNA stock, or TE_{low} buffer to bring the stock to a final concentration of 1 ng / *u*l.

Trial PCR was conducted with three volumes of the 1 ng / *u*l stock DNA for final amounts of 1 ng, 5 ng, and 10 ng DNA / reaction. Banding patterns resulting from these runs were compared with the pattern previously determined for the particular primer / population combination for amplification of 5 ng of DNA. At DNA concentrations too low or too high for optimum amplification, bands became faint and disappeared. At DNA concentrations too high for optimum amplification, some bands disappeared (failed to amplify), while other bands became disproportionately intense. For a 1ng / *u*l solution, 3 ng, 5 ng, and 7 ng gave identical banding patterns; therefore, banding profiles produced with 1ng, 5 ng, and 10 ng of DNA were very similar, if not identical, to one another. For a 1 ng / *u*l solution, this range of DNA concentrations gave repeatable and consistent banding patterns. A final concentration of 1 ng / *u*l was confirmed in a PCR

experiment with these three concentrations of DNA, by comparing consistency of banding patterns.

This final stock solution was then aliquoted into 2 to 4 sterile 1.5-ml clear, Eppendorf tubes. One tube from each sample was placed in styrofoam box and stored at 4° C for analysis with PCR. The remaining tubes were placed in another styrofoam box, and stored at -20° C (in a non-frost free freezer) until needed for PCR.

DNA cleaning

To check the purity and amount of contaminants of the DNA, and any possible effect these may have on amplification, 30 samples of DNA were processed with Prep-A-Gene DNA Purification System. (see Appendix E). 'Processed' and 'unprocessed' samples were amplified side-by-side in a PCR reaction plate. Amplification products of the treatments were compared on agarose gels.

Polymerase chain reaction (PCR)

The PCR method was adapted from Ausubel et al. (1992), with modifications based on literature and experimentation (Penner et al., 1993) (Appendix I). The optimum combinations of different DNA and magnesium concentrations were determined, and then tested with different cycling parameters. The DNA concentrations tested were 1 ng, 5 ng, and 10 ng. The Mg concentrations tested were 1 mM, 2 mM, 5 mM, and 10 mM. After amplification parameters were optimized, six cycling profiles were tested. The 6 profiles were designed to include all possible combinations of initial denaturation time (3 minutes or 5 minutes) and the final extension time (3 minutes, 7 minutes, or 10 minutes). After the optimal time for each step was established, three

concentrations of Taq polymerase were compared to determine the minimum concentration needed for optimum amplification. Twenty-five *u*l reaction mixtures (including DNA) were tested with 0.5 units, 1 unit, and 5 units of Taq polymerase.

Polymerase chain reaction was conducted on a 96-well thermocycler (PTC-100, MJ Research, Inc.), in 96-well, polycarbonate, V-bottom microassay plates, "Concord" design. Plates were washed before use (Appendix F). Sixty primers were screened for variability in these eight populations (Kits W, M, C; Table 12). To insure that a substantial amount of variability in each population was included, screening was conducted with a 'cocktail' made of DNA from 10 individuals in each population, combined into 1 ng DNA / *u*l stock solution. Polymorphism for a RAPD generated genetic marker is manifested as a band at a specific kilobase size that shows both character states of 'presence' and 'absence' in the populations of interest, i.e., a specific band is present in some individuals, and absent in 5% or more individuals, or vice versa.

For data collection, samples from each of the 8 populations, with two primers and a negative control (i.e., all reaction components included, but without the DNA) were included on each plate. This procedure allowed simultaneous analysis of 45 to 47 samples per primer per plate for each of two primers. A minimum of two cycling procedures was performed with each sample-primer combination.

Data analysis

Data were analyzed by using two computer packages designed to handle RAPD data: RAPDistance Package, Version 1.04 (Armstrong et al., 1994), and POPGENE Version 1.2 (Yeh and Boyle, 1997). Because the data set exceeded the maximum

number of both individuals and primers that can be evaluated with the RAPDistance program, the data were entered in six different sets: two sets of individuals, each for three sets of primers. Each of the two data sets of individuals was analyzed separately for each of the three groupings of primers. The grouping of primers into the three sets was arbitrary and followed the order in which each primer was completed for the first set of individuals. The complete data set was combined and analyzed with the POPGENE package. This program calculates genetic distance estimates, and dendrograms, based on both Nei's original measures (1972) and Nei's unbiased measures (1978) of genetic distance. Nei's (1978) genetic distance and genetic identity were estimated for all data sets. Dendrograms were drawn for each analysis in the POPGENE package using UPGMA clustering method with Nei's unbiased measures of genetic distances. This program is an adaptation of J. Felsenstein's program NEIGHBOR of PHYLIP. This latter program constructs a maximum likelihood tree for individuals based on similarity / dissimilarity for nucleic acid sequence data.

RESULTS

DNA preparation and amplification

Concentrations of DNA determined with agarose gels ranged from 0.01 *u*g / *u*l to 0.7 *u*g / *u*l (Appendix K). Determining optimum conditions for all variables proved difficult because many variables had to be tested simultaneously, and lack of results was not obviously due to a particular factor or parameter. For 25 *u*l total reaction volume, the optimum quantity of DNA for amplification was determined to be 5 ng; optimum Mg concentration was 5 mM. Due to the expense of Taq polymerase, the optimum

concentration of Taq polymerase was the minimum amount required to amplify the maximum number of reproducible bands observed in these experiments. A concentration of 1 unit Taq polymerase / 25 *u*l reaction was determined to give optimum amplification.

The cycling profile which produced the maximum number of consistently resolvable bands is given in Appendix J. Important specifications in this profile included an initial denaturation step of 95° C, for 5 seconds, followed by 3.10 minutes at 93° C, 42 cycles, and a prolonged final extension time (total 10 minutes, 72° C).

Quality of DNA cleaned with Prep-A-Gene was evaluated. There was a slightly detectable improvement in the clarity of banding in PCR products, but no increase or decrease in the number of bands amplified. However, there was only about an estimated 20% recovery of the total amount of DNA when compared with estimates of nanogram DNA / *u*l whole blood obtained with phenol / chloroform extraction. Therefore, given the loss of DNA, expense and time required in using this procedure, and the lack of meaningful improvement in data, use of this procedure for all individuals was rejected. Similarly, the Guanidine-HCl DNA extraction procedure gave tighter, cleaner banding than the phenol/chloroform extraction procedure when evaluated on agarose concentration gels. However, there was low recovery of DNA in samples with total blood volumes less than about 0.2 ml. The total blood volume for a number of samples did not exceed 0.2 ml, rendering this technique inappropriate for these samples. Therefore, in order to standardize the extraction process for all samples, this technique was rejected.

Early runs demonstrated that the volume of the reaction mixture affected the outcome of the PCR runs. A total reaction volume mixed for 96 samples did not consistently amplify. One explanation for this inconsistency in amplification with size of the experiment is the inherent error in pipetting. Optimum repeatability required that no more than 50 samples be set up for one primer reaction. Therefore, this design required each population be analyzed in two sets of individuals, and each experiment for a primer contained individuals in similar proportions from each population.

Twenty-seven of the 60 primers resulted in production of polymorphic and reproducible bands (45%) over all eight populations and taxa. With these 27 primers, a total of 302 reproducible, polymorphic bands was amplified (Table 12). Initially, after the first two sets were analyzed, 308 bands were identified as polymorphic and scorable. However, after all individuals were analyzed for all primers, 6 of these polymorphic bands were evaluated as non-reproducible. These were bands that were monomorphic or showed a high frequency for presence of a specific marker in a population in the first two sets, but the band failed to amplify in the last two sets of individuals. Due to the poor reproducibility of these 6 bands, they were discarded from the analysis.

Among the five spotted bass populations, 178 of these bands were polymorphic, and 124 of these bands were present in all five spotted bass populations (i.e., for 60 loci, the presence of a band was not observed in one or more spotted bass populations). Over all eight populations for these twenty-seven primers, 288 of the bands amplified were polymorphic (95.4%). The variable primers, with the total number of bands amplified by each primer, and the number of bands amplified by each primer that were variable over

all nine populations and just over the *M. punctulatus* species group are given in Table 12. Variable primers are followed by an asterisk; primers for which a band was discarded are marked a with a second asterisk.

Fourteen bands, 4.6% of the total number of amplified bands from these twenty-seven primers, were present in all individuals in all eight populations (Table 13). A total of 217 bands was amplified with the 27 primers in *M. punctulatus* and *M. treculi*. Thirty-nine of these bands were seen in all individuals in the two species comprising the *punctulatus* complex, i.e., 18.0% of the total number of bands amplified in the five spotted bass populations was seen in all spotted basses (Table 13). Nineteen of these alleles were unique to the spotted basses, and not seen in any of the outgroups. Four of the *punctulatus* populations had one or more alleles unique to that population (Table 14): *Micropterus treculi* showed the largest number of population specific alleles among this species group, with nine; the Alabama population was next with six population specific alleles. There were two population specific alleles in the Kentucky population, while the Tickfaw River (LE) samples showed one population specific allele. There were no population specific alleles seen in the sample from the Atchafalaya Basin (LW). The numbers of species - specific alleles seen in the three outgroups were: 19 in *M. salmoides*, 21 in *M. notius*, and 11 in the Chipola bass (Table 15).

Genetic distance

The 159 samples were initially analyzed with the RAPDistance program (Armstrong, et al., 1994) in the two sets of samples of 78 and 81 individuals. Primers were grouped into three sets of 8, 12, and 7 primers for data entry. Each set was

analyzed separately, and in various combinations with the other two sets, to determine if the results changed with the addition of more samples or primers. All data were combined into one data set and analyzed with the computer package POPGENE, a program designed for population genetic data analysis (Yeh and Boyle, 1997).

The clustering of individuals was first evaluated with the RAPDistance program and Excoffier's measure of genetic distance (Excoffier et al., 1992), and the phenogram was produced in NTSys (Fig. 6). The individuals from each population formed single clusters on separate branches for each population. Genetic distance between these individuals was also calculated with Dice's coefficient (Dice, 1945). The pattern for individuals grouping at a branch terminus did not change with this analysis, but the arrangement of individuals within a population changed in a few cases.

Nei's (1978) genetic distances and genetic identities were calculated by population for the total data set (159 individuals, 27 primers) with the POPGENE program (Table 16). Within the spotted black bass complex for the entire data set, genetic distances ranged from 0.0506 (between Atchafalaya Basin and Kentucky) to 0.1968 (between the Guadalupe and Alabama populations). The east (Tickfaw River) and west (Atchafalaya Basin) Louisiana populations, and the Kentucky population show very close genetic relationships (ranging from 0.048 to 0.091), with Atchafalaya (LW) and Kentucky showing the closest relationship (D = 0.0506). The Alabama population was more closely related to the two Louisiana populations than to the other two populations, with genetic distances of 0.1367 (Atchafalaya) to 0.1421 (Tickfaw River). The Guadalupe bass (TX) was the most distinct of this group, with the consistently

Table 12. Banding with three sets of primers (C, M, W) screened across all eight populations and taxa. Primers which amplified polymorphic and reproducible bands are followed by an asterisk. For primers with ambiguous or indistinct banding patterns, the number of total bands is given in parentheses; '0' designates primers producing no discrete or readily definable bands. The designation '*Mp*' refers to both *M. punctulatus* and *M. treculi*. A second asterisk designates a primer for which one band was originally scored, then found to have poor reproducibility.

Primer	Total number bands	# of bands variable over all populations	# of bands variable in *Mp* populations
W- 1*	8	8	5
2*	18	17	9
3*	10	10	7
4**	10	10	9
5*	16	15	8
6	11	0	0
7*	14	14	5
8	12	0	0
9**	7	7	4
10*	7	7	7
11	7	0	0
12	7	0	0
13	10	0	0

(table cont.)

Primer	Total number bands	# of bands variable over all populations	# of bands variable in *Mp* populations
14	0	0	0
15	0	0	0
16*	8	6	3
17*	22	21	10
18	8	0	0
19*	10	10	3
20	11	0	0
C - 1	8	0	0
2	(10)	0	0
3	6	0	0
4*	15	15	10
5*	12	10	4
6*	12	12	9
7	8	0	0
8*	11	10	6
9*	6	6	5
10*	14	14	10

(table cont.)

Primer	Total number bands	# of bands variable over all populations	# of bands variable in *Mp* populations
C-11	8	0	0
12	6	0	0
13*	15	14	9
14	(5)	0	0
15	(7)	0	0
16**	9	8	5
17	6	0	0
18	0	0	0
19	8	0	0
20	7	0	0
M - 1	5	0	0
2	7	0	0
3	7	0	0
4**	9	9	6
5*	7	6	6
6	6	0	0
7	8	0	0

(table cont.)

Primer	Total number bands	# of bands variable over all populations	# of bands variable in *Mp* populations
M - 8	3	0	0
9*	8	8	7
10*	9	7	3
11	9	0	0
12	9	0	0
13	7	0	0
14**	8	8	5
15	4	0	0
16*	22	21	14
17	5	0	0
18*	8	8	4
19	3	0	0
20*	9	8	6

Table 13. Conserved alleles in study populations and taxa. 'Band' is in kilobase (kb) size. *Ms* = *M. salmoides*; *Mn* = *M. notius*; Ch = Chipola bass; TX = *M. treculi*.

Primer	band	present in all populations	present in *M. punctulatus* and *M. treculi*	
C-05	900		+	(plus *Ms*)
C-05	1500	+	+	
C-05	1850	+	+	
C-06	2400		+	
C-08	1450		+	
C-08	2000	+	+	
C-13	1200		+	
C-13	1800	+	+	
C-16	1050	+	+	
C-16	1780		+	(plus Ch)
M-04	1400		+	(plus *Mn* and Ch)
M-04	3500		+	
M-05	1550	+	+	(2 TX=0)
M-10	1200		+	(plus *Ms* and *Mn*)
M-10	1400	+	+	
M-10	1900	+	+	

(table cont.)

Primer	band	present in all populations	present in *M. punctulatus* and *M. treculi*
M-14	705		+
M-14	1000		+
M-16	2000	+	+
M-18	1550		+
M-18	1630		+
M-20	870		+
W-01	680		+
W-01	910		+
W-02	520		+
W-02	700	+	+
W-03	600		+
W-03	1250		+
W-05	600	+	+
W-07	780		+
W-07	1400		+
W-09	600		+
W-16	800	+	+
W-16	1000		+ (plus *Mn* and Ch)

(table cont.)

Primer	band	present in all populations	present in *M. punctulatus* and *M. treculi*
W-16	1500	+	+
W-17	750		+
W-17	900	+	+
W-17	1200		+
W-19	820		+ (plus *Ms* and *Mn*)

Table 14. Population-specific alleles in the *M. punctulatus* species group. Population designations explained in the text. 'Band' is in kilobase (kb) size.

Population	Primer	Band
Kentucky	M-09	1620
(KY)	W-03	2100
Tickfaw	W-16	920
(LE)		
Lake Jordan Reservoir	M-09	1800
(AL)	M-14	1610
	M-16	980
	M-18	1000
	M-20	650
	W-17	1610
Guadalupe River	C-04	800
(TX)	M-14	1450
	M-16	1000
	M-16	1160
	W-02	2400
	W-03	1000
	W-10	1080
	W-17	550

(table cont.)

Population	Primer	Band
Guadalupe River	W-17	1520
Alleles shared exclusively by only two, or three populations.		
Kentucky and Guadalupe	W-19	1000
Atchafalaya (LW) and Guadalupe	C-13	1500
Guadalupe, Atchafalaya (LW), and Tickfaw (LE)	M-16	1550
Guadalupe and *M. salmoides* (LMB)	C-13	3000

Table 15. Species-specific alleles in the two 'outgroups', *M. salmoides* and *M. notius*, and the Chipola bass. Band is in kilobase (kb) size.

Population	Primer	Band
M. salmoides	C-05	1450
	C-06	650
	C-08	1100
	C-13	1100
	C-16	2700
	M-10	900
	M-16	3900
	M-16	1130
	W-01	1000
	W-02	520
	W-02	1800
	W-02	3000
	W-04	960
	W-05	1900
	W-07	520
	W-07	790
	W-07	1500
	W-09	900

(table cont.)

Population	Primer	Band
	W-17	700
M. notius	C-05	830
	C-05	880
	C-08	1400
	M-10	1650
	M-14	1850
	M-16	700
	M-16	1300
	M-16	1620
	W-02	2600
	W-05	1095
	W-05	1540
	W-05	2500
	W-07	1900
	W-17	720
	W-17	1000
	W-17	1300
	W-17	1400
	W-17	1650

(table cont.)

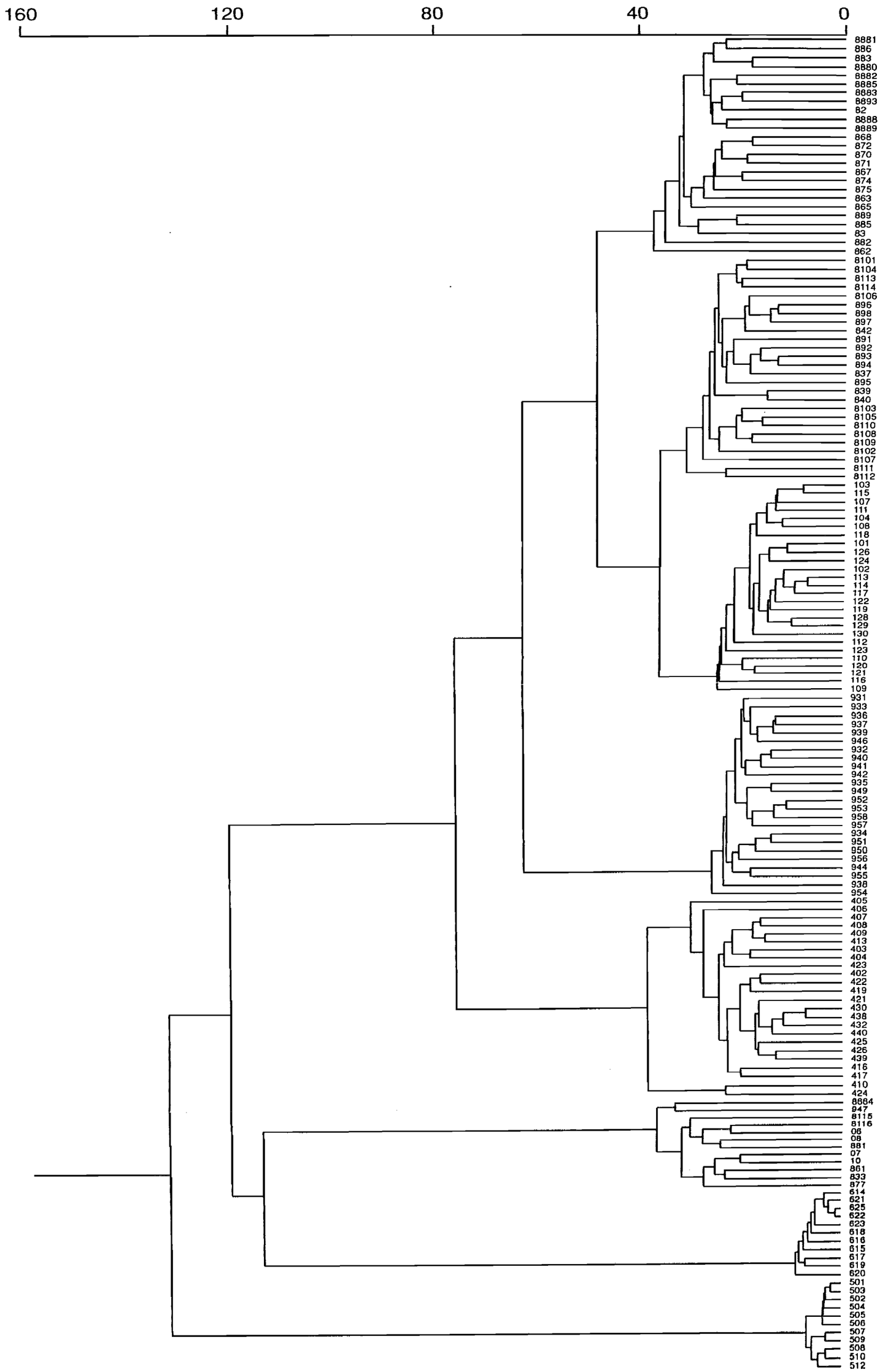
Population	Primer	Band
	W-19	460
	W-19	1590
	W-19	1900
Chipola bass	C-05	600
	C-06	725
	C-08	1250
	M-10	1250
	W-03	2500
	W-04	630
	W-04	850
	W-05	970
	W-05	1480
	W-07	2500
	W-17	1510
Species (or population-)-specific alleles shared exclusively by two 'outgroup' populations.		
M. salmoides and	C-05	850
the Chipola bass	C-16	2800

(table cont.)

