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GENETIC VARIATION AND SPECIATION IN NEW WORLD CICHLIDS

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In both the New and Old World, endemism in freshwater fishes of the family Cichlidae presents novel problems in differentiation and speciation (Fryer and Iles, 1972; Trewavas et al., 1972; Greenwood, 1974). In the New World, at least three undescribed forms are endemic to the Cuatro Cienegas Basin, Coahuila, Mexico (Taylor and Minckley, 1966; Minckley, 1969; La Bounty, 1974). The most abundant and widely distributed of the endemic forms possesses very fine pharyngeal teeth and light supporting musculature, and feeds primarily upon algae and detritus. Occurring with less abundance and more limited distribution, is a form characterized by thick heavy pharyngeal teeth used primarily for feeding upon snails. Upon external examination, the two forms are indistinguishable. The distribution and abundance of a third endemic is very restricted. It is presumably predatory in habit, and differs markedly in shape and dentition from the other two types (Taylor and Minckley, 1966; La Bounty, 1974). The occurrence of these endemic fishes within a limited geographic area is reminiscent of certain African lake cichlids (Fryer and Iles, 1972). Although the ancestoral form is not known, a widely distributed species, *Cichlasoma cyanoguttatum* (Baird and Girard) has invaded portions of the Cuatro Cienegas Basin in recent years, and occurs sympatrically with the endemic species in several places. Hybrids between *C. cyanoguttatum* and the two endemics are rare (La Bounty, 1974).

Annual variation of temperature in the aquatic environments of Cuatro Cienegas is extremely low when compared to streams and rivers of arid regions owing to constant circulation from underground thermal springs. Although some ponds are shallow, and possibly subject to the effects of variation in air temperature and seasonal water runoff, most are fairly deep with relatively little surface area.

Temporal measurements of temperature are not available for the lagunas studied here, but data exist for many contiguous or close-by lagunas that demonstrate marked temperature invariability. Twenty-two measurements taken over a ten year period in Laguna West Mojarral ranged between 33.5 and 34.0 C (Arnold, 1972). Maximum observed annual variation at the water surface was 5.0 C. Taylor (1966) provides temperature data for three additional lagunas in Cuatro Cienegas whose annual range of variation is 0 to less than 3.0 C.

Moreover, since we are dealing with a mobile species, should suboptimal environmental conditions prevail at the water surface of marshy edges, fishes will move deeper into the laguna towards the source of the water inflow (this is, in fact, where they are generally observed). *Cichlosoma cyanoguttatum*, by contrast, inhabits extremely variable aquatic environments. Water temperatures in the Rio Grande near Mission, Texas seasonally range from a low of 14.2 C (Hubbs, 1951) to well over 25 C. Periodic flooding conditions dramatically alter other physical characteristics of the water mass such as nutrient content and suspended matter.

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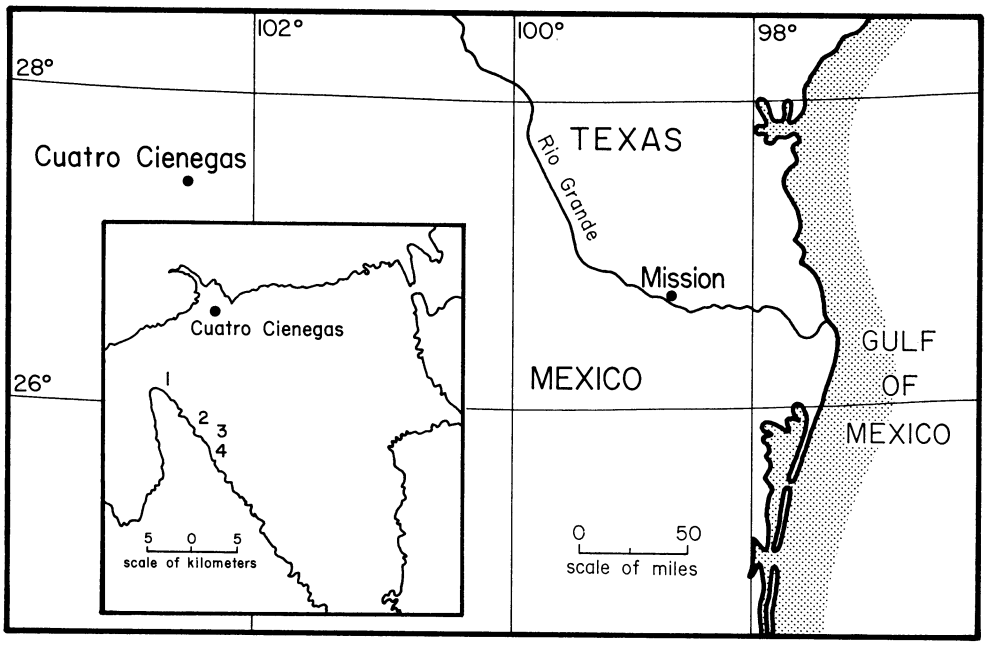

FIG. 1. Sample localities of *Cichlosoma cyanoguttatum* at Mission, Texas, and cichlids within the Cuatro Cienegas Basin (adapted from Minckley, 1969).

Related species, experiencing different degrees of environmental heterogeneity, are important to studies attempting an understanding of selective forces that maintain genetic variation in natural populations. Assuming different adaptations of alleles, environmental heterogeneity (or some component thereof) can act as a factor in the maintenance of genetic variability (Levene, 1953; Levins and MacArthur, 1966; Prout, 1968; Levins, 1968; Bryant, 1974; Gillespie and Langley, 1974; but see Sabath, 1974 and Templeton and Rothman, 1974). Support of this hypothesis derives from laboratory studies of *Drosophila* in which higher average heterozygosity and number of alleles per locus occurred in populations maintained in more heterogeneous environments (Powell, 1971; McDonald and Ayala, 1974). Optimal evolutionary responses to heterogeneity in abiotic factors of an organism's environment were inferred from identical patterns of isoenzyme variation in two sympatric marine bivalves along an environmental gradient (Koehn and Mitton, 1972). More recently, Levinton (1973) demonstrated correlation between allelic diversity at two loci and depth in substrate of several infaunal marine bivalvia. Since general support for the heterogeneity-heterozygosity hypothesis is suggested by these studies, it is compelling to use this cichlid fauna to further test this relationship. It is also of general interest to examine the over-all levels of genetic variability and differentiation among these endemically speciated fishes, particularly as their evolution may resemble that of cichlids in the great African lakes.

MATERIALS AND METHODS

Forty specimens of *C. cyanoguttatum* were collected in October 1971 from the Rio Grande near Mission, Texas, and shipped live to Stony Brook, N.Y. Seventy-nine male specimens of the algae-eating species and thirty-nine males of the snail-eating species were collected by hook and line in July 1971 from three localities within the basin of Cuatro Cienegas (Fig. 1

TABLE 1. *Electrophoretic and staining methods.*

Enzyme	Tissue[1]	Buffer[2] system	Stain reference
Acid Phosphatase (AcPh)	L	TCW	Allen and Weremink (1971)[3]
Aminopeptidase (AP)	L	LiOH	Lewis (1970)
Esterase (EST)	L	LiOH	Shaw and Prasad (1970)[4]
General Protein (GP)	M	LiOH	Amido Black
Glutamate oxaloacetate transaminase (GOT)	L	LiOH	Shaw and Prasad (1970)
Lactate dehydrogenase (LDH)	M	LiOH	Selander et al. (1971)
Malate dehydrogenase (MDH)	M	TCW	Selander et al. (1971)
Phosphoglucomutase (PGM)	L	TCW	Selander et al. (1971)
Tetrazolium oxidase (TO)	L	TB	Wright and Shaw (1969)[5]
Xanthine dehydrogenase (Xdh)	L	TB	Selander et al. (1971)

[1] L = liver, M = muscle.
[2] LiOH: Discontinuous pH 8.4 LiOH buffer of Selander et al. (1971).
TB: Tris-borate EDTA pH 8.7 buffer of Whitt and Horowitz (1970).
TCW: Tris-citrate pH 6.9 buffer of Whitt (1970).
[3] Stain modification: 0.1 M Tris-malate pH 5.0 buffer.
[4] Stain modification: 0.5 M Phosphate pH 7.0 buffer.
[5] Stain modification: 0.1 M Phosphate pH 7.0 buffer. White bands appear when stained for glycerol-3-phosphate dehydrogenase.

and Appendix). External morphology and pharyngeal teeth of the two common Cuatro Cienegas species and the Rio Grande Cichlid are illustrated in Fig. 3. Because sample sizes were very small for females, frequency comparison between sexes was not performed. Only males are considered in all following analyses.

Serum samples were obtained by the method of Koehn (1969). The liver and a small piece of ventral skeletal muscle were excised from each specimen and placed in centrifuge tubes containing an equal volume of deionized water. In field sampling, tubes were cooled on ice, and returned to a local freezer. The tissue samples were later stored at −60 C for up to nine months with no loss of activity.