Population	Primer	Band
	M-16	1200
	M-16	1580
	M-16	1610
	M-16	2000
M. salmoides and *M. notius*	W-07	1100

Fig. 6. Cluster analysis of individual bass for the total RAPD data set. Scale is Excoffier's genetic distance. Population association of individuals by branching order and including sample numbers is:

M. p. punctulatus:
first 25 branches: Tickfaw River, La. (82, 83, 862 - 889, 8880 - 8893),
following 26 branches: Atchafalaya Basin, La. (8101 - 8112, 837 - 842, 891 - 898),
following 26 branches: Lake Herrington, Ky. (102 - 130),
M. p. henshalli:
following 24 branches: Lake Jordan Reservoir, Al. (931 - 958),
M. treculi:
following 24 branches: Guadalupe River, Tx. (402 - 440),
M. salmoides:
following 12 branches,
Chipola bass:
following 11 branches (614 - 625),
M. notius:
following 11 branches (501 - 512).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861
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877
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512

highest four genetic distance measures for comparisons within this group (0.1678 with the Tickfaw River population, to 0.2178 with Kentucky).

The two outgroups, *M. salmoides* and *M. notius*, included to provide a root for the phenogram, were relatively distant from the five spotted bass populations, i.e., the *punctulatus* species group, with LE, LW, KY, AL, TX. *M. salmoides* was genetically closer to the *punctulatus* group (average D = 0.3853) than to *M. notius* (D = 0.4383). The Suwanee bass, *M. notius*, consistently showed the greatest distances from all other populations in this study (0.4383 with the Chipola bass, to 0.5045 for Kentucky). The proposed new species, *'M. cataractus'*, the Chipola bass, showed similar genetic affinities with the largemouth bass and the *punctulatus* group (average D from members of the *punctulatus* group, D = 0.3836, and from *M. salmoides*, D = 0.3702, Table 16).

Phenogram

The computer package POPGENE was used to produce phenograms based on Nei's genetic distances (Nei, 1978) derived from this RAPD generated data for these eight populations. The analysis of the total data set (Fig. 7) grouped the three currently classified *M. punctulatus punctulatus* populations into a cluster, with the Atchafalaya (LW) and Kentucky (KY) populations showing the closest genetic relationship (D = 0.0506). The Tickfaw River (LE) population branched from this pair with an average genetic distance from the two populations of 0.0877. The subspecies *M. p. henshalli* from Lake Jordan Reservoir, Alabama was the next population to branch from this

Table 16. Nei's genetic identities (above diagonal) and genetic distances (below the diagonal) for eight black bass populations. Measures are based on data obtained with RAPD-PCR analysis with 302 variable RAPD markers. Population designations are described in text; LM = *M. salmoides*; SU = *M. notius*; Ch = Chipola bass.

pop ID	KY	AL	LE	LW	TX	LM	SU	Ch
KY	0.0000	0.8464	0.9134	0.9530	0.8503	0.6673	0.5926	0.6468
AL	0.1668	0.0000	0.8709	0.8673	0.8533	0.7183	0.6288	0.6915
LE	0.0906	0.1382	0.0000	0.9267	0.8796	0.7283	0.6383	0.6846
LW	0.0481	0.1424	0.0761	0.0000	0.8628	0.6672	0.5980	0.6672
TX	0.1621	0.1587	0.1283	0.1476	0.0000	0.7183	0.6446	0.7034
LM	0.4045	0.3917	0.3170	0.4047	0.3309	0.0000	0.6535	0.6936
SU	0.5232	0.4640	0.4490	0.5142	0.4392	0.4254	0.0000	0.6382
Ch	0.4357	0.3689	0.3789	0.4047	0.3518	0.3659	0.4491	0.0000

cluster, with an average distance from these three populations of D = 0.1519. The most distinct of the spotted bass complex was the *M. treculi* population from the Guadalupe River. The average D for *M. treculi* from these four *punctulatus* populations was 0.1970.

The most divergent of the three 'outgroups' was *M. notius* (D's range from 0.4441 (LE) to 0.5045 (KY), for the spotted bass complex, Table 16, and Figure 7). *M. notius* was approximately equally distant from *M. salmoides* (D = 0.4383) and the Chipola bass (D = 0.4344), as it was from the *punctulatus* complex (average D = 0.4738). The specimens of uncertain affinity from the Chipola River, FL., most closely aligned with the largemouth bass (D = 0.3702), but were only slightly more divergent from populations in the spotted bass complex (D's range from 0.3562 (AL) to 0.4246 (KY), with an average D from all five populations equal to 0.3814). This species actually showed the closest genetic affinities to AL and TX (D = 0.3686, Table 16).

The effect on the analysis of the inclusion of different individuals vs different primers was evaluated from comparison of the results from the six different data set analyses (Figures 8, 9). All estimates derived from Nei's (1972) original measures of genetic distance for these six data sets produced the same topology for the five *M. punctulatus* complex and *M. treculi* populations. Only one analysis using Nei's (1978) unbiased measures of genetic distance gave a different topology: with the Atchafalaya (LW) and Tickfaw River (LE) populations forming the tightest cluster, and the Kentucky (KY) population branching off this.

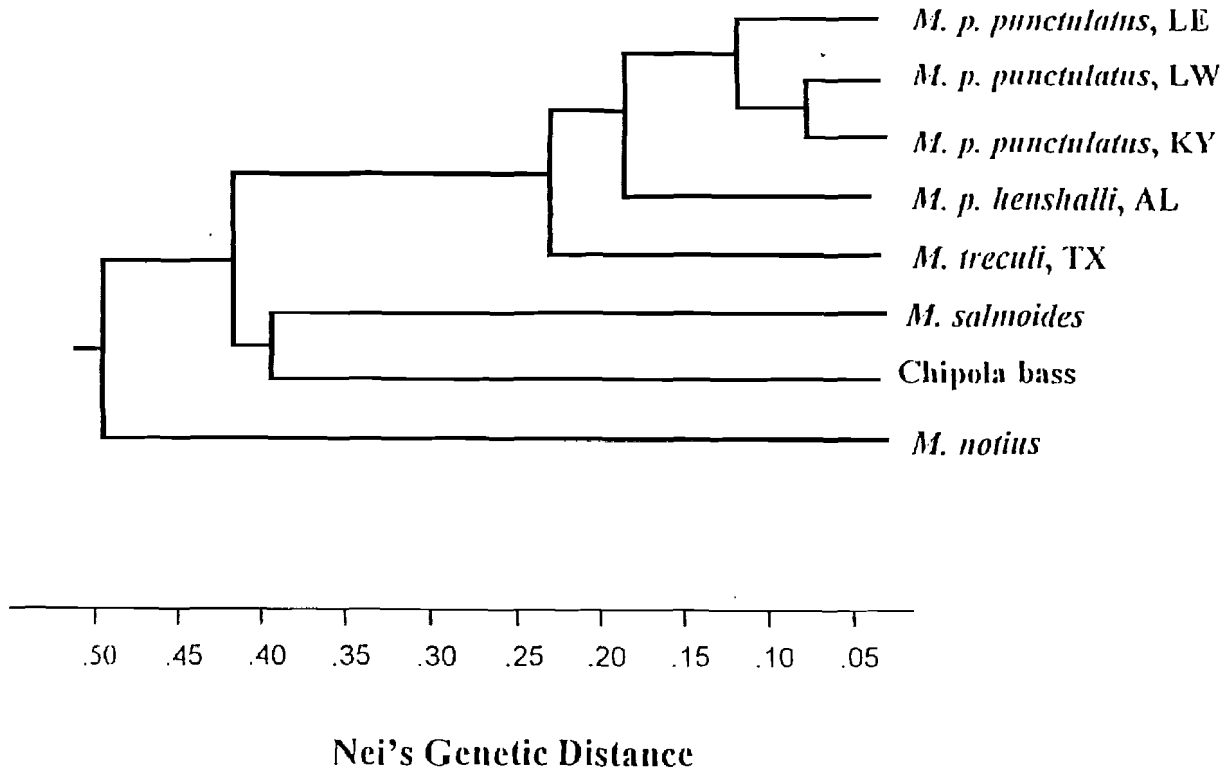


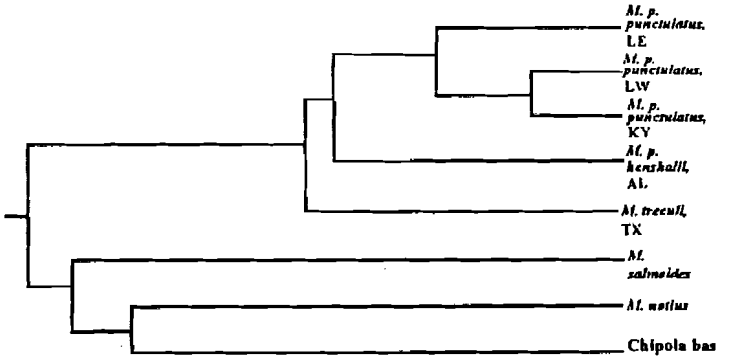
Fig. 7. Cluster analysis (UPGMA) of eight black bass populations based on RAPD-PCR data analysis; data are the complete data set. Genetic distance is Nei's (1978) unbiased estimate.

The effect on branching order with the three outgroups with these six different data sets was more profound. Affinities among these three changed depending on the data set in the analysis. In 11 of the 12 analyses for both Nei's original and unbiased, *M. notius* was the most divergent. Only one analysis placed *M. salmoides* as the most divergent.

DISCUSSION

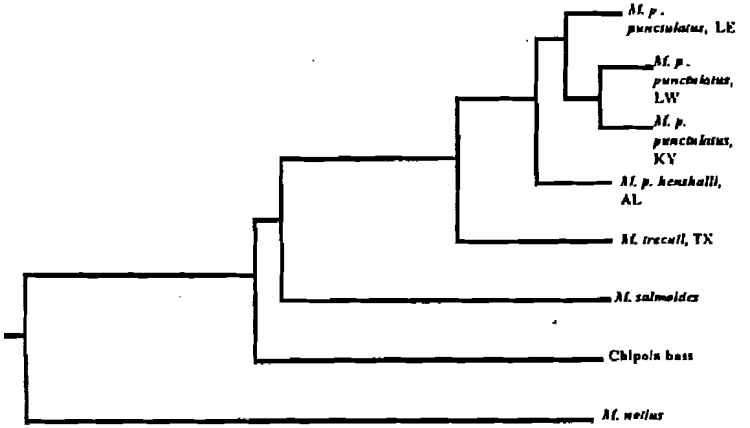
I have evaluated polymorphisms detected with the polymerase chain reaction using 27 random-sequence primers, in eight populations of black basses. Five populations were spotted basses, four belonging to the *Micropterus punctulatus* species complex, plus *M. treculi*. The remaining three populations comprised related species, also in the genus *Micropterus*. The primers were chosen from 60 screened primers, based first on presence of polymorphic bands displayed over the eight populations, and secondly on clarity and reproducibility of bands.

Other studies employing RAPDs in fish have uncovered different amounts of variability. In this study, forty-five percent of primers screened showed reproducible polymorphisms across the five species; this percentage was not inconsistent with some previous studies in fishes and other vertebrate species. Bielawski and Pumo (1997) screened 40 primers in five populations of striped bass (*Morone saxatilis*) from different rivers, and found that 33 primers produced amplification products, and 31 showed intense and consistent banding patterns. However, only eight of these primers were

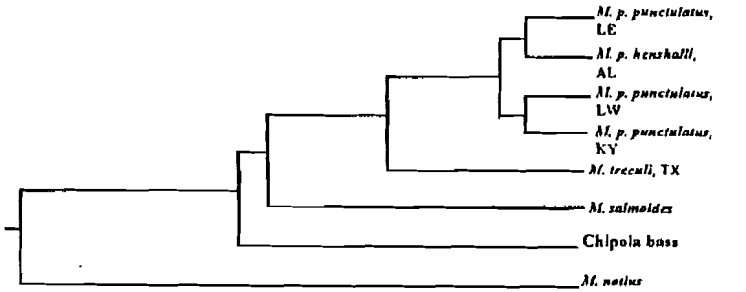


81 fish; 20 primers

78 fish, 20 primers

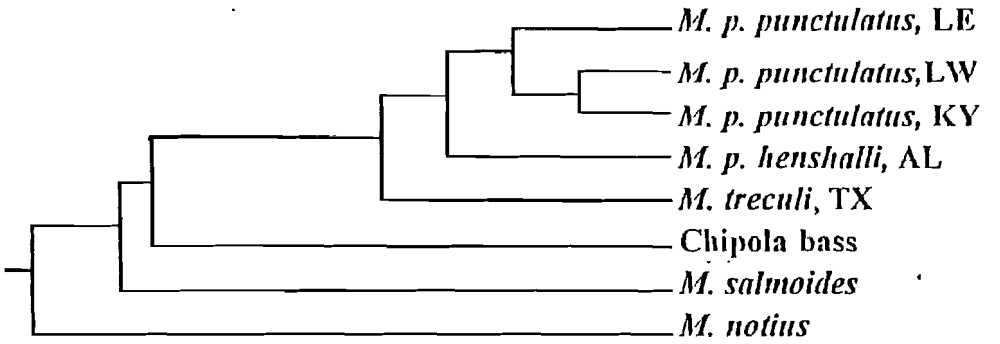


81 fish, 7 primers



78 fish, 7 primers

Fig. 8. Phenograms based on RAPD analysis of partial data sets for black basses. Data are for different numbers of individuals (78 and 81) and primers (20 and 7). Species and locale sampled are given at each terminal branch. Scale is based on Nei's (1978) genetic distance.



78 fish, 27 primers

81 fish, 27 primers

Fig. 9. Phenograms based on RAPD analysis for partial data sets. Data are for different individuals for the total data set of 27 primers. Species and locale sampled are given at each terminal branch. Scale is based on Nei's (1978) genetic distance.

polymorphic. With these eight primers, a total of 53 amplification products were produced, with 33 of these fragments showing polymorphisms. Bardakci and Skibinski (1994) chose 13 primers to assay polymorphisms in three species, and four subspecies, of tilapia (*Oreochromis* sp., and *O. niloticus* ssp.). All 13 primers produced species-specific RAPD patterns. RAPD markers were used to examine genetic changes in sea bass (*Dicentrarchus labrax*) following acclimation to fresh water (Allegrucci et al., 1995). Fifteen of 40 primers produced detectable polymorphisms and clarity in banding, for a total of 126 fragments that could be scored.

In this present study of black basses, a total of 302 amplification products were observed with the 27 primers which amplified one or more variable loci; 295 of the bands produced with these 27 primers were polymorphic. Construction of the dendrograms incorporates frequency differences observed at these polymorphic loci. Perhaps more informative than the frequencies of alleles shared across these widely distributed congeneric populations, is the distribution of population specific alleles and the number of species-specific alleles, i.e., alleles found in only one population or species. Reproductive isolation over time is predicted to lead to fixation of alleles in reproductively isolated populations. As stochastic processes are predicted to be partly responsible for genetic differentiation, the amount of divergence and number of fixed alleles is proportional to time since populations were last part of a population experiencing gene exchange (Nei, 1975).

The five populations currently representing two species of spotted basses were clustered relative to the three outgroups by the number of alleles shared among only

these five populations (19 alleles). The number of species-specific alleles seen in each of the two outgroup species was equal to or slightly larger than the number of alleles shared within only the spotted basses (*M. salmoides*, 19; *M. notius*, 21); the Chipola bass showed 11. Therefore, these five populations do not display genetic differentiation among each other as great as seen among any of the outgroups included in this study.

With the high amount of variation observed at RAPD loci (45% variable loci), it was not surprising that a number of population-specific, as well as species-specific, bands were observed with these widely distributed populations. Each population, except the Louisiana-West population from the Atchafalaya River Basin, showed at least one population-specific allele (Table 14). However, very few population-specific alleles (three) were observed when comparing the three *M. p. punctulatus* populations. These results were not surprising under the current taxonomy. These two populations (TX and AL) are currently classified as the most distinct from the other *M. punctulatus* species. The Texas population has been elevated to species status (*M. treculi*, Hubbs and Bailey, 1942); , and the Alabama population is regarded as a separate subspecies (*M. p. henshalli*, Hubbs and Bailey, 1940).

However, the affinity of the five populations in this species complex was affirmed upon examining band sharing in this group compared to the outgroups. Thirty-nine alleles were conserved in all members of the *punctulatus* group, i.e., all *M. punctulatus* sp. and *M. treculi* (Table 13). Nineteen of these alleles were shared among only the five spotted basses and not seen in any of the outgroups (Table 14). Five additional alleles were conserved across all the *punctulatus* group, and shared with one or two of the

species included as outgroups. The most distinct spotted bass population, *M. treculi* (the Guadalupe bass), shared four alleles exclusively with one or two of the other populations: one allele was shared with the Atchafalaya (LW) population only, one with both Louisiana populations, one with Kentucky only, and the fourth allele with *M. salmoides*. If it is accepted that band sharing represents primitive characters retained from an ancestral relationship, then this species represents one that branched early from the line leading to the current group of *M. punctulatus* species, soon after diverging from the common ancestor shared with the *M salmoides* lineage. This hypothesis was also supported by placement of these groups in the phenogram derived from this entire data set (Figure 6): *M. treculi* was the first to diverge from the branch leading to the remaining *M. punctulatus* populations.