Tissue samples were prepared for electrophoresis by ultrasonic cell disruption for approximately ten seconds. Homogenates were centrifuged at 0 C for twenty minutes at 15,000g and the supernatant used immediately for electrophoresis.

Separation of liver and muscle isoenzymes was performed by horizontal starch-gel electrophoresis as described by Koehn and Rasmussen (1967). Electrophoretic buffers were identical for all species (see Table 1 for buffer systems and staining references). Starch gels were incubated at 37 C in the appropriate staining solution, photographed, and fixed in a 5:5:1 mixture of methanol, water and acetic acid.

RESULTS

Zymograms for all studied isoenzymes are shown in Figure 2. Sample sizes, number of loci postulated, and gene frequencies are given in Table 2. Enumeration of loci is difficult for multi-banded phenotypes when intraspecific variation and interspecific mobility differentiation do not exist. In such instances, we estimate the number of controlling gene loci in the most conservative manner. Our general conclusions concerning a relationship between genic heterozygosity and environmental heterogeneity are not dependent upon knowledge of the absolute number of loci analyzed. For example, although the three-banded general protein (GP) phenotype could be controlled by three independent gene loci, only one locus is postulated since all three species exhibit identical mobility of bands. Single banded systems are consistent with a single locus (e.g., AP, PGM, TO, and XDH). Occasionally, diffuse

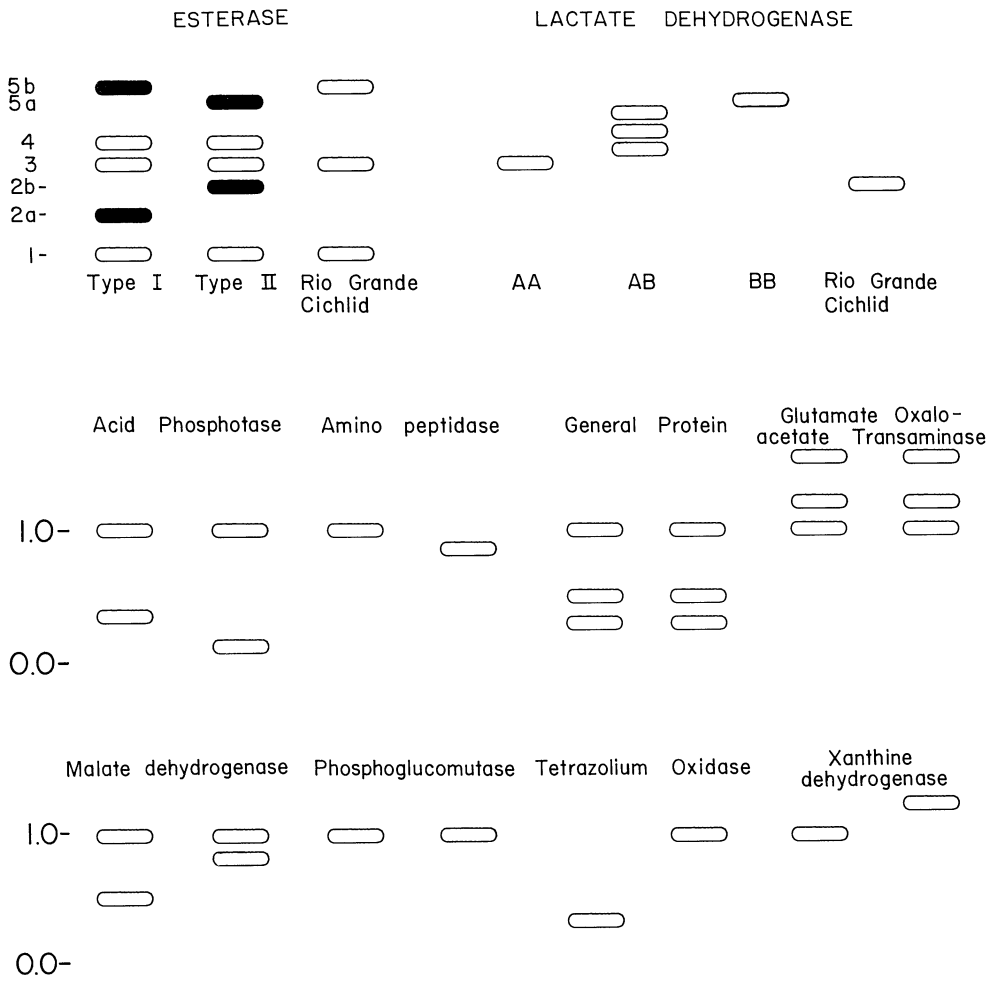

FIG. 2. Zymograms of polymorphic and monomorphic isoenzymes. Polymorphic systems are shown above. Observed electrophoretic phenotypes are shown for 10 monomorphic protein systems of *C. cyanoguttatum* (left) and Mexican cichlids (right). Banding was identical for both Cuatro Cienegas forms. Black esterase bands were heavier bands on the starch gel.

cathodal bands were associated with TO and AP in both endemic species. These were most probably artifactual as presumed dimeric heterozygotes were never observed.