With increasing genetic distances seen with the three outgroups, when compared to the *punctulatus* species complex as well as each other, discrimination with RAPD markers became unambiguous. This genetic distinctness was manifested in the number of species-specific alleles observed in these three most distant species (Table 15). *M. salmoides* showed 19 species-specific alleles; the Chipola bass showed 11. However, these two basses are linked to the other by six alleles seen exclusively within their two populations.

The putative most 'primitive' group of the black bass (based on morphometrics and meristics), *M. notius*, showed 21 species-specific alleles. The number of unique characters that separate populations, is expected to increase with time since divergence of one population from another or with time since a population split from a lineage (Nei,

1987). Primitive characters are those present in an ancestral population at the time this population splits into two or more lineages. Therefore, these characters may or may not be retained in subsequent lineages, thus increasing the number of unshared characters with the more ancestral population. Derived characters arise in a lineage after splitting from the ancestral population, and are shared with only those taxa that are descendent from that lineage posterior to an earlier split. Therefore, taxa diverging at later time are linked by such characteristics (Nei, 1978). The observation of a large number of unique alleles in *M. notius* compared with these other seven populations would further support the hypothesis that *M. notius* displays the most primitive characteristics of this group. These populations would appear to have been reproductively isolated long enough to allow fixation of very different RAPD profiles. The banding pattern on the gels for each of these 27 polymorphic primers for these four species groups (the spotted basses, *M. punctulatus* plus *treculi*, *M. salmoides*, *M. notius*, and the Chipola bass), produced a distinctive pattern for each species group relative to each of the other species. The populations within the *punctulatus* complex did not show these kind of clear pattern differences, even in comparisons including *M. treculi.*

The phenogram with the 159 individual fish based on Excoffier's coefficient (Fig. 6), forms a branching topology of individuals in each population that mirrors the one produced when the analysis is run with individuals assigned to one of the eight populations (Fig. 7). All individuals within a specific population fall in a cluster on a single terminal branch. The analysis was based on the genetic distances calculated with Excoffier's distance measure. This measure is analogous to Wright's Fst. Another

genetic distance, Dice's coefficient, is sometimes used for these comparisons, and has the advantage over some measures in that it considers not only the bands shared between two taxa, but also the number of bands seen in only one taxon, and absent from the other (Dice, 1926; Nei and Li, 1979). However, Excoffier's coefficient is less dependent on specific assumptions than some other coefficients, including Dice's. With Excoffier's coefficient, at the intraspecific level, the structure of the genetic clustering of taxa or individuals is not significantly affected by information about the phylogenetic relationships among the genetic markers being evaluated. Therefore genetic distance is independent of the site in the genome sampled (Excoffier, et al., 1992).

The RAPDistance computer package contains a program that evaluates the distance matrix calculated for each band with how well it correlates to the distance matrix produced from the overall data set. That is, it identifies the bands that provide the most, or least, information useful for distinguishing species groups and individuals. The two sets of samples (with 78 and 81 individuals) were analyzed with this program separately for the 302 bands (because this program will handle a maximum of 100 samples). The results for the two data sets were similar for the bands identified as informative. Only 34 of the 302 bands significantly correlated to the distance matrix in one data set, and not in the other. Among the entire data set of 302 bands over all samples, 118 bands provided little information, i.e., the pattern of distribution of allele frequencies across the populations for that band correlated poorly with the distance matrix generated from the total data set. The informative bands, i.e., those that produced a pattern of population relationships that did correlate with the distance matrix

from the total data set, were distributed over all 27 primers. No primer lacked a band showing a presence / absence pattern that significantly correlated with the calculated genetic distances between samples. Bands that correlated with the matrix at the 0.0001 level of significance were seen for the following 14 primers (number of bands that were significant for each primer is in parentheses): C-5 (2), C-8 (2), C-13 (4), C-16 (2), M-10 (2), M-16 (3), M-18 (3), W-1 (2), W-2 (5), W-3 (1), W-5 (5), W-7 (4), W-17 (7), and W-19 (5).

Based on the information obtained from my data and analyses, an individual fish can be placed in one of the classes of spotted basses with a RAPD banding profile determined with four primers: M-16, W-3, W-16, W-17. In addition, band presence for population specific alleles (psa) can reliably place a fish in a specific population, and is seen for the following populations and primers:

KY: W-3, 1-psa;

LE: W-16, 1-psa;

AL: W-17, 1-psa, and W-16, 1-psa;

TX: W-1 7, 2-psa, and M-16, 2-psa.

Alleles found exclusively in only two or three populations are seen for the following primers and populations: one at M-16 (in LE, LW, and TX), and one at W-17 (KY and TX).

The statistical significance of the dendrogram that included all populations and PCR data was evaluated by bootstrap analysis with the computer package Phylogenetic Analysis Using Parsimony (PAUP, Swofford, 1996). Bootstrap analysis is a method of

numerical resampling, that is used to approximate the distribution of the original parameter estimator, which can then be used to derive an estimate of statistical confidence of the estimator and each branch in the tree. This technique operates by drawing random samples with replacement, determined with Monte Carlo generated random numbers, from the original data set. The size of the redrawn sample is equal to the size of the original sample (Weir, 1996). A bootstrap analysis (UPGMA) with 1000 replicates was performed with this data (Fig. 10). As can be seen, individuals in each population cluster on a terminal branch, so that members of each population form a single, cohesive cluster on the tree (with the exception of one individual from the Chipola bass population, which was distinct from all populations). The percentage of replicates that support each branch containing all individuals from a complete population range from 72 to 100. The LE population branch pattern shows the lowest number for this replication value, but this branch is supported by 72% of the trees. Each branching pattern for the remaining seven populations is supported by 95% or more of the simulated trees. Six of the branch nodes are supported by 100% of the trees. Therefore, the dendrogram generated using the RAPD data represents a statistically well supported tree.

Although the RAPD technology has only recently been applied to population studies, its utility, as well as drawbacks, have been demonstrated in a growing number of studies. As mentioned earlier, Bielawski et al. (1997) applied this technique to Atlantic coast striped bass, a species with very low nuclear DNA variation when evaluated with standard techniques such as isozymes and isoelectric focusing. Of the primers that could

be scored, 75% were monomorphic, i.e., only one allele was seen in all striped basses, no other alleles were present in the populations for that locus. Although the variation uncovered using RAPDs was also low, this technique did reveal nuclear DNA variation in this genetically conserved species. RAPD markers disclosed subdivision between populations from two river systems when mtDNA analysis failed to detect differences. In another study of fishes, Bardakci and Skibinski (1994) found RAPDs offered advantages over both isozymes and mtDNA for examining differences in three species, and four subspecies of tilapia and their hybrids. Isozymes could discriminate between species, and mtDNA could discriminate subspecies, but neither technique demonstrated substantial variation between populations. Intrapopulation variation was detected with each of the 13 primers used in this study. In addition, the assumption of only female transmission of mtDNA may not always hold. There is evidence that inheritance of the mtDNA genome can be biparental (Magoulas and Zouros, 1993). The conclusions regarding genetic relationships among the three tilapia species based on this RAPD data differ from the widely accepted taxonomy which groups *Oreochromis aureus* and *O. niloticus* in a subgenus separate for *O. mossambicus*. Their RAPD data suggested a closer relationship between *O. mossambicus* and *O. niloticus*. Since intrapopulation variation was detected with all primers, RAPD analysis may be more sensitive and useful for studies of intrapopulation variation than mtDNA, as well as, for studies where interpopulation variation is low.

Baruffi et al. (1995) found in six wild populations and five laboratory strains of the medfly (*Ceratitis capitata*) that RAPDs revealed larger amounts of genetic variation

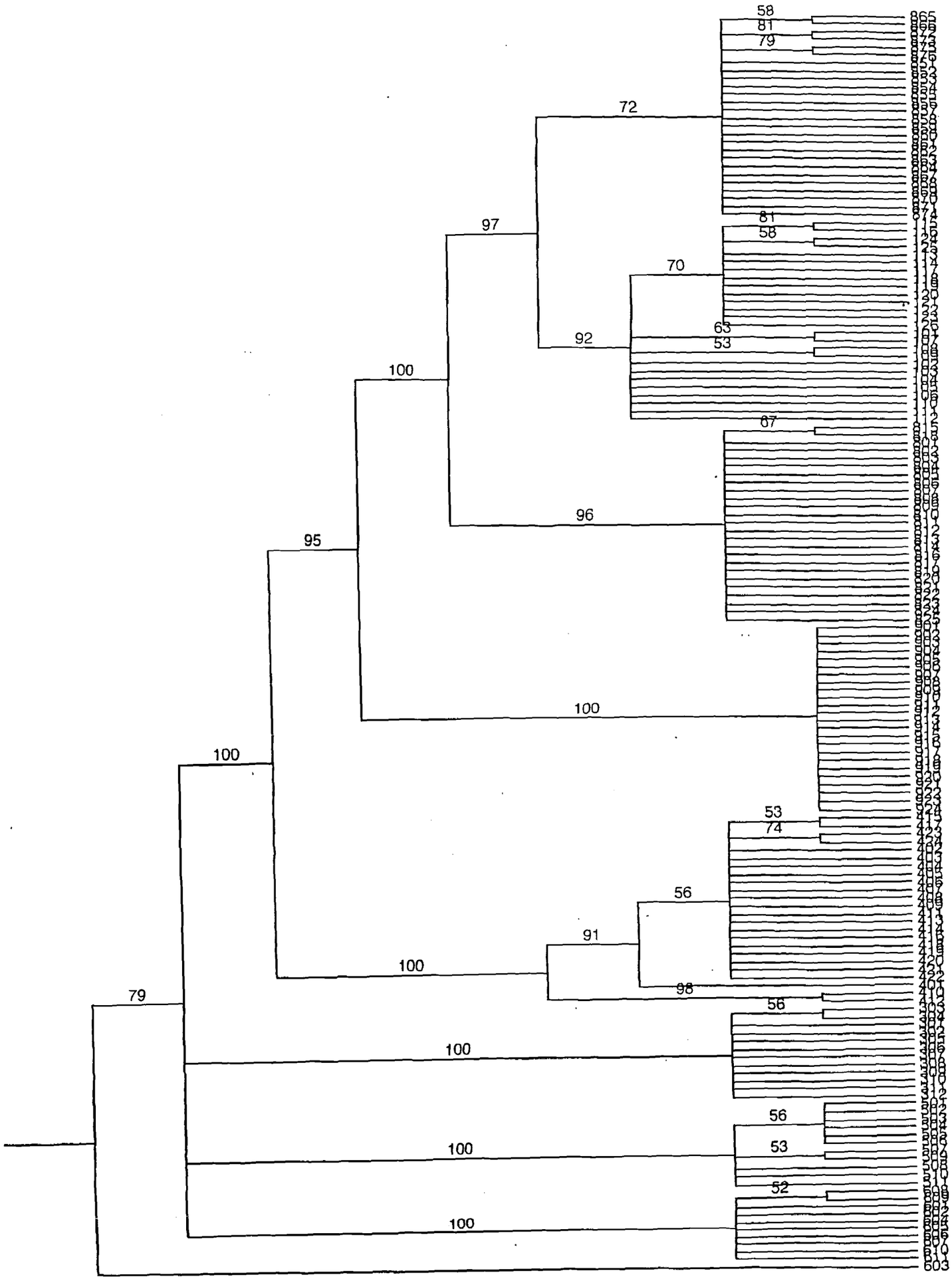


Fig. 10. Bootstrap analysis (UPGMA) of total RAPD data set, 1000 replicates. Individual fish within a population are represented as follows:

Tickfaw River, LE: 851 - 875, the first 25 branch lengths,
Lake Herrington, KY: 101 - 126, the following 26 branch lengths,
Atchafalaya Basin, LW: 801 - 826, the following 26 branch lengths,
Lake Jordan, Reservoir, AL: 901 - 924, the next 25 branch lengths,
Guadalupe River, TX: 401 - 424, the next group of 24,
M. salmoides, largemouth bass: 301 - 312,
M. notius, Suwanee bass, Sante Fe, River, FL.: 501 - 511;
Chipola bass, Chipola River, FL.: 601 - 611.

than isozymes, despite the suspected tendency of RAPDs to underestimate heterozygosity due to dominance. Their study used four primers that produced 175 polymorphic bands out of a total 176 bands amplified. However, estimates of relationships using the two different techniques correlated, which was not surprising given the assumption that molecular markers are affected similarly by factors such as population size and drift during the colonization process. The reduced levels of variability at the isozyme level were possibly due to unequal rates of chromosome evolution. Different parts of the chromosome can evolve at different rates, and it is possible that regions amplified with RAPD-PCR evolve at higher rates than those areas assayed using isozymes, especially if these are microsatellite or minisatellite regions.

The utility of RAPD markers in estimating population genetic parameters and the problem of dominance inherent with RAPD markers was investigated by two researchers (Lu and Rank, 1996). The problem of dominance was overcome by studying a haplo-diploid insect. Haploid males in five geographic isolates of the leaf-cutting bee (*Megachile rotundata*) were examined. Three measures of gene diversity were estimated within and between populations: heterozygosity, nucleotide divergence, and Nei's genetic distance. These three measures of genetic diversity showed similar trends as the RAPD data in all five populations. They found that these measures of genetic diversity were about ten times greater than previous estimates based on allozyme data. The authors concluded that the problem of dominance can be overcome with use of an adequate sample size, and RAPDs can be an efficient tool for evaluating genetic divergence in diploids, also.

Studies are accumulating that apply RAPDs to the study of population and taxonomic questions (Hunt and Page, 1992, honey bee, *Apis mellifera*; Stothard and Rollinson, 1996, nine species of freshwater snails, *Bulinus*; Johnson et al., 1994, zebrafish, *Brachydanio rerio*; Patwary et al., 1993, 1994, marine red algae, *Gelidium vagum,* and bivalves, *Placopecten magellanicus*; Caswell-Chen et al., 1992, nematodes, *Heterodera* sp.; Yeh et al., 1995, trembling aspen, *Populus tremuloides*; Marilla and Scoles, 1996, barley, *Hordeum* sp.). These studies and others are showing the utility and limitations of RAPD technology. For RAPDs to be useful for estimating nucleotide divergence, the true nucleotide sequence divergence should not exceed 10% (Clark and Lannigan, 1993; Stothard and Rollinson, 1996). The problem of dominance can lead to bias in parameter estimation, but this can be minimized by sampling large numbers of individuals per population (Lynch and Milligan, 1994), or a large number of markers (Hedrick, 1992). Nei (1978) recommends for systematic studies that examining a large number of loci rather than a large number of individuals per locus will reduce sampling error, but when possible a large number of individuals and a large number of loci is preferable for reducing errors in parameter estimation.

In my study, the problem of minimum sample size and number of loci necessary to produce confidence in the results was approached by sequential analysis of subsets of the data. The level considered adequate was that at which the genetic distances and overall topology of the dendrogram did not change with additional samples or loci. Even with the random selection of primers in this study, information obtained with only seven primers (96 markers)in the two different data sets of individuals produced overall similar

trees to the final tree (with one exception in the relationships among the three most distant outgroups). The number of loci and sample sizes used in the final analysis in this study exceeded the levels at which the genetic distances calculated among the populations appeared to stabilize. However, I could not be confident in this numerical stability until I had attained a large sample size for both primers and individuals.

CHAPTER 4

SUMMARY AND CONCLUSIONS

Despite the widespread geographical distribution for the five populations of *M. punctulatus* spp. and *M. treculi*, these populations retain an overall genetic cohesiveness that clusters them into a coherent group. This is especially obvious with the inclusion in the analysis of the three congeneric outgroups. Even though genetic affinities have been previously demonstrated between one of the outgroups *M. salmoides*, and especially *M. treculi*, *M. salmoides* is very distant from this cluster, relative to the largest distance seen within the cluster. Therefore, the relatively small genetic distances, physical similarities, habitat preferences, and ability to produce interfertile offspring among these five bass populations warrant including these five geographic populations in one species, *M. punctulatus*. The genetic distances estimated from both the PCR and isozyme studies support retaining subspecies status for the two most divergent among these populations: the Alabama spotted bass, *M. p. henshalli*, and the Guadalupe bass, as *M. p. treculi*. The central U.S. populations, including Louisiana and Kentucky populations, should retain the current subspecies designation, *M. p. punctulatus*.

The outgroups examined with this study also fit expectations of genetic alignment. All three were relatively distant from the *M. punctulatus / treculi* grouping, with *M. salmoides* being the closest to this group of five. As expected, the predicted most 'primitive' form *M. notius* was the most genetically distant. The genetic relationship of the proposed new species from Florida (the Chipola bass,

proposed species designation, *M. cataractus*, Dr. J. Williams, personal communication), to several of these congeneric species, would indicate reproductive isolation. Thus, the Chipola bass deserves recognition as a distinct species, based on the genetic distances derived from the RAPD analysis.

The assessment of genetic variation at the molecular level and the significance of that variation to systematic relationships of biota has been a primary goal of population geneticists. Lewontin and Hubby (1966) first applied starch gel electrophoresis to the analysis of genetic differentiation within and among populations. Different levels of genetic variability and genetic differences were detected across species. The significance of this observed variation and levels of differentiation among taxa has been a major subject of debate in population genetics. New molecular tools have been developed and applied over the last 30 years, which assay a wider scope of variability than possible with isozyme analysis. Information obtained from these new techniques allows calibration of different levels of 'taxonomic' divergence with observed measures of genetic differentiation, and has given insight into the genetic change occurring at different systematic levels.

The most widely applied molecular tool for assaying genetic differences for species discrimination and systematic studies has been isozyme analysis, which detects variation at the structural protein level. The genetic markers produce a product (a structural protein or enzyme), for which at least part of the sequence and structure must be responsive to selection pressures. Therefore, there is constraint on the rate at which changes can accumulate in the DNA encoding such products (Allegrucci et al., 1995).

Lu and Rank (1996), conducting comparative studies on the leaf-cutting bee (*Megachile rotundata)*, estimated that the genetic diversity measured with RAPDs is about 10 times greater than previous estimates based on isozyme studies in this species. As discussed in the introduction, a number of investigators have compared results obtained with the two techniques for population and systematic application. Baruffi et al. (1995) assayed colonizing populations of the medfly (*Ceratitis capitata*) and correlated results obtained with both techniques (isozymes and RAPDs). Overall results were similar, which was expected, since these molecular markers should be similarly affected by factors such as population size, and drift during the colonization process.

This study with black basses also obtained genetic differentiation estimates by using both techniques. My findings discussed here present different phenetic relationships among the five populations of spotted basses. The results obtained with my isozyme analyses differed from the traditional classification of these five groups. However, as discussed, this was primarily due to fixation of a relatively rare allele at a single locus. Population genetic theory predicts such outcomes as a consequence of genetic drift in small, reproductively isolated populations (Nei, 1975). However, based on such a limited study of structural proteins, it is impossible to attribute a single cause to the occurrence of this fixed rare allele, whether it is a product of founder effect, drift, or selection.

Genetic markers generated in RAPD analysis, theoretically sample a wider array of loci, i.e., from conserved to highly variable regions of the genome, that may, or may not, be part of a structural gene that is constrained by selective pressures. In addition, a

far larger number of putative genetic loci can be assayed at one time. Therefore, the information obtained from RAPDs should be a more complete and less biased sampling of the genome. And with this technique, the overall systematic relationships among the spotted basses and other black basses were generally supported. The Louisiana and Kentucky populations showed very close genetic relationships (average G.D. = 0.074). The results of the RAPD-PCR analysis obtained in this study support the current status of the Alabama subspecies, *M. p. henshalli*, and the more divergent status of the Texas population, currently classified as a distinct species, *M. treculi*.

M. treculi was the most divergent among the five populations of spotted basses. The average genetic distance to three spotted bass populations, based in four polymorphic isozyme loci, and excluding Kentucky, was 0.0333. The average distance calculated using these data, and including Kentucky but without Mpi, was 0.0285. The average distance between TX and the remaining four spotted bass populations based on the RAPD-PCR data was 0.1943. However, with neither technique (RAPD or isozyme) did the Texas population, or any spotted bass population, stand out as being notably distant relative to the others.

As can be seen, *M. salmoides*, *M. notius*, and the Chipola bass were relatively distant from the group of five spotted bass populations (genetic distances range from 0.3245 to 0.5028), and from each other (genetic distances of 0.3263 to 0.4346). With these outgroups included in the PCR analysis, *M. treculi* formed a relatively tight cluster with the four *M. punctulatus* species. In the isozyme analysis including Mpi, but omitting the Kentucky population, the average genetic distance of *M. treculi* to the other

three was 0.033; with all four populations excluding Mpi, the average genetic distance was 0.0285. As mentioned earlier, Imsiridou (p. 26) found genetic distances among populations within a single species of river chub ranged from 0.002 - 0.063, with no evidence of speciation. The Texas population did not show a genetic distance this great in any of the isozyme analyses. When the genetic distances estimated from these two molecular studies are compared with distances estimated in similar studies with other species, the Guadalupe bass, currently classified as *M. treculi*, does not warrant species recognition.

Both *M. notius* and the Chipola bass are from relatively small geographic areas with limited distributions. The banding patterns observed with RAPD-PCR in the nine individuals assayed from each population were remarkably uniform within each of these two populations, compared to variation observed among individuals within any of the other six taxa. This lack of variation was not due to the small sample size in *M. notius* and the Chipola bass, as the number of individuals assayed for *M. salmoides* was approximately the same. The same consistency in banding pattern as seen in these two species of limited distribution was not seen in *M. salmoides*. Such results are expected from population genetic theory; genetic variability is expected to be lower in small, isolated populations. Therefore, this observed low genetic variation in populations from a limited range would suggest that the populations of *M. notius* and the Chipola bass are relatively small and not experiencing gene flow from peripheral populations.

Populations of spotted basses collected across the taxon's range not only give an interesting picture of their genetic relationships, but also demonstrate the utility of

spotted basses for examining past and ongoing population genetic processes. Such stream dwelling fishes are subject to varying degrees of reproductive isolation and restricted gene flow, which can result in reduced effective population size and genetic bottlenecks. Genetic differentiation can occur as a result of selection to local environmental differences, genetic drift, or both. Genetic differentiation is then, in part, both a product and an indicator of the level of gene flow between populations. Thus, current genetic differences reflect the genetic history of a group of potentially interbreeding populations and can be useful in assessing genetic changes associated with further disruptions in natural distributions, which may affect migration patterns and differentiation among subpopulations, populations, or species.

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APPENDIX A

SELECTED RECIPES FOR PCR

10X reaction buffer:

500 mM KCl
100 mM Tris, pH 8.4
100 mM $MgCl_2$

10X TBE

89 mM Tris Base	108 gm
89 mM Boric acid	55 gm
0.5 M EDTA, pH 8.0	40 ml

(adjust pH with 10N NaOH)
bring to 1 L with dH_2O

50X TAE

40 mM Tris-acetate

Tris base	242 gm
Glacial acetic acid	57.1 ml
2 mM $Na_2EDTA{\cdot}2H_2O$, pH 8.5	37.2 gm

bring to 1 L with dH_2O

Tris-EDTA buffer (TE_{low}), low concentration EDTA (0.1 mM EDTA)

10 mM Tris-Cl, bring to pH 8.0, with HCL	121.1 mg
EDTA	3.72 mg

bring to 100 ml with dH_2O.

6X loading buffer

40% sucrose	4 gm
0.25 % bromophenol blue	25.0 ml

bring to 10.0 ml with dH_2O
autoclave, cool, aliquot into sterile
2.0-ml Eppendorf tubes

10X loading buffer

60% sucrose	6.0 gm
0.25% bromophenol blue	25.0 mg
0.25% xylene cyanol	25.0 mg

bring to 10 ml with water
Autoclave, cool, aliquot into 2.0-ml tubes.

APPENDIX B

STOCK dNTPs

2'-Deoxynucleoside 5'-Triphosphates (Pharmacia Biotech)

10 mM stock solution of dNTPs:

deoxyadenosine nucleoside triphosphate (dATP)

deoxyguanidine nucleoside triphosphate (dGTP)

deoxycytosine nucleoside triphosphate (dCTP)

deoxythymidine nucleoside triphosphate (TTP)

1) For kit containing 100 mM stock solution of each dNTP:

Add: 25 *u*l dATP

25 *u*l dGTP

25 *u*l dCTP

25 *u*l dTTP

To 900 *u*l sterile, distilled, deionized water.

2) For kit containing 25 mM stock solution of each dNTP:

Add: 100 *u*l dATP

100 *u*l dGTP

100 *u*l dCTP

100 *u*l dTTP

To 600 *u*l sterile, distilled water.

3) Filter sterilize: with a syringe and a sterile, Nalgene 0.45 *u*m syringe filter (acetate membrane, disposable).

4) Decant into five sterile, 1.5 ml Eppendorf tubes. Freeze at -20° C.

APPENDIX C

AGAROSE GELS

Materials:

Agarose: Gibco-BRL Ultrapure

Buffer, e.g., 1X-TBE or 1X-TAE

Horizon 20-25 Gel Electrophoresis Apparatus, contains:

20 X 25 cm gel bed

Electrophoresis tank, 2000 ml

two 20-tooth comb sizes: 1mm, 2mm

1) securely tape gel molds on ends, to form barrier, for fluid retention; seat appropriate size comb in mold
2) gels are made by percentage: wt:vol.
3) combine agarose with correct volume of buffer in appropriate sized flask, heated 2-3 min. in a microwave, swirled, and heated an additional 1.5 min., or until boiling vigorously
4) add appropriate volume of ethidium bromide
5) cool gel: a water bath is constructed by adding water to a 2000ml beaker, placed on a stir plate, flask and gel with stir bar is placed in beaker, and gel stirred until a temperature of 45°C is reached
6) pour gel
7) remove combs and tape on ends, after gel has solidified
8) place gel in gel tray with buffer

APPENDIX D

DNA EXTRACTION: GUANIDINE-HCl

Materials:

8 M Guanidine-HCl 76.42 g Guanidine-HCl / 100 ml (add about 50 ml H_2O),

(make fresh each time used for extraction)

2 M Potassium acetate 19.63 g $KC_2H_3O_2$ / 100 ml H_2O

100% ethanol

1. Add 0.5 ml blood to a centrifuge tube
2. Add 5 ml 8M Guanidine-HCl
3. Add 3 drops potassium acetate
4. Vortex
5. Add 2.5 volumes of ethanol
6. Spool DNA with glass rod
7. Redissolve DNA on rod in 5 ml fresh Guanidine-HCl, plus 3 drops K-acetate
8. Vortex
9. Add 2.5 volumes ethanol
10. Spool DNA onto a clean glass rod
11. Dissolve DNA in 100 - 300 *u*l TE (low EDTA) buffer

APPENDIX E

PREP-A-GENE PROTOCOL

1. Materials:

   Prep-A-Gene (PAG) matrix

   PAG Binding buffer:

   6M Na perchlorate

   50 mM Tris-HCl, pH 8.0

   10 mM EDTA

   PAG Wash buffer

   20 mM Tris-HCl, pH 7.5

   2 mM EDTA

   PAG Elution buffer

   10 mM Tris-HCl, pH8.0

   1 mM EDTA

2. Vigorously vortex Prep-A-Gene (PAG) matrix bottle to resuspend matrix; invert bottle several times.
3. Add Binding buffer: volume of Binding buffer = 3 times the total volume of the matrix plus DNA solution.
4. Add 10 *u*l PAG matrix for each 2.0 *u*g of DNA.

   (For < 2 *u*g DNA, add 5 *u*l PAG matrix.)
5. Incubate mixture at room temperature for 10 minutes.
6. Centrifuge 10 seconds.

Pipet off supernatant.

7. Rinse pellet: resuspend pellet in Binding buffer approximately 50X the volume of matrix.

   Vortex.

   Centrifuge and discard supernatant.

8. Repeat step #7.

9. Remove all liquid: pipet off supernatant

   Recentrifuge

   Pipet off supernatant.

10. Resuspend matrix pellet in $\geq$ 1 pellet volume of elution buffer.

11. Incubate mixture at 37° - 50° C, for 5 minutes.

12. Centrifuge.

13. Pipet off supernatant, transfer to a clean tube.

14. Wash pellet: add 1 volume of elution buffer.

    Incubate at 37° - 50° C, for 5 minutes.

15. Centrifuge, transfer supernatant to a clean tube, and recentrifuge.

16. Carefully remove supernatant with a pipet; transfer to a clean tube.

17. Quantify, or freeze at -20° C.

APPENDIX F

REACTION PLATES / PROCEDURE FOR WASHING

1. Add 5 drops of clear Ivory soap to 1 liter of distilled water in a 1000-ml beaker.
2. Place 4 microassay plates in beaker with detergent and water.
3. Place beaker in an ultrasonic a water bath (Branson 2200 UltraSonic Cleaner), for 10 minutes.
4. Rinse each plate with deionized water: rinse each side of the plate a minimum of five times.
5. Rinse both sides of each plate with Nannopure water (>17 ohms).
6. Place the four plates in a 1000 ml beaker of 95% ethanol; place beaker with plates in ultrasonic water bath for 10 minutes.
7. Shake excess fluid off; lean upright in a ventilated hood, until dry.
8. Store in a 'Ziploc' baggie until use.

APPENDIX G

PRIMERS

--Primers are obtained from Operon Technologies, in kits of 20 primers/kit

--each primer in this study is a 10-mer, i.e., 10 nucleotides of random base order

--primers are packaged desiccated in 1.5 ml tubes

--picomoles for each primer is given, with kit information

--each primer is diluted to a stock solution concentration of 5 *u*M, in sterile dH_2O;

dilutions are calculated for approximately 1 ml stock solution with the following formula, where *p*M is the given *p*M concentration of each primer:

$$\text{ml } H_2O = ((p\text{M} \times (10^{-6}\ u\text{M} / p\text{M})) \times 1000 \text{ ml / L}) / (5\ u\text{M / L}).$$

APPENDIX H

PCR PROTOCOL

1. Reaction mix: All steps in setting up the reaction plate are carried out in a LabConco Purifier Clean Bench, all surfaces washed with 70% ethanol. The following reaction components are combined in a 2.0-ml Eppendorf tube, minus the Taq polymerase; volume for 1 reaction, multiplied by the number of reactions, plus 10 reactions:

H_2O	14.0 *u*l
10x buffer	2.5 *u*l
25 mM $MgCl_2$	5.0 *u*l
10 mM dNTPs	2.0 *u*l
Primer	1.0 *u*l
Taq polymerase	0.5 *u*l

2. Add to separate well of reaction plate: 5.0 *u*l of 1.0 ng / *u*l stock DNA, discard pipet tip after each sample, and use a clean, sterile pipet tip with filter barrier for each sample.
3. Add Taq Polymerase to reaction mix.

   Vortex mixture and centrifuge.
4. Add 20 *u*l of reaction mix to each well, with an octopet. Mix the solution with DNA well by pipetting this mixture up and down 5 times.

5. Overlay 25 *u*l of sterile mineral oil* in each well, by slowly dripping oil down side of well. Add an additional 25 *u*l of mineral oil to each well, for a total of 50 *u*l mineral oil, covering fluid in each well.

   *mineral oil is filtered sterilized with a 50-ml sterile disposable syringe, and a sterile Nalgene 0.45-*u*m acetate membrane syringe filter.

6. Cover plate with a plastic wrap (e.g., Saran Wrap), insuring surface is smooth and there are no air pockets between reaction plate and wrap.
7. Add glycerol to each well in the thermocycler (about 3 drops); seat reaction plate in thermocycler.
8. Start thermocycler.

APPENDIX I

CYCLING PROFILE

Temperature, ° C	Time, minutes
1. 95.0	0:05
2. 92.0	1:55
3. 95.0	0:05
4. 92.0	1:08
5. 37.0	1:08
6. 72.0	2:10
7. 42 times to #3	
8. 72.0	8:00
9. 4.0	indefinitely

APPENDIX J

AMPLIFIABLE AND VARIABLE PRIMERS, IN BLACK BASSES

PRIMER	SEQUENCE
C-04	CCGCATCTAC
C-05	GATGACCGCC
C-06	GAACGGACTC
C-08	TGGACCGGTG
C-09	CTCACCGTCC
C-10	TGTCTGGGTG
C-13	AAGCCTCGTC
C-16	CACACTCCAG
M-04	GGGAACGTGT
M-05	GGGAACGTGT
M-09	GTCTTGCGGA
M-10	TCTGGCGCAC
M-14	AGGGTCGTTC
M-16	GTAACCAGCC
M-18	CACCATCCGT
M-20	AGGTCTTGGG
W-01	CTCAGTGTCC

(table cont.)

PRIMER	SEQUENCE
W-02	ACCCCGCCAA
W-03	GTCCGGAGTG
W-04	CAGAAGCGGA
W-05	GGCGGATAAG
W-07	CTGGACGAGT
W-09	GTGACCGAGT
W-10	TCGCATCCCT
W-16	CAGCCTACCA
W-17	GTCCTGGGTT
W-19	CAAAGCGCTC

APPENDIX K

STOCK DNA CONCENTRATIONS

DNA concentrations in stock solutions of study fish; concentration quantified after first extraction with the phenol-chloroform procedure. Concentrations are estimated from comparisons with DNA standards on agarose gels. DNA dilutions are in sterile Tris-EDTA (0.1mM EDTA) buffer.

Sample	[DNA], ng / *ul*	Sample	[DNA], ng / *ul*
LE			
862	120	886	600
863	160	889	100
865	70	8-880	50
867	160	8-881	200
868	180	8-882	130
970	80	8-883	70
871	100	8-885	18
872	120	8-888	200
874	160	8-889	7
875	400	8-893	100
882	15	82	30
883	180	83	200
885	160		
LW			
837	160	8-103	280
839	120	8-104	350
840	400	8-105	200
842	80	8-106	300
891	160	8-107	300
892	160	8-108	200
893	160	8-109	200
894	160	8-110	200
895	300	8-111	160
896	340	8-112	120

(table cont.)

Sample	[DNA], ng / *u*l	Sample	[DNA], ng / *u*l
897	300	8-113	100
898	200	8-114	400
8-101	120		
KY			
101	400	116	330
102	300	117	330
103	300	118	400
104	300	119	500
107	1000	120	500
108	500	121	400
109	1000	122	240
110	1000	123	400
111	650	124	240
112	650	126	30
113	330	128	30
114	300	129	100
115	330	130	200
TX			
402	300	419	100
403	200	421	100
404	240	422	200
405	240	423	100
406	200	424	250
407	50	425	250
408	450	426	150
409	300	430	30
410	400	438	50
413	40	439	60
416	75	440	35
417	20		

(table cont.)

Sample	[DNA], ng / *ul*	Sample	[DNA], ng / *ul*
AL			
931	200	944	30
932	300	946	150
933	400	949	180
934	180	950	150
935	180	951	130
936	200	952	180
937	200	953	150
938	200	954	160
939	180	955	180
940	160	956	180
941	150	957	40
942	150	958	60
LM			
8-884	18	881	20
8-115	200	06	180
8-116	200	08	500
833	180	947	120
861	200	07	180
877	150	10	200
SU			
501	100	507	180
502	140	508	600
503	100	509	400
504	190	510	300
505	140	512	200
506	180		
CH			
614	90	620	400
615	80	621	400
616	20	622	80

(table cont.)

Sample	[DNA], ng / ul	Sample	[DNA], ng / ul
617	140	623	20
618	100	625	200
619	220		

APPENDIX L

PCR DATA

DATA FILE:
8 BASS POPULATIONS / 159 INDIVIDUALS
POPULATION:
1 = Tickfaw River, La. (LE), 801 - 825
2 = Atchafalaya Basin, La. (LW), 851 - 876
3 = Guadalupe River, Kerrville Hatchery, Tx. (TX), 401 - 424
4 = Lake Herrington, Ky. (KY), 101 - 126
5 = Lake Jordan Reservoir, Al. (AL), 901 - 924
6 = Largemouth bass, Atchafalaya Basin (LM), 301 - 312
7 = Suwanee bass, Sante Fe River, Fl. (SU), 501 - 511
8 = Chipola bass, Chipola River, Fl. (CH), 601 - 611.

PCR Bass data.
Bands and scores for individual fish:
Score is '1' or '0', for presence or absence of a band. Data points for each individual are listed in the order that the primers and bands amplified by each primer are given below.

Bands:

C-4.1	C-5.6	C-9.2	C-13.2	C-16.7
C-4.2	C-5.7	C-9.3	C-13.3	C-16.8
C-4.3	C-5.8	C-9.4	C-13.4	C-16.9
C-4.4	C-5.9	C-9.5	C-13.5	C-16.10
C-4.5	C-5.10	C-9.6	C-13.6	M-16.1
C-4.6	C-5.11	C-10.1	C-13.7	M-16.2
C-4.7	C-5.12	C-10.2	C-13.8	M-16.3
C-4.8	C-6.1	C-10.3	C-13.9	M-16.4
C-4.9	C-6.2	C-10.4	C-13.10	M-16.5
C-4.10	C-6.3	C-10.5	C-13.11	M-16.6
C-4.11	C-6.4	C-10.6	C-13.12	M-16.7
C-4.12	C-6.5	C-10.7	C-13.13	M-16.8
C-4.13	C-6.6	C-10.8	C-13.14	M-16.9
C-4.14	C-6.7	C-10.9	C-13.15	M-16.10
C-4.15	C-6.8	C-10.10	C-16.1	M-16.11
C-5.1	C-6.9	C-10.11	C-16.2	M-16.12
C-5.2	C-6.10	C-10.12	C-16.3	M-16.13
C-5.3	C-6.11	C-10.13	C-16.4	M-16.14
C-5.4	C-6.12	C-10.14	C-16.5	M-16.15
C-5.5	C-9.1	C-13.1	C-16.6	M-16.16

M-16.17
M-16.18
M-16.19
M-16.20
M-16.21
M-16.22
C-8.1
C-8.2
C-8.3
C-8.4
C-8.5
C-8.6
C-8.7
C-8.8
C-8.9
C-8.10
C-8.11
M-4.1
M-4.2
M-4.3
M-4.4
M-4.5
M-4.6
M-4.7
M-4.8
M-4.9
M-4.10
M-5.1
M-5.2
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M-14.1
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M-20.1
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M-20.3
M-20.4
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M-20.7
M-20.8
M-20.9
W-3.1
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W-05.2
W-05.3
W-05.4
W-05.5
W-05.6
W-05.7
W-05.8
W-05.9
W-05.10
W-05.11
W-05.12
W-05.13
W-05.14
W-05.15
W-05.16
W-07.1
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W-19.6
W-19.7
W-19.8
W-19.9
W-19.10
M-10.1
M-10.2
M-10.3
M-10.4
M-10.5
M-10.6
M-10.7
M-10.8
M-10.9

PCR data: Data are listed by population. Population is designated at beginning of each data block. Individual sample number is given in parentheses, preceding the first datum. Sample numbers were changed to accommodate spacing requirements of this program; the order of samples below corresponds to the order in Appendix K, [DNA]'s.

Tickfaw River, La. (LE):
(801)0101111100010001111110100001111101100101111100110110111001000110l
0011010000010111100010000100001000001100110010001011111010011001111l1
1000010100001111101110100000010100001011101101111110010111101101l0100
0100101111101111111010101010101000011001010001010000011010011000000l0
1001001000000001000100000l0010111011
(802)0101111100010001101110100001011100000101110100110000011001000110l
0011000000010111000010000100000000011100110010001011111010011001111l1
1001011100001111101110100010010110001011101101011101010111101101l0100
0100101111101111011010101010100000011001010001011000011010011000000l0
100100100000100100010000010010111011
(803)0001011100000001101110100001111100000101111100110000011001000100l
0011000000010011000010000100001000001100110010001001110010110001110l1
1001010100001111101110100010010100001011101001011100010111101100l0101
0100101111101111011010101011100000011001010001010000011000011000000l0
100100100000100100010000010010011011
(804)0001011100000001101110100001011101100101111100110000011001000100l
0011000000010011010010000100001000001100110010001001110011101001101l1
1001010100001111100110100000010100001011101101011110010101101101l0100
0100101111101111011010101000100000011001010001011000011000011000000l0
100100100000100100010000010010111011
(805)0001111000010001101110100001111110100101110100110110001001000100l
0011000000010111100010001100001000001100110010001101110010101001111l1
1000010100001111100110100000010100001011101101111100010011101101l0100
0100101111101111011000101011100000011001010001011000011000011000000l0
100100100000100000010000010010111011
(806)0001111100010001111110100001001100100101111100110000101001000000l
0011010000010101110010000100001000001100110010001011110010001001111l1
0000010100001111101110100010010100001011101101111010010101101101001 00
0100101111101111011010101010100000011001010001011000011000011000000l0
100100100000100100010000010010111011
(807)0001111100010001101110100001101111100101111001110110101001000100l
0011010010010111110010001100001000001100110010001111101010111001110l1
1001010100001111101110100000010100001011101100111010010011101101l0100
0100101111101111011000101101101000011001010001011000011010001000000l1
100100100000000100010000010010111011

(table cont.)

(808)00011111000100011011101000010111010001011111001100100110010001001
001100000001011100001000010000100000110011001000110110101011100111101
100101010000111110111010001001010000101110110110111001001110110110101
010010111110111101101010111110000001110101000101000001100000100000010
100100100000100000010000010010111011
(809)00011101000100011011101000011111011001001111001000001110010001101
001100000001100100001000110000100000110011001000111111001010100111001
100101010000111110111010000001010000101110110110101001010110110110100
010010111110111111101010101110000001100101000101100001100000100000011
100100100000100100010000010010111011
(810)00011111000100011011101000011111011001011111001100000010010001101
001100000001001101001000010100100000110011001000111111101101100101101
100001010000111110111010001001011000101110110101100001011110010110100
010000111110111101101010100110000001100101000101100001100001100000010
100100100000100100010000010010111011
(811)00011111000100001001101000010011011001011111001100000100010001001
001100000001001101001000110000100000110011001000110111101101100111101
100001010000111110111010000001010000101110110010111001011010110110001
010010111110111101100010100110000001100101000101100001100001100000010
100100100000100100010000010010111011
(812)00011111000100011011101000011111111001001110001100001110010001101
001100000001011110101000110000100000111011001000111111101011100111111
100001010000111110111010000001011000101110110100101001001010110110100
010010111110111111100110111110000001100101000101100001101001100000011
1001001000000000100010000010010111011
(813)01011111000100011011101000011111001001001101001101000110010001101
001101000001011101001000010000100000111011001000111111101110100111111
100001010000111100111010000001010000101110110111111001011110110110100
010010111110111101100010101110000001100101000101100001100001100000010
100100100000100000010000010010011011
(814)000100010001000110111010000101110000001001101001100001100000001101
0011000100010111010000000010000100000111011001000110111001110000111111
100101010000111100011010011001011000101110010111111001011110010010000
010010111110111101100010110110000001000100000101100001100001100000011
100100100000100100010000010010011011
(815)00011111000100011011101000011111111001011111001101000110010001101
0011000010010111111101000010100100000110011001000111111101100100111011
100001000000111100111010001001011000101110010111111001001110110110100
010010111110111111100010111110000001110101000101100001101001100000011
101100100000100100010000010010011011

(table cont.)

(816)000101110011000111111010000101111100010111010011001001100100011010011000110010111110010000101001000001100110010001111111011101001111111001010100001111000110100010010110001011100101111110010110100010001011111011110110001011111000000101000100010110000011000011000000110010010000010010001000001001011101
(817)000101110001010110111010000111111100010011010011000001100100011010011000110010111010000010111111010000100001000001100110010001111111011101001001011010010100001111001010100010010110001011100101111110010011101101101000100010111110111011000101111100000001000001000101100000110000110000001100100100000100100010000010010111011
(818)000101110001000111111010000111111100010011110011010001100000011010011000110010111010000010111111010000100001000001100110010001111111011101001001011010010100001111001010100010010110001011100101111110010011101101101000100010111110111011000101111100000001000001000101100000110000110000001100100100000100100010000010010111011
(819)000011110001010110111010000111101110010011010010000011100100010100110010001111111011111001010111101000010000100001100110010001111111011101001001011010010100001111001010100010010110001011100101111110010011101101101000100010111110111011000101111100000001000001000101100000110000110000001100100100000100100010000010010011011
(820)000111100001000111111010000101111100010010010011011011100100010100110010001111111011111001011111001000010000100001100110010001111111011101001001011010010100001111001010100010010110001011100101111110010011101101101000100010111110111011000101111100000001000001000101100000110000110000001100100100000100100010000010010111011
(821)000011000001000111111010000101111110010110010011010010100100010100110010001111111011101001011111001000010000100001100110010001111111011101001001011010010100001111001010100010010110001011100101111110010011101101101000100010111110111011000101111100000001000001000101100000110000110000001100100100000100100010000010010011011
(822)000111110001000111111010000111111110010111110011011010100100010100110010001111111011101001111111001000010000100001100110010001111111011101001001011010010100001111001010100010010110001011100101111110010011101101101000100010111110111011000101111100000001000001000101100000110000110000001100100100000100100010000010010011011
(823)000111010001010110111010000101111110010011110011010000100100010100110010001111111011101001111111001000010000100001100110010001111111011101001001011010010100001111001010100010010110001011100101111110010011101101101000100010111110111011000101111100000001000001000101100000110000110000001100100100000100100010000010010011011

(table cont.)

(824)00011111000100011001101000010111110001011111001101100010010001101
0011000000010111111010000100001010001100110010001111111011101001011111
100001100000111100111010000001011000101110010110011001011110110110100
010010111110111001101010100110000001010001000101100001100001100000010
101100100000100100010000010010111011
(825)01011111000100011011101000010011010001011101001100000110010001101
001100001001011101001000010000100000110011001000110111101101100111111
100101011000111100111010001001010000101110010110101001011010110110000
0100101111101111101100010101110000001100101000101100001100000100000010
1001001000001000000010000010010011011

Atchafalaya Basin, La. (LW)

(851)01011111001101011111001010011110011001001110001101101010010001101
001101001001010111001000000100100001110011001000111111101011100111111
100101110000111010111010011001111000101110110110111001001110010110000
010011110110110011101010110100100001100101100100100001101001100000011
1011001000001001000100000010010011011
(852)01001101000101011001001000010101111001001110001101001010011001101
0011010110010101111010000000100101001110011001000111111101001100101110
100101111000111000111010011001111000101110110110101001011100010111100
010010110110110011101010110100100001100101100101100001101001100000010
1011001000001001000100000010010111011
(853)01011111000101011011001000011101011001001110001101101010010001101
001101010001010111001000100100100001110011001000111111101110100101111
100101010000111010111010011001111000101110110100011001001110010111101
010011110110111011101010110100100001100101100101100001101001100000011
1011001000000000100010000010010011011
(854)01001111000101011011001000011110111001001110001001001010011001101
0001010110010101110010000000100100001111011001000111111101111100101101
100100110000101000111010011001111000101110110100101001001100010111100
010010110110110011101010111110100001100101100101100001101001100000011
1011001000000000100010000010010011011
(855)01011111000101011011001000011110010001001101001100001110010001101
0011000010010001010010000000100100001110011001000111111001011100111101
100101010000111010111010011001111000101110110110111001001100010110101
010010110110111011101010111100100001100101100101100001101001100000011
1011001000001001000100000010010011011

(table cont.)

(856)01001111000101011011001000011111011001001110001100001110010001101
0011010110010001010010000000001000011110110010001111111011111001111 10
10010111000011100011101001100111100011111011011000100100111001011 0101
0100111101101110111010101101001000011001011001011000011010011000000 10
1001001000001001000100000100100 11011
(857)01011111000101011011001000011111111001001110001001101010011001101
1000010010010101111010001001001000011110110010001111111010111001010 00
1001011000001110001110100110011110001111101101101110010010100101100 00
0100101101100110111010101111101000011101011001001000011010011000000 11
101100100000000100010000010010011011
(858)01011111000101011011001000010111011001001110001001101110010001101
0011010010011111111010000000100101001110011001000111111101001100101 000
1001011110001110001110100110011110001011101101101010010010100101101 00
0100101101100100011010101101101000011001011001011000011010011000000 11
101100100000000100010000010010111011
(859)01011101000101011011001000011110011001001101001101100010011001101
0011010010010111111010000000100101001111011001000111111101010100101 000
1001011100001110001110100110011110001011101101101010010010100101101 00
0100101101100100111010101101001000011001011001001000011010011000000 11
101100100000100100010000010010111011
(860)01001111000101011011001000011110111001001111001001101010011001101
0011010010010111111010000000100101001111011001000111111101111100101 001
1001011110001110001110100110011110001111101101101010010010100100101 00
0100101101101100111010101100001000011001011001011000011010011000000 10
101100100000000100010000010010011011
(861)01001111001101011011001000011111111001001110001001101010010001101
0011010010010111111010000000100101000110011001000111011101010100101 011
1001011110001110001110100110011110001011101101101110010010100101100 00
0100101101101110111010101111001000011001011001001000011010011000000 11
101100100000100100010000010010111011
(862)01001111000101011011001000011110011001001101001001101010010001101
1011010110010111111010000000100100000110011001000111011101001100111 011
1001011100001110001110100010011110001011101101101110010010100101101 00
0100111101101110111010101111001000011001011001011000011010011000000 10
101100100000100100010000010010011011
(863)01011111001100011011001000010110110001001110001101101010010001101
1001010110010101111010000100001010011100111010001111111010011001110 10
1001011100001111001110110110011110001011101101010110010001100101110 00
0100111101101110111010101101000000011001011001011000011010011000000 11
101100100000100100010000010010011011

(table cont.)

(864)0101111100010101101110100001011111000100111100110110101001000110
1011010010010111111010000001001010011100111010001111111011111001110 01
1001011110001111001110110110011111000111110110101111001001110010111100
0100111101101110111010101001000000011001011001011000011010011000000011
1011001000000001000100000100100110 11
(865)0100110100010101101100100001111011000100111000110110011001000110 1
1011010010010111111010000001001010011100110010001111111011101001111111
1001011110001111001110110110011111000101110110101111001001110010111100
0100111101101110111010101111101000011001011001011000011010011000000011
1011001000000001000100000100100110 11
(866)0101111100010001100100100001011011000100111000110100011001000110 1
1011010010010111111010000001001010011100111010001111111011101001010 11
1001011110001111001110110110011111000111110110101011001001110010111100
0100111101101110111010101101101000011001011001011000011010011000000011
1011001000000001000100000100100110 11
(867)0101110100110001101110100001011111000100110100110010111001000110 1
0011010110010111111010000101001010011100111010001111111011111001111111
1001011110001111001110110110011111000111110110101111000001110010111100
0100101101101110111000101011101000011001011001010000011010011000000011
1011001000000001000100000100101111011
(868)0101111100110001101110100001111001100100111000110000111001000110 1
0011010010010111110010000001001000011100110010001111111011111001111111
1001011110001111001110110110011111000111110110101111000001100010111100
0100101101101111111010101111101000011001011001010000011010011000000011
1011001000001001000100000100101110 11
(869)0000111100010101101110100001111101000100110100110000011001000110 1
0011010010010111111010000001001000011100110010001111111011111001111111
1001011110001111001110110110011111000111110110101111001001110010111100
0100101101101111011010101111101000011001011001010000011010011000000011
1011001000001001000100000100101110 11
(870)0101111100010001101100100001111001100100111100110000111001000110 1
0111010010010111111010000001001000011100110010001111111011111001111101
1001011110001111001110110110011111000101110110101111001011110010111100
0100101101101110111010101111101000011001011001010000011010011000000011
1011001000000001000100000100100110 11
(871)0101110100010101101100100001011101000100110100110100001001000110 1
0001010010010101111010000101001010011100111010001111111010111001111111
1001011110001110001110100110011111000101110110101011001001110110111100
0100101101101110111010101111100000011001011001011000011010011000000011
1011001000000001000100000100100110 11

(table cont.)

(872)000011010001010110010010000111111110010011100011010011100100011010
0011010010010101111010000001001010010100111010001111111010011001111111
1001011100001111001110110110011110001111101101011110010011100101110000
0100111101101110111010100001101000010001011001011000011010011000000111
1001001000001001000100000100100110111
(873)0000111000010101100100100001111001000100111000110100111001000110101
0011010010010101111010000001001010011100111010001111111010011001110111
1001011100001111001110110110011110001011101101011110010011000101110000
0100111101101110111010100011100000011001011001011000011000011000000111
1011001000000001000100000100100110111
(874)0001111100010001101100100001111101100100111100110110111001000110101
1011010010010101111010000001001010011100110010001111111010111001110011
1001011100001111001110110110011110001011101101011110010011000101100000
0100101101100110111010101111101000010001011001011000011010011000000111
1011001000001001000100000100100110111
(875)000110010001000110010010000111100110010010010011010000100110011010
1001010010010101111010000000001010011100111010001111111011111001011011
1001011110001111001110110110011110001111101101011110010010000101111100
1100111001101110111010101011101000011001011001011000011010011000000111
1011001000001001000100000100100110011
(876)010110010001000110010010000101110110010111110011010010100100011010
0011010010010101111010000001001010010100111010001111111011101001111111
1001011100001111001110110110011110001011101101011110010010100101110100
1100111001101110111010101111101000011001011001011000011000011000000101
1011001000001001000100000100101110011

Guadelupe River, Kerrville hatchery, Tx.

(401)0001111100110011111100100001011011100101010100110010011011010000 1
1001000010011111011000001100010011001010110010001101110011001011111111
0001011100011011000110100001010110001011111110101100100111101100000101
1100111001101100111000101111000110010100011011011000101011011000100101
1011001010100100000110000100101110111
(402)0000110000010101111100100001011011100101100100100100001001110110 1
1001000000011101011010001100010011001100110010001100111010011011010111
1000011000011011000010100001010110001011111100101100000111101101000101
1100111001101100111000101111000110010100011011011000101011011000100101
1011000010000100000100000100101110111

(table cont.)

(403)00011110001101011111001000010111111001011101001101100010010101101
100100001001110101101000110001001100110011001000110011101000101101101
100001110001101100111010000101011000101111111010110001010110110100010
110011100110110011100010101100011001010001101101100010100101100010010
100100101010010000011000010010111011
(404)00011110000101011111001000010111111001011101001101100110010101101
10010001100111110110100011000100110010101100100011111110100011101111
100001110001101100111010000101011000101111110010110000011110110100010
110011100110110011100010101100011001010001101101100010101101100010010
100100101010010000011000010010111011
(405)00001111000101011101001000010110111001011101001101100010010101101
100100000001111101101001010001001100111011001000111011101000101101101
000001110001101100111010000101011000101111110010110000011110110100010
110011100110110011100010101100011001010001101101100010101101100010010
100100001000010000011000010010011011
(406)00001110000101011101001000010110111001011001001101000010010101101
100100000001110101101000110001001100111011001000111111101000101101101
100001110001101100111010000101011000101111110010110000011110110100010
110011100110110011100010101100011001010001101101100010101101100010010
101100101010010000010000010010111011
(407)00011111001100011111001000010110111001011101001001100000010101101
100000000001110101101001100001001101111011001000110011101000101101101
100101110001101100101010000101011000101111110010110000011110110000010
110011100110110001100010101100011001010001101101100010101101100010010
100100101010010000011000010010111011
(408)00011110000101011111001000010111111101011101001001100110010101101
10010100100111110110100110000100110011001100100011001110100011101101
100001110001101100101010000101011000101111111010110000011110110100010
110011100110110001100010101100011001010000101100100010100001100010010
101100101010010000011000010010111011
(409)00011111000101001101001000010110111001011101001001100110010101101
100100000001111101101001110001001100110011001000110011101000101101001
100101100001101100101010000101011000101111111010110000011110110100010
110011100110110011100010101100011001010000101100000010101101100010010
101100101010010000010000010010111011
(410)00010110000001101111001000010111111001010101001101000100000100101
101100010001110101101001100001001100110011001000110111001000101011001
000100110001101100001010000101011000101111111010110000011110110100010
110010100110110011100010011100011001010001101101100010100001100010010
100100001000010000011000010010111011

(table cont.)

(411)000011100001010111110010000101101110010111010011001000100101011011
10010001000111110110100110000100110011101100100001001110100011111111
10010111000110110010101000010101100010111111111011000000101011010001
0110011100100110001100010101100011001010000101101100010100101100010010
10110010101001000001100011001011 1011

(412)0001011000110011111100100001011111100101010100110100010001001 1101
0001010100011100011000011000010011001110110000001111111010001010110 01
0000001100011011000010100001010110001011111110101100000111101101000 10
1100111001101100011000100011000110010100001011001000101000011000100 10
100100001000010000011000010010111011

(413)0001111100110101111110010000101101110010111010010011001100101 01101
1001000100010101011010001100010011001110110010001100111011001011111 11
1001011100011011001110100111000110001011111110111100000101101101100 10
1100111001101100111000101011000110010100011001011000101011011000100 10
100100101010010000011000010010011011

(414)000111110011000111111001000010111111000101110100100110010001010 1101
1001000000011101011010001000010001011110110010001100111010001011111 01
1000011100011011001110100111010110001011111100111100000111101101000 10
1100111001101110111000101001000110010100010001011000101011011000100 10
100100100000010000011000010010111011

(415)000101110001010111111001000010111110001011101001101100110010101 101
1001000100011101010010001100010011011110110010001100111010001011111 11
1001011100011011001110100111010110001011111100111100000111101101000 10
1100111001101110111000101011000110010100011011011000101011011000100 10
101100101010010000011000010010111011

(416)000111110001010111111001000010111110001011101001101100010010101 101
1001010110011101011010001100010011001100110010001101111011001011111 11
1000011100011011001110100111010110001011111110111100000011101101000 10
1100111001101110111000101011000110010100001011011000101011011000100 10
101100101010010000011000010010111011

(417)000011110001010111111001000010111110001011101001100100110010101 101
1001000100011101011010001100010011001110110010001101111011001011111 11
1001011100011011001110100111010110001011111100111100000111101101000 10
1100111001101100111000101011000110010100011011011000101011011000100 10
101100101000010000011000010010111011

(418)000111010001010111111001000010110110001011101001000100100010101 101
1001010100011101011010001100010001001110110010001101111011001011111 11
1001011100011011001110100111010110001011111110111100000111101101000 10
1100111001101100011000101111000110010100001011011000101011011000100 10
101100101010010000011000010010111011

(table cont.)

(419)000110000011000111010010000101101100010111010011010000000101011101
100100001001110101101000110001001101110011001000110011101100101111101
000101110001101100111010011101011000101111011010110000011110110110010
110011100110111011100010101100011001110001000101100010101001100010010
100100101010010000011000010010111011
(420)000101000011010111110010000101111100010111010011010000100101011101
100100001001010101001000110001001101111011001000110011001100001111101
100101110001101100111010011101011000101011111011110000011110110110010
110011100110110001100010101100011001010001101101100010101101100010010
100100100010010000011000010010111011
(421)000101110001010111110010000101101100010111010011010001000101010011
100100000001110101001000110001001101111011001000110011101100101111101
100101110001101100111010011101011000101111111011110000011110110100010
110011100110110001100000100100011001010001100101100010101001100010010
100100101010010000011000010010111011
(422)000111010001010111110010000101111100010111010010010000100101010011
100100001001110101101000110001001100111011001000110011101100001111111
100101110001101100111010011101011000101111111001111000000111011010010
110011100110110001100010100100011001010001101101100010101101100010010
101100001010010000011000010010111011
(423)000111000001000111110010000101111110010111010011011001100101010011
100100000001110101101000110001001100111011001000110011101100101111111
100101110001101100111010011101011000101111101001111000001101011010010
110011100110110111100010101100011001010001000100100010101101100010010
100100101010010000011000010010111011
(424)000111010011000111110010000101101110010111010010011001100101010110
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100101110001101100101010011101011000101111101001111000001101011010010
110011100110111011100010101100011001010001100100100010101101100010010
101100101010010000011000010010111011

Lake Herrington, Ky.

(101)000011110001010110110010000101111010010011010011000011100100111101
001101001001011111110100000010010100101001110100011111111010111001011101
100111111000111100111011001001111001101110110110111001011110010111100
010010110110110111110101011011010000100010100010110000110100110000001 1
101100100000100000011100110010011011

(table cont.)

(102)0000111100110101101100100000101010010010011010011000011100100101010011010010010111111010000000100101001111011101000111111101011100101001100111110000111100111010001001111001101110110100111001011010010110101010010110110111010101001101000010001010001011000011010011000000011011001000001000000111001100100110110

(103)0000111100010101101100100000101110010010011010011000011100100011010001010010010111111010000000100100001010001101000111111101011100101101100111111000111100111011001001111000101110110100101001011110010111100010100101101101111010101111101000011001010001011000011010011000000011011001000001000000111001100100110110

(104)0000111100110101101100100000111110010010011010011000011100100011010001010010010111111010000000100100001010001101000111111101011100101001100111110000111100111011001001111000101110110100101001011110010111100010100101101101111010101011101000011001010001011000011010011000000011011001000001000000111001100100110110

(105)0000111100010101101100100000101011010010011010011000011100100011010001010010010111111010000000100101001010001101000111111101011100101001100111110000111100111011001001111000101110110100101001011110010111100010100101101101111010101101101000011001010001011000011010011000000010011001000001000000111001100100110110

(106)0000111100010101101100100000101110010010011010011000011100100111010001010010010111111010000000100100001010001101000111111101001100101001100111110000111100111011001001111000111000111011001001111000111111011011011100101101001011110010111100010100101101101111000010100100000000100010100010010000110100100000010011001000001000000111000100100110110

(107)0000011100110101101100100000101011010010011010011000011100100111010001010010010111111010011001001100111110000111100111011001001111000111000111011001001111000111111011011011100101101100001011110001010010110101101111010000110010100010110000110100110000001101100100000100000011100110010011011

(108)0000101100010101101100100000101010010010011010010011010100111011010001010010010111111010000000100101001010001101000111111100100110010100110011110000111100111011001001111000111111011011011100101101001011110010111100010100101101101111010000100010100010110000110100110000001011001000001000000111001100100110110

(109)0000111100010101101100100000101010010010011010011011010100111011010001010010010111111010000000100101001010001101000111111100100110010100110011110000111100111011001001111000111111011011011100101101001011110010101011000100101101101111010000111010100010110000110100110000001101100100000100000011100010010011011

(table cont.)

(110)0000111000110101101100100000111010010010011010011001011100101011010011010111111010000001001000011110110010001111111010111001110010001111110001110011101100110111100111111101101001110010110100101001011011011011110001010010010000100010100010110000110100110000001110110010000010000001110001001001101
(111)0000111000110101101100100000101010010010011010011000011100100010010011010111111010000001001010010100111010001111111010111001111011001011110001111001110110010011110011011101100101111001011110010111100010010110111011011011110101010011010000110010110010110000011000011000000011101100100000100000011100010010011011
(112)0000111100110101101100100000101010010010011010010011011100100010010011010111111010000001001010010100111010001111111010111001010011001111010001111001110110010011110011011101100101111001011110010011000010010110111011011110101011111010000110010110010110000011010011000000011101100100000100000011100110010011011
(113)0000011100110101101100100000101110111001001101001100001110010001101001101011111010000001001010011110110010000111111010011001111010010111001110110010011110011011101100101111001011110010111100010010110111011011110101011111010000110010110010110000011010011000000011101100100000100000011100110010111011
(114)0100011100010101101100100000101110100010011010011000011000100011010011010111111010000001001010011100111010001111111010011001011010010111001110110010011110011011101100101111001011110010111100010010110111011011110101011011000000010001011001011000011010011000000011101100100000100000010000010010111011
(115)0000111100110101101100100000101111100010011010011000011100100011010011010111111010000001001010011110111010001111111010011001110110010111001110110010011110011011101100101111001011110010111100010010110111011011110101011011000000010001011001011000011010011000000011101100100000100000011100010010111011
(116)0000011100010101101100100000101110100010011010011000011100100011010011010111111010000001001010011110111010001111111010011001111010010111001110110010011110011011101100101111001011110010111100010010110111011011110101011011000000010001011001011000011010011000000011101100100000100000011100010010111011
(117)0000111000110101101100100000101111100010011010011000011100100011010011010111111010000001001010011110111010001111111010011001110110010111001110110010011110011011101100101111001011110010110000010010110111011011110001010110100000100000110010110000011010011000000011101100100000100000011100110010111011

(table cont.)

(118)000001110011010110110010000101110110010011010011000011100100011 01
001101001001011111101000000100101001111011101000111111010011 00101101
000111111000111100111010011101111001101110110101111001011110010111100
010010110110110111100010111110100001100001100101100001101001100000011
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(119)000001110011010111110010000101100100010011010011001011100100011 01
001101001001011111101000000100100001111011101000111111010011 00101101
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100100100000100000011100010010111001
(120)000011110001010110110010000101110100010011010011000011100100011 01
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010010110110110111101010110110000001100101100101100001101001100000011
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(121)000011000001010110110010000101100110010011010011000011100100011 01
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100111111000111100111010011101111001101110110101111001011110010111100
010010110110110111101010110110100001100101100101100001101001100000010
100100100000100000011100010010011011
(122)000011110001010110110010000111110100010011010011000010100100011 01
001101001001011111101000000100101001111011101000111111010011 00111101
100011111000111100111010011101111001101110110101111001011110010111100
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(123)000011010001010110110010000101110100010011010011000010100100011 01
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(124)000011110001010110110010000101100100010011010011000011100100011 01
001101001001011111101000000100100001111011101000111111010011 00111101
100101101000111100111010011101111001101110110111111001011110010111000
010010110110110101101010110110100001000101100101100001101001100000011
101100100000100000011100010010111001
(125)000011110011010110110010000101100100010011010011000011100100011 01
001101001001011111101000000100101001010011101000111111010011 00111101
000111101000111100111010011101111001101110110111111001011110010111000
010010110110110111101010111110100001100101100101100001101001100000011
101100100000100000011100010010111001

(table cont.)

(126)0000111000110101101100100001111001000100110100110000111001000110
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1001111100001111001110100111011110001011101101011110010111100101110000
0100101101101101011010101101101000011001011001011000011010001000000010
1011001000001000000111000100101110001

Lake Jordan, Reservoir, Al.

(901)0001110000010001111100100001011011100101111001100110001001000110
1
0001010010011101011010001100000000100100110010001101111011111001011111
1010011110101110011010110110010111001001101101000101011111100101101000
0100111001101100011010000001100010011001010001011000011011011000000010
1001001000010000000101000100101110111
(902)0001110000010001111100100001011101100101111001000110001000000110
1
0001010010011101011010001100000000100100110010011101111011111001011000
1010011110101110011010110110010111001001101101000100001111110010110100
0100111001101100011010101011110101001100101100100100001101101100000010
1001001000110000000101000100101110111
(903)0001110000010001111100100001011010100001111001000110001000000110
1
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0100111001101110011010101001100010011001010001001000011010011000000010
1001001000010000000101000100101110111
(904)0000110000010001111100100001011011100101111001100110001000000110
1
0001010100011101011010001100000000110100110010001101111011001001011100
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0100111001101110011010101111100010011001011001001000011010011000000010
1011001000010000000101000100101110111
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1
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0100111001101100011010101111101010011001011001011000011011001000000010
1001001000110000000101000100101110111
(906)0000110000010001111100100001011011100101111001100110001000000110
1
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1011001000010000000101000100101110111

(table cont.)

(907)00011100000100011111001000010110111001011110011001100010010001101
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010011100110111111100010111110101001100101000101100001101101100000010
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010011100110111001100010101110101001100101000101100001101000100000010
100100100011000000010100010010111011

(909)00011100000100011111001000010011111001011110011001100010010001101
000100001001110111101000110000000010010011001000110111101110100101100
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010011100110110001101010101110101001100101000100100001101101100000010
100100100001000000010100010010111011

(910)00011100000100011111001000010111111001011110011001100010000011101
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101001111010111001101011011001011100100110110111100001001010010110100
010011100110110111101010101110101001100101100101100001101101100000010
101100100001000000010100010010011011

(911)00001100000100011111001000010011011001011110011001100000000001101
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010011100110110001100010101110001001100101000100100001101101100000010
100100100001000000010100010010011011

(912)00001100000100011111001000010111111001011110011001100010000001101
000100011001110101001000110000000010000011101000110111101110100101100
101001111010111001101011011001011100100110111001100001001010010110000
010011100110111011101010100110101001100101100001100001101101100000010
101100100001000000010100010010111011

(913)00011100000100011111001000010110110001011110011000100010000001101
001100011001110101101000010000000010010011001000110111101110100111111
101001111010111001101011011001011100000110110111101001011110010110100
010010100110111001101010111110101001100101000101100001101101100000010
101100100011000000010100010010111011

(914)000110000001000111110010000101101100000011110011001100010000001101
000100001001110101101000010000000011010011001000110111001110101101111
101001101010111001001011011001011100100110110111111001011110010110100
010011100110110101101010101110101001100101100101100001101100100000010
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(table cont.)

(915)0001110000010001111100100001011111000101111001100010011000001101
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1010011110101110011010110110010111000001100101011110010111100101101 00
0100111001101111011000101011100010011001011001011000011010001000000 10
1001001000010000000101000100101110 11

(916)0000110000010001111100100001011001000101111001100010001000000 1101
000101001001110101101000010000000011010011001000110111101100010101111
1010011110101110011010110110010111000001101101011000010111100101101 00
010011100110111101101010111110101001100101100101100001100101100000010
10010010000100000001010001001011 1011

(917)0001110000010001111100100001011011100001111001100110011000000 1101
00110100100111010110100001000000001101001100100011011110110010010 1111
101001101010111001001011011001011100100110110111100001011110010110100
010011100110111101101010111110101001100101100101100001100101100000010
10010010001100000001010001001011 1011

(918)0001110000010001111100100001011001000101111001000110111000000 1101
0001010010011101011010000100000000110100110010001101111010101001011 01
101001101010111000001011011001011100100110110101100001011110010110100
010011100110111101101010101110101001100101000101100001100101100000010
1001001000110000000101000100101110 11

(919)0000110000010101111100100001011110100101111001100110001000000 1101
0011000010011101011010000100000000100100110010001101111011000001011 01
101001111010111001001011011001011100100110010111100001011010010110000
010011100110111001101010111110001001100101100001000001101001100000010
1011001000010000000101000100101110 11

(920)0000110000010001111100100001001001000101111001000110011000000 1101
000101001001110101101000010000000011010011101000110111101100100111101
101001111010111001001011011001011100100110010011110001011110010110000
010011110110111111101010101110101001000101000100100001101100100000010
1001001000110000000101000100101110 11

(921)0000110000010101111100100001011101000101111001100110001000000 1101
0011010010011101011010000100000000110100110010001101111011001001011 01
101001101010111001101011011001011100100110110011100001011110010110100
010011110110111001101010111110001001000101100101100001101000100000010
1001001000010000000101000100101100 01

(922)0000110100010001111100100001010011100101111001100110001000000 1101
0001000010011101011010000100000000110100111010001100111011101001011 01
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010011100110111101101010111110001001000101100100100001100001100000010
1011001000010000000101000100101110 11

(table cont.)

(923)0000110100010001111100100001011001000101111001100110001000000001101
00010000010011101011010000100000000010010011001000110111101110100101111
101001101010111001001011011001011100100110110101111001011110010110100
010011110110111111101010101110101001100101000101100001101001100000010
100100100001000000010100010010111011
(924)0001110000010001101100100001011001000101111001100110001000000001101
000101001001110101101000010000000011010011001000110111101000100101101
101001111010111000001011011001011100100110110011101001011110010110000
010011100110111101101010101110101001000101100101100001100001100000010
100100100011000000010100010010111011

Largemouth Bass, Atchafalaya Basin, La.

(301)10010000000011000111111101000111101001101100110111100100000000001
000110100100110100111010000010010000100111101010110000001101110101001
000011010000110010010100111110011000010110111011010101010111011100100
011000100010011011100110101001000011000000001001110101010100111001010
1001000000100010000110110000110011011
(302)101100000000110001111111000001001110011011001101110001100100100001
000110100110110000011010000010010000100111101010110011001100111001101
000101010000100010000100011110010000010110110010010101010111011100000
011000100011100001100110101001000011000000000101110101011100110001010
1001000000100010000110010100110011011
(303)1011000000001100011011111000001011010011011001101110000111000000001
0001101011001101001110110000100100001000111010101100111011111111101001
000011010000100010010100011110010000111110111011010101010111001100100
011000100011100001100010001001000011000000000100110101011100110001010
1001000000100010000110010100110011011
(304)101100000000110001111111000001111000011010001101110000111100100001
000110101100110100111010000010010000100111101011110011101110101101001
000101010000110010010100010110010000111110111011010101010111001100100
011000100010100001100110001001000011000000001100110101011000111001000
1001000000100010000110000100110011011
(305)101100000000110001011111010001011110011011001101111000111100100001
000110101010110100111011000010010000100111101010110011101100100101001
000101011000111010010100011010011000110110111011010101010111011100100
011000100010101001100010101001000011000000000001110101011010111001000
1001000000100010000110010100110011011

(table cont.)

(306)10110000000110001011111000001011010011011001101110001111100100001
00011010010011010011101100001001000010011110101011001100111011010100 1
00011101000011001001010011111001100011011011101101010101001101110010 0
01100010001110010110001010100100001100000000010111010101110000100101 0
10010000010001000011001010011001 1
(307)10110000000110001111111000001111010011011001101110000110100100001
00011010011011010011101100001001000010011110101111000100010111110100 1
00011100000011001001010011111001100010011011001001010101011100110010 0
01100010001010010110001010100100001100000000010011001010101000110010 1
00100000100010000110110100010011011
(308)10100000000110001101111010001111010011010001101110001111100100001
00011010111011010011101100001001000010011110101111000000010111110100 1
00011100000011001000010001011001100011011011001101010101011101110010 0
01100010001010000110001010100100001100000000011011001010111001110010 0 0
10010000100010000110010100110011011
(309)00110000000110001111111000001100110011011001101100000111000100001
00011010110011010011101100001001000010011110101111000110110101110100 1
00010100000011001000010001111001100001011011101101010101011101110010 0
01100010001111010110001010100100001100010100110111010101110000100101 0
10010000100010000110110100110011011
(310)10110000000110001111111010001111110011010001001101000110100100001
00011010000011010011101100001001000010001110101111000100010101110100 1
00001101000011001001010011111001100010001011101101010101000101010010 0
01100010001100011110011010100100001100000100100111010101010000100100 0
10010000100010000110110100110011001
(311)10110000000110001011001000001111010011011001101110000111100100001
00011010110011010011101100001001000010011110101111000100110011110101 1
00011101000011001001010011111001100010011011101101010101011101010010 0
01100010001010010110011010100100001100000100100010010101001000100100 0
10010000100010000110110100110011011
(312)10110000000110001111101000001110010011011001101110000111000100001
00011010111011010011101100001001000010011110101111000100110110110100 1
00011101000011001000010011111001100010011001001101010101011100110010 0
01100010001010000110001000100100000100010100100011010101110000100101 0
10010000100010000110110100010011011

(table cont.)

Suwanee bass, Sante Fe River, Fl.

(501)00111100110100001011000101001100000000111101101000010010000000011
01011000000010110010110000100000000000011101000101000100111100010100 1
10000010010000110000000001110000000010011011001001000101011010001000 0
01011111001000000011000001111010010101011000101000101010111000010011 0
11001100011000100111000100101011010 1
(502)00111100110100001011000101001100000000111101101000010010000000011
01011000000010110010110000100000000000011101000101000100100100010100 1
10000010010000110000000001110000000010011011001001000101011010001000 0
11011111001000000010000001111010010101011000101000101010111000010011 0
11001100011000100111000100101011010 1
(503)00111100110100001011000101001100000000111101101000010010000000011
01011000000010110010110000100000000000011101000101000100100100010100 1
10000010010000110000000001110000000010011011001001000101011010001000 0
01011111001000000011000001111010000101011000101000101010111000010011 0
11001100011000100111000100101011010 1
(504)00111100110100001011000101001100000000111101101000010010000000011
01011000000010110010110000100000000000011101000101000100100100010100 1
10000010010000110000000001110000000010011011001001000101011010001000 0
01011011001000000011000001111010010101011000101000101010111000010011 0
10001100011000100111000100101011010 1
(505)00111100110100001011000101001100000000111101101000010010000000011
01011000000010110010110000100000000000011101000101000100000100010100 1
10000010010000110000000001110000000010011011001001000101011010001000 0
01011111001000000011000001101010010101011000101000101010111000010011 0
10001100011000100111000100101011010 1
(506)00111100110100001011000101001100000000111101101000010010000000011
01011000000010110010110000100000000000011101000101000100100110010100 1
10000010010000110000000001110000000010011011001001000101011010001000 0
01011111001000000011000001111010010101011000101000101010111000010011 0
10001100011000100111000100101011010 1
(507)00110100110100001011000101001100000000111101101000010010000000011
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10000010010010110000000001110000000010011011001001000101011010001000 1
01011111001000000011000001111010010100011000101000101010111000010011 0
11001100011000100111000100101011010 1
(508)00110100110100001011000101001100000000111101101000010010000000011
00011000000010110010110000100000000000011010001010001001101100101001 1
10000010010000110000000001110000000010010001001001000101011010001000 1
01011111001000000011000001111010010101011000101000101010111000010011 0
11001100011000100111000100101011010 1

(table cont.)

(509)00111100110100001011000101001100000000111101101000010010000000011
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01011111001000000011000001111010010100011000101000101010110000010011 0
11001100011000100111000100101011010 1
(510)00111100110100001011000101001100000000111101101000010010000000011
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10000010010000110000000001110000000010011001001001000101011010001000 1
01011111001000000011000001111010010101011000101000101010111000010011 0
11001100011000100111000100101011010 1
(511)00111100110100001011000101001100000000111101101000110010000000011
00011000000010110010110000100000000000011101000101000100110100010100 1
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01011111001000000011000001111010010101011000010100010101011100001001 1
0
11001100011000100111000100101011010 1

Chipola bass, Chipola River, Fl.

(601)00011100000100011011000010100000110100011101000000110010000000011
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11001111000010110000010011110001101000000100011010011110110010011100
01001010001001010110110100110000000100100111000000001010000000000010
10010001000010001000000101100010110 11
(602)00011100000100011011000010100000110100011101000000110010000000011
00001000010111010110101100001000000110001100110001000101110001010101 0
11001111000010110000010011110001101000001101100110100100101100100111 00
01001110000001000110110100110000000100100111000000001010000000000010
10010001000010001000000101100010110 11
(603)00011100000100011011000010100000110100011101000000110010000000011
00001000010111010110101100001000000110001100110001000101110001010101 0
11001101000010110000010011010001101000000011001101001111011001001110 0
01001010000001010110110100110000000100100111000000001010000000000010
10010001000010001000000101100010110 11
(604)00011100000100011011000010100000110100011101000000110010000000011
00001000010111010110101100001000000010001100110001000101110001010101 0
11001101000010110110010011110001101000000011101101001111011001001110 0
01001110000001010110110100110000000100100111000000001010000000000010
10010001000010001000000001000101101 1

(table cont.)

(605)000111000001000110110000101000001101000111010000001100100000000110000100001011101011010110000100000011000110011000100010111000101010101100111100001011000001001111000110100000000111011010011111011001001100001001111000100100011011010011000000010010011100000000010100000000000001010010001000010001000010000010110001011011
(606)000111000001000110110000101000001101000111010000001101100000000110000100001011101011010110000100000011000110011000100010111000101010101100111100001011010110010011110001101000000010010011100000000010100000000000001010010001000010001000010000010110001011011
(607)000111000001000110110000101000000101000111010000001001100000000110000100001011101011010110000100000011000110011000100010111000101010110100111010000010110000010011110001101000000010010011100000000010100000000000001010010001000010001000010000010110001011011
(608)000111000001000110110000101000001101000111010000001100100000000110000100001011101011010110000100000011000110011000100010111000101010111001111000010110100000100111100011010000000110011010011111011001001110001001010101101101001100000001001001110000000010100000000000001010010001000010001000010000010110001011011
(609)000111000001000110110000101000001101000111010000001100100000000110000100001011101011010110000100000011000110011000100010111000101010111001111000010110100000100111100011010000000110011010011111011001001110001001010101101101001100000001001001110000000010100000000000001010010001000010001000010000010110001011011
(610)000111000001000110110000101000001101000111010000001100100000000110000100001011101011010110000100000011000110011000100010111000101010111001111000010110100000100111100011010000000110011010011111011001001110001001010101101101001100000001001001110000000010100000000000001010010001000010001000010000010110001011011
(611)000111000001000110110000101000001101000111010000001100100000000110000100001011101011010110000100000011000110011000100010111000100010101001111000010110100000100111100011010000000110011010011111011001001110001001010101101101001100000001001001110000000010100000000000001010010001000010001000010000010110001011011

APPENDIX M

ISOZYME DATA

Number of populations = 5
Number of loci = 17
Locus name, followed by abbreviation and the Enzyme Commission number:

Phosphoglucose isomerase-1 (Pgi-1, E.C. 5.3.1.9)
Phosphoglucose isomerase-3 (Pgi-3)
Esterase, napthol AS-D acetate substrate (NADA, E.C. 3.1.1.1)
Mannose-6-phosphate isomerase (Mpi, E.C. 5.3.1.8)
Isocitrate dehydrogenase (Idh, E.C. 1.1.1.42)
Lactate dehydrogenase (Ldh, E.C. 1.1.1.27)
Malate dehydrogenase (Mdh, E.C. 1.1.1.37)
Glycerol-3-phosphate dehydrogenase (Gpdh, E.C. 1.1.1.8)
Phosphoglucomutase (Pgm, E.C. 2.7.5.1)
Glutamate-oxaloacetate transaminase (Got, E.C. 2.6.1.1)
6-Phosphogluconate dehydrogenase (6Pgd, E.C. 1.1.1.44)
Superoxide dismutase (Sod, E.C. 1.15.1.1)
Creatine kinase (Ck, E.C. 2.7.3.2)
Adenosine deaminase (Ada, E.C. 3.5.4.4)
Alkaline phosphatase (Alp, E.C. 3.1.3.1)
Aldolase (Ald, E.C. 4.1.2.13)
Alcohol dehydrogenase (Adh, E.C. 1.1.1.1)

Order of isozyme scores* in data list:
Pgi-1 Pgi-3 NADA Mpi Idh Ldh Mdh Gpdh Pgm Got 6Pgd Sod Ck Ada
Alp Ald Np
*Score for samples for which the band was not scorable is marked by '--'.

Tickfaw River, La. (LE)
MS MM MM HH MM MM MM MM MM MM MM MM MM MM MM MM MM
MM MM HH HH MM MM MM MM MM MM MM MM MM MM MM MM MM
MM MM FM HH MM MM MM MM MM MM MM MM MM MM MM MM MM
MM MM FF HH MM MM MM MM MM MM MM MM MM MM MM MM MM
MM MM FH HH MM MM MM MM MM MM MM MM MM MS MM MM
MM MM FM HH MM MM MM MM MM MM MM MM MM MM MM MM MM
MM MM FM HH MM MM MM MM MM MM MM MM MM MM -- MM
MS MM MM HH MM MM MM MM MM MM MM MM MM FH MS MM MM
MM MM MM HH MM MM MM MM MM MM MM MM MM MM MS MM MM
MM MM FM HH MM MM MM MM MM MM MM MM MM MM MM MM

(table cont.)

(LE, cont.)
MM MM MM HH MM MM MM MM MM MM MM MM MM FF FF MM MM
MM MM MM HH MM MM MM MM MM MM MM MM MM MS MM MM MM
MM MM FF HH MM MM MM MM MM MM MM MM MM MM MM MM MM
MM MM FM HH MM MM MM MM MM MM MM MM MM MM MM MM MM
MM MM FM HH MM MM MM MM MM MM MM MM MM FS MM MM MM
MS MR MM HH MM MM MM MM MM MM MM MM MM FM MM -- MM
SS MM MM HH MM MM MM MM MM MM MM MM MM MS MM MM MM
MM MR MM HH MM MM MM MM MM MM MM MM MM MS MM MM MM
MM MM FM HH MM MM MM MM MM MM MM MM MM MM MM MM MM
MM MM MM HH MM MM MM MM MM MM MM MM MM MM MM -- MM
MM MM MM HH MM MM MM MM MM MM MM MM MM MM MM -- MM

Atchafalaya Basin, La. (LW)
MM MR FM HH MM MM MM MM MM MM MM MM MM MM MS MM MM
MM MR MM HH MM MM MM MM MM MM MM MM MM MS SS MM MM
MM MM MM HH MM MM MM MM MM MM MM MM MM MM MS MM MM
MM MM MM HH MM MM MM MM MM MM MM MM MM MM MS MM MM
MM MM MM HH MM MM MM MM MM MM MM MM MM MM MM MM MM
MM MR MM HH MM MM MM MM MM MM MM MM MM MM SS MM MM
MM MM MM HH MM MM MM MM MM MM MM MM MM MM MS MM MM
MM MM MM HH MM MM MM MM MM MM MM MM MM MM MM MM MM
MM MM MM HH MM MM MM MM MM MM MM MM MM FM SS MM MM
MM MM MM HH MM MM MM MM MM MM MM MM MM MM MS MM MM
MM MM MM HH MM MM MM MM MM MM MM MM MM MM SS MM MM
MM MM MM HH MM MM MM MM MM MM MM MM MM FM SS MM MM
MM MM MM HH MM MM MM MM MM MM MM MM MM MS MM MM MM
MM MM FM HH MM MM MM MM MM MM MM MM MM MM SS MM MM
MS MM FH HH MM MM MM MM MM MM MM MM MM MS MM MM MM
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MM MM FF HM MM MM MM MM MM MM MM MM MM MM MS MM MM
MM MM FM HH MM MM MM MM MM MM MM MM MM MM MM MM MM
MM MS MM HH MM MM MM MM MM MM MM MM MM MS MM MM MM

Lake Herrington, Ky. (KY)
MM MM MM MM MM MM MM MM MM MM MM MM MM MS MM MM MM
MM MM MM MM MM MM MM MM MM MM MM MM MM MS MS MM MM
MM MM HH MM MM MM MM MM MM MM MM MM MM SS MM MM MM
MM MM FM MM MM MM MM MM MM MM MM MM MM MM MM MM MM
MM MR FM MM MM MM MM MM MM MM MM MM MM SS MS MM MM

(table cont.)

(KY, cont.)
MM MM FM MM MM MM MM MM MM MM MM MM MM MS SS MM MM
MM MM MM MM MM MM MM MM MM MM MM MM MM MM SS MM MM MM
MM MM FF MM MM MM MM MM MM MM MM MM MM MM MM MM MM MM
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MM MM FF MM MM MM MM MM MM MM MM MM MM MM MM -- MM
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MM MM FM MM MM MM MM MM MM MM MM MM MM MM MM MM MM MM

Lake Jordan Reservoir, Al. (AL)
MM MM FF HH MM MM MM MM MM MM MM MM MM MM MM MM MM MM
MM MM MM HH MM MM MM MM MM MM MM MM MM MM MM MM MM MM
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MM MM HM FH MM MM MM MM MM MM MM MM MM MM MM MM MM MM
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MM SS HM HH MM MM MM MM MM MM MM MM MM MM MM MM MM MM
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MM MS MM FF MM MM MM MM MM MM MM MM MM FS MM -- MM
MM MS MM FH MM MM MM MM MM MM MM MM MM FF MM MM MM
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MM MM FH HH MM MM MM MM MM MM MM MM MM MS MM MM MM
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MM MM HM HH MM MM MM MM MM MM MM MM MM MM MS MM MM
MM MM MM HH MM MM MM MM MM MM MM MM MM FM MM -- MM
MM MM MM HH MM MM MM MM MM MM MM MM MM FM MS MM MM
MM MM MM HH MM MM MM MM MM MM MM MM MM FM MS MM MM
MM MM MM FF MM MM MM MM MM MM MM MM MM MM MM -- MM
MM MM MM HH MM MM MM MM MM MM MM MM MM MM MM MM MM MM
MM MM HH HH MM MM MM MM MM MM MM MM MM MM MM MM MM MM
MM MM MM HH MM MM MM MM MM MM MM MM MM SS MM MM MM
MM MM HM FS MM MM MM MM MM MM MM MM MM MS MM MM MM

(table cont.)

Guadalupe River, Heart of the Hills Hatchery, Tx. (TX)
(These samples are not scored for Ada and Alp.)
MS MM FM HS MM MM MM MM MM MM MM MM MM -- -- MM MM
MS MM MM HH MM MM MM MM MM MM MM MM MM -- -- -- MM
MM MM MM MS MM MM MM MM MM MM MM MM MM -- -- MM MM
MS MM MM HH MM MM MM MM MM MM MM MM MM -- -- MM MM
MS MM MM HH MM MM MM MM MM MM MM MM MM -- -- MM MM
MM MM MM HH MM MM MM MM MM MM MM MM MM -- -- -- MM
MM MM MM HH MM MM MM MM MM MM MM MM MM -- -- MM MM
MM MM MM HH MM MM MM MM MM MM MM MM MM -- -- MM MM
MM MM MM HM MM MM MM MM MM MM MM MM MM -- -- MM MM
MM MM MM HH MM MM MM MM MM MM MM MM MM -- -- -- MM
MM MM FM HH MM MM MM MM MM MM MM MM MM -- -- MM MM
MM MM MM HH MM MM MM MM MM MM MM MM MM -- -- MM MM
MM MM FM MM MM MM MM MM MM MM MM MM MM -- -- -- MM
MM MM MM MM MM MM MM MM MM MM MM MM MM -- -- MM MM
MM MM MM FF MM MM MM MM MM MM MM MM MM -- -- MM MM
MS MM MM HH MM MM MM MM MM MM MM MM MM -- -- -- MM
MM MM MM HH MM MM MM MM MM MM MM MM MM -- -- MM MM
MM MM MM HH MM MM MM MM MM MM MM MM MM -- -- MM MM
MS MM FM HH MM MM MM MM MM MM MM MM MM -- -- MM MM
MS MM MM HH MM MM MM MM MM MM MM MM MM -- -- MM MM
MM MM MM HH MM MM MM MM MM MM MM MM MM -- -- MM MM
SS MM MM HM MM MM MM MM MM MM MM MM MM -- -- MM MM
MS MM MM HM MM MM MM MM MM MM MM MM MM -- -- -- MM
MM MM MM MM MM MM MM MM MM MM MM MM MM -- -- -- MM
MM MM MM MM MM MM MM MM MM MM MM MM MM -- -- MM MM
MM MM MM HM MM MM MM MM MM MM MM MM MM -- -- MM MM
MM MM MM HM MM MM MM MM MM MM MM MM MM -- -- MM MM
MM MM MM HH MM MM MM MM MM MM MM MM MM -- -- MM MM
MS MM MM FH MM MM MM MM MM MM MM MM MM -- -- MM MM
MM MM MM HH MM MM MM MM MM MM MM MM MM -- -- MM MM
MS MM MM HH MM MM MM MM MM MM MM MM MM -- -- MM MM
MM MM MM HH MM MM MM MM MM MM MM MM MM -- -- -- MM
MM MM MM FF MM MM MM MM MM MM MM MM MM -- -- MM MM
MM MM MM HH MM MM MM MM MM MM MM MM MM -- -- MM MM
MM MM MM HH MM MM MM MM MM MM MM MM MM -- -- MM MM
MM MM MM HH MM MM MM MM MM MM MM MM MM -- -- -- MM
MS MM MM HH MM MM MM MM MM MM MM MM MM -- -- MM MM
MM MM FM HH MM MM MM MM MM MM MM MM MM -- -- MM MM
MM MM MM HM MM MM MM MM MM MM MM MM MM -- -- MM MM

APPENDIX N

SOURCE OF SELECTED CHEMICALS AND COMPUTER PROGRAMS

Electrostarch Co.
Lot # 307
P.O. Box 1294
Madiosn, Wi. 53701

Sigma Starch
#105H9527
Sigma Chemical Co.
P.O. Box 14508
St. Louis, Mo. 63178

Taq Polymerase
#M1866
Promega
2800 Woods Hollow Rd.
Madison, Wi. 52711-5399

COMPUTER PACKAGES:

The RAPDistance Package, Version 104
Authors: John Armstrong, Adrian Gibbs, Rod Peakall, Georg Weiller
Research School of Biological Sciences, Institute of Advanced Studies
P.O. Box 475
Australian National University, Canberra, Australia
A.C.T., 2601
contact 1st author: JohnA@rsbs-central.anu.edu.au

POPGENE Version 1.2
Authors: Francis C. Yeh and Timothy Boyle
Department of Renewable Resources
University of Alberta
Edmonton, AB Canada T6G 2H1
email: fyeh@rr.ualberta.ca

NTSys-pc: Numerical Taxonomy and Multivariate Analysis System, Version 1.80
Author: F. James Rohlf
Exeter Software
100 North Country Rd., Building B
Setauket, NY 11733

APPENDIX O

GEL PICTURES

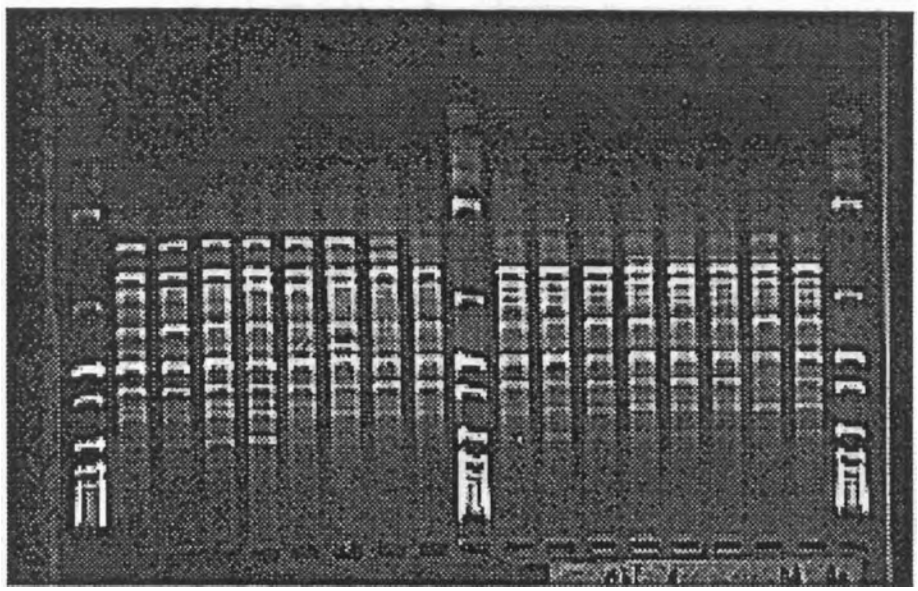

Figure 11. Gel of RAPD products, primer M-20. Lanes 3-8: AL; 9: LW; 10, 12-19: KY.

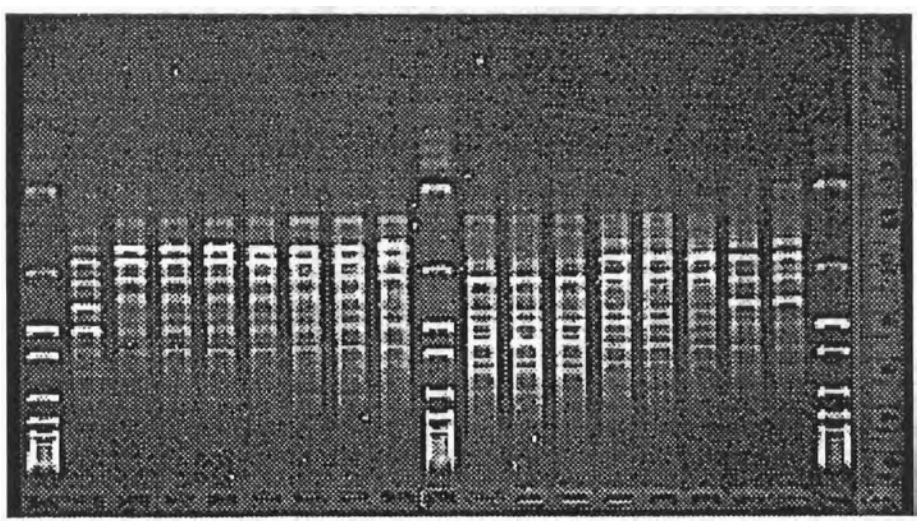

Figure 12. Gel of RAPD products, primer M-20. Lane 2: *M. salmoides*; 3-9: *M. treculi*; 10-12: *M. notius*; 13-15: Chipola bass; 16,17: *M. salmoides*.

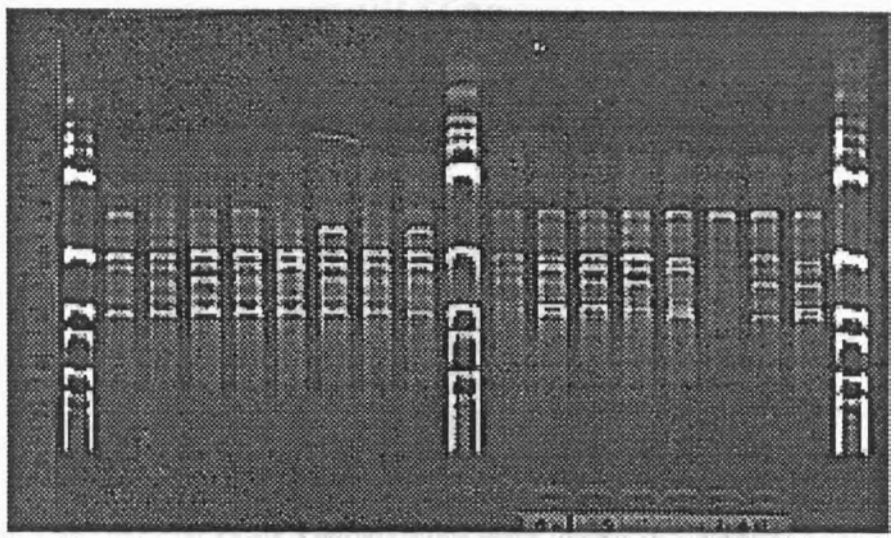

Figure 13. Gel of RAPD products, primer W-04. Lanes 3-10: LW; 12-19: LE.

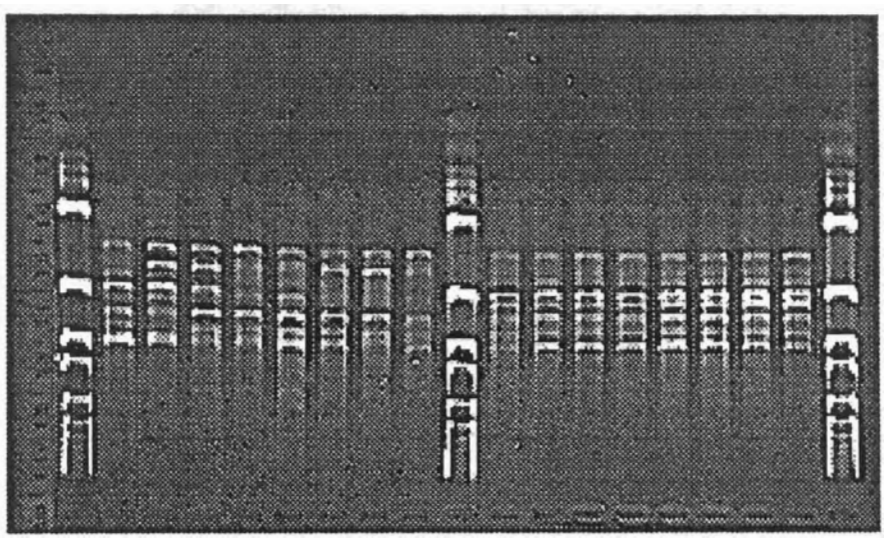

Figure 14. Gel of RAPD products, primer W-04. Lane 3: KY; 4: LW; 5-10: AL; 12-19: KY.

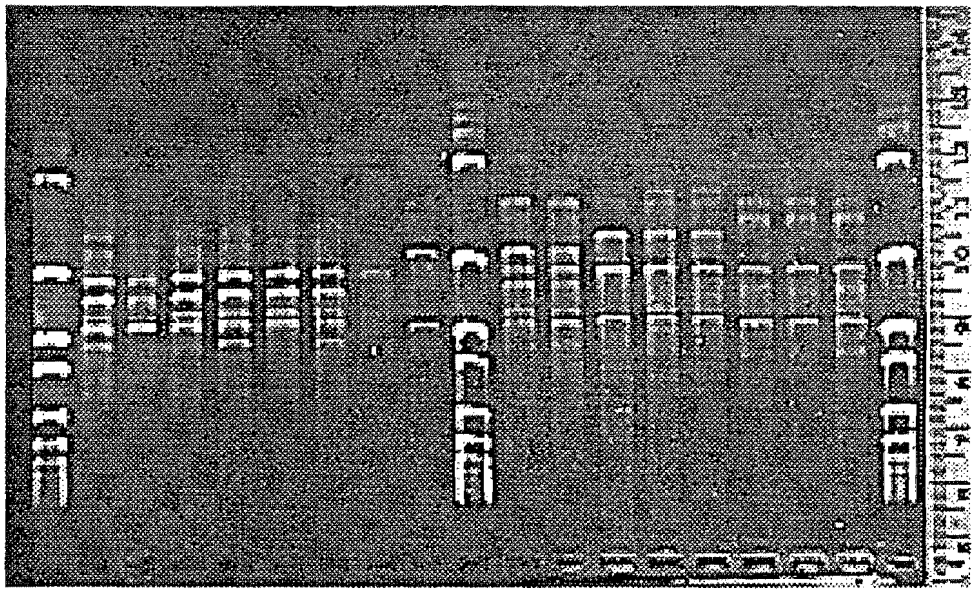

Figure 15. Gel of RAPD products, primer W-04. Lanes 2-8: *M. treculi*; lanes 9, 11-12: *M. salmoides;* 13-15: Chipola bass; 16-18: *M. notius;* 20: primer control..

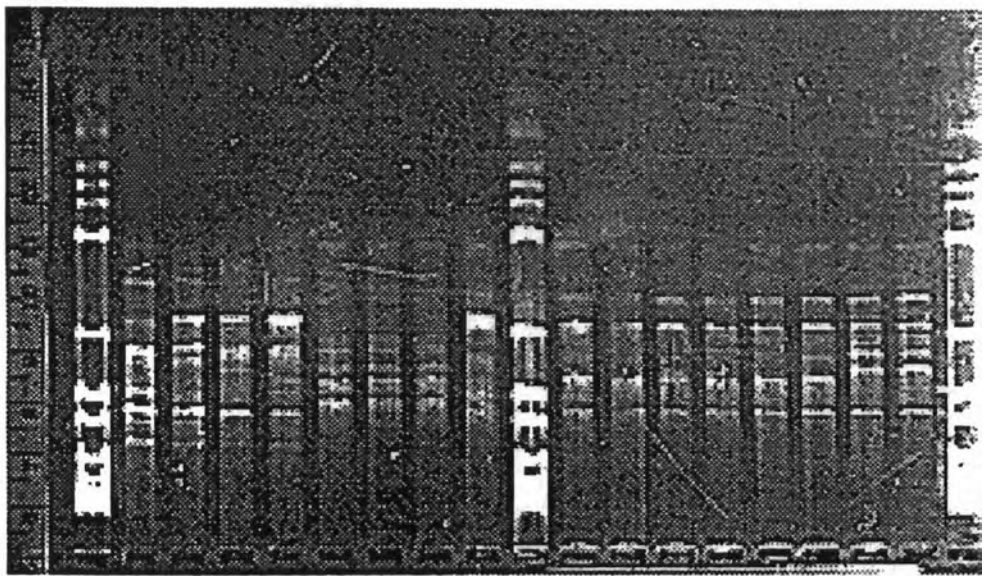

Figure 16. Gel of RAPD products, primer M-16. Lanes: 3: *M. salmoides* (Tickfaw River, La.); 4-6: *M.salmoides* (Atchafalaya Basin, La.); 7-9: *M. notius*; 10: Chipola bass; 12-17: *M. p. henshalli* (AL); 18,19: *M. p. punctulatus* (KY).

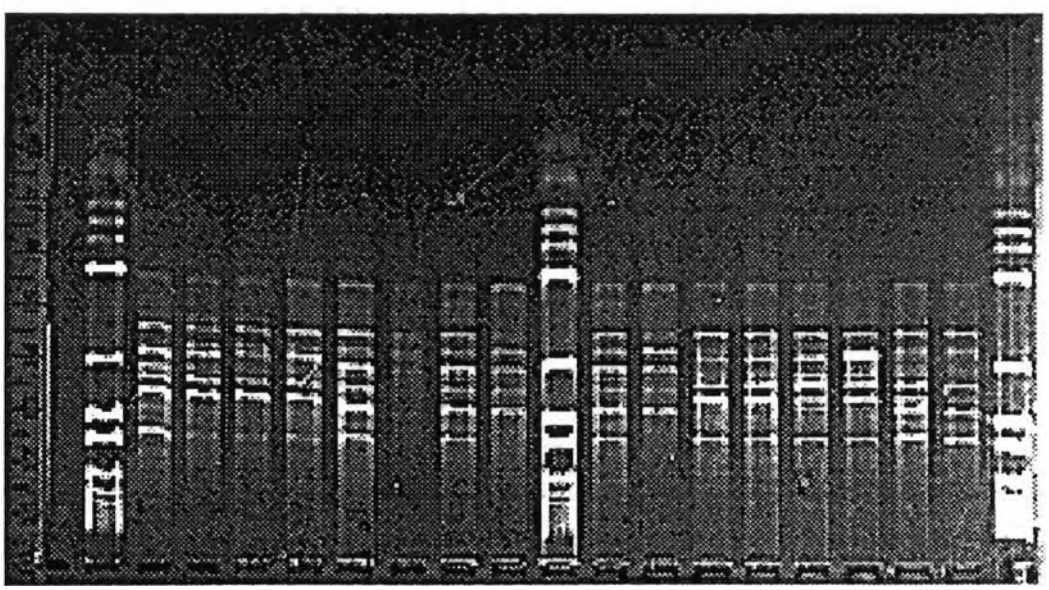

Figure 17. Gel of RAPD products, primer M-16. Lanes 3-6: KY; 7-10, 12-13: TX; 14-19: LW.

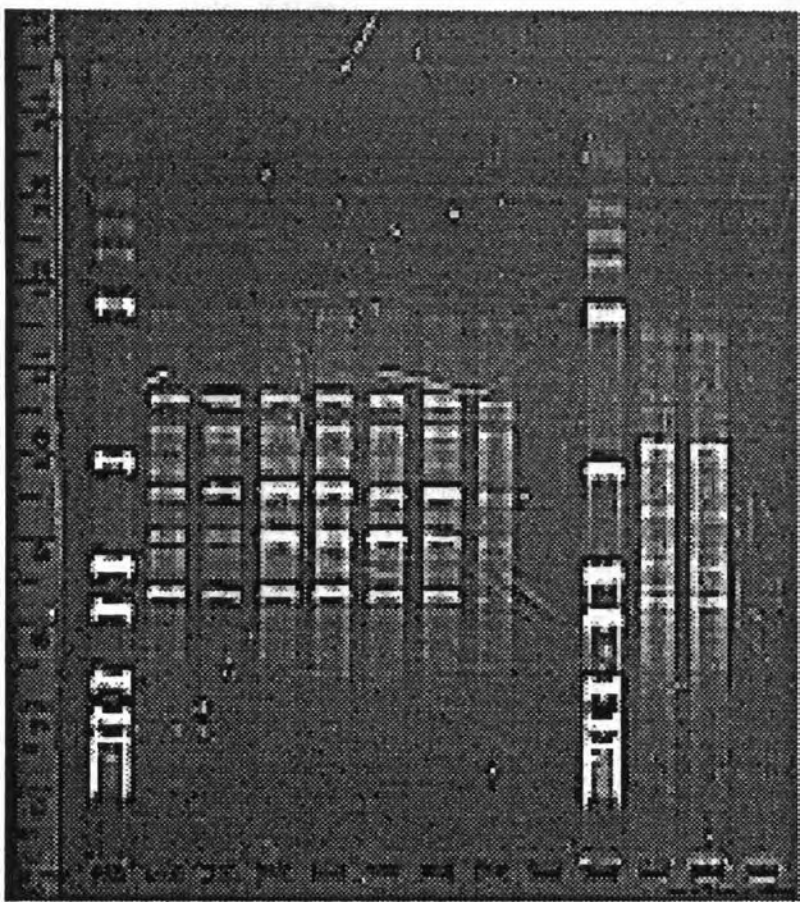

Figure 18. Gel with RAPD products, primer M-16. Lane 3: LW; 4-9: LE; 10: primer control; 12,13: Chipola bass.

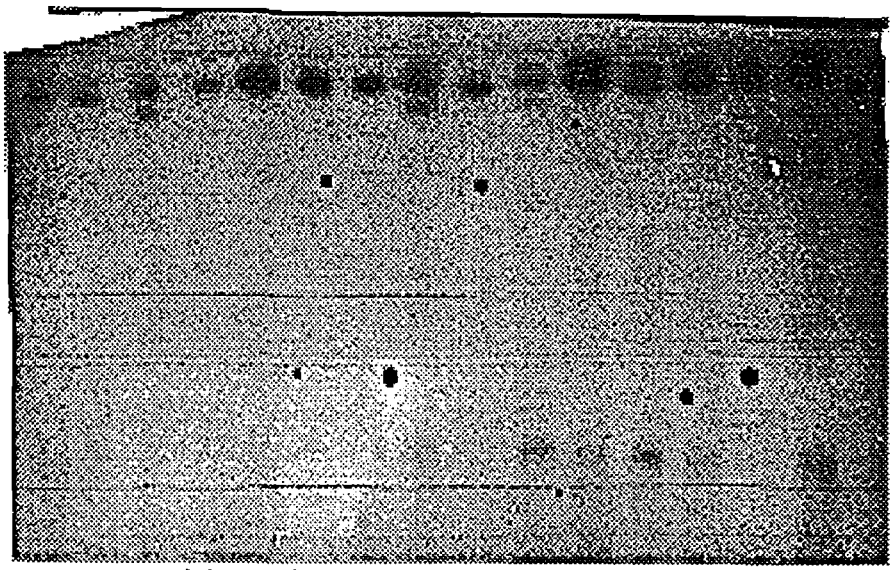

Figure 19. Example of a zymogram stained for mannose-6-phosphate isomerase. Two currrently classified species of spotted basses are included on this gel: *M.treculi*, and two Louisiana populations of *M. p. punctulatus* (LE represents a population from the Tickfaw River, east of the Mississippi River; LW represents a population sampled from the Atchafalaya Basin, west of the Mississippi).

VITA

Belinda Fuller is originally from Shreveport, Louisiana. She received a bachelor's degree in 1975 from the University of Houston, and continued her education at Houston, receiving a master's in biology in 1977. The focus of her research during her master's studies was population genetics involving a grass shrimp found along the Gulf coast. Subsequent to finishing this, she worked in the medical field, in clinical cytogenetics, and in genetics education. With a desire to return to more basic science in a field involving more outdoor work, she accepted a job in Hawaii, and the responsibility of designing and implementing a genetics improvement program for a shrimp farm. After the farm failed, she spent time in Alaska and Seattle, before deciding to return to school to pursue a degree in some aspect of fisheries genetics. This circuitous route has led back to her home state, and in a field very similar to what she started out in, in her early college years. After finishing here, she hopes to work in some area combining natural fisheries and genetics, and human resource management.