Although malate dehydrogenase (MDH) exists as a conformational dimer in fishes (Markert and Whitt, 1968), a mobility band characteristic of the heterodimer was not observed in our material. However, since mobility of the cathodal band was interspecifically divergent, and both bands consistently exhibited equal staining intensity, two loci were postulated for this enzyme.

Variation at the lactate dehydrogenase (LDH) locus most probably involves two alternate alleles. Although a five-banded heterozygote would be expected for a functionally tetrameric isoenzyme, usually only three, and infrequently four bands were observed for this enzyme (Fig. 2). In view of restricted subunit assembly (Whitt and Horowitz, 1970), a smaller number of bands is commonly observed in fishes.

TABLE 2. *Sample sizes (N) and gene frequencies for two species of endemic cichlids from localities within the Cuatro Cienegas basin and for* Cichasoma cyanoguttatum *from Mission, Texas. Locality designations are listed in the appendix.*

Locus \ Locality	Endemic detritus feeder			Endemic snail feeder		*C. cyanoguttatum*
	1 (16)	2 (40)	3 (23)	1 (17)	2 (22)	Texas (38)
AcPh-1	1.00	1.00	1.00	1.00	1.00	1.00
AcPh-2	1.00	1.00	1.00	1.00	1.00	1.00
AP	1.00	1.00	1.00	1.00	1.00	1.00
EST-1	.200 ± .071	.200 ± .044	.304 ± .067	.294 ± .078	.157 ± .055	1.00
EST-2	1.00	1.00	1.00	1.00	1.00	1.00
GP	1.00	1.00	1.00	1.00	1.00	1.00
GOT	1.00	1.00	1.00	1.00	1.00	1.00
LDH	.937 ± .042	.925 ± .029	.978 ± .030	.941 ± .040	.931 ± .038	1.00
MDH-1	1.00	1.00	1.00	1.00	1.00	1.00
MDH-2	1.00	1.00	1.00	1.00	1.00	1.00
PGM	1.00	1.00	1.00	1.00	1.00	1.00
TO	1.00	1.00	1.00	1.00	1.00	1.00
XDH	1.00	1.00	1.00	1.00	1.00	1.00

LDH varied in a manner consistent with a simple genetic model and all samples (Table 2) corresponded to Hardy-Weinberg expectations.

The esterase patterns of liver tissues could be due to a maximum of five loci, but consistent with conservative estimation, two loci are postulated. The esterase variation, though most probably genetic, did not conform to any simple pattern (Fig. 2). At this variable locus, the frequency of the most common phenotype was considered to be the homozygote.

The electrophoretic phenotypes at all studied loci were identical between the two endemics. Allele frequencies at the two variable loci (LDH, EST-1) were homogeneous among samples and individuals were pooled among localities for variability estimates. All individuals ($N = 39$) of *C. cyanoguttatum* were invariant for every locus and were therefore considered monomorphic. The average percentage of loci heterozygous per individual was calculated by assuming Hardy-Weinberg equilibrium (see Lewontin and Hubby, 1966). Average heterozygosity per individual was 3.63 in the endemic detritus feeder ($N = 79$), 3.43 in the endemic snail eater ($N = 39$), and 0.00 in *C. cyanoguttatum*. Since the number of loci is estimated conservatively, all heterozygosity estimates are maximal.

GENETIC VARIATION

Heterozygosity estimates for the two species of endemic cichlids are low relative to those for other fishes (Frydenberg and Simønsen, 1973; Avise and Smith, 1974) and vertebrates in general (Selander and Johnson, 1973).

The relatively homozygous genome of the Cuatro Cienegas species is consistent with our *a priori* expectations of organisms evolving in a fairly constant environment. Since *C. cyanoguttatum* inhabits a highly variable environment, particularly when compared to the Cuatro Cienegas lagunas but is by our estimate genetically monomorphic, support is denied for the environmental heterogeneity strategy suggested by recent studies of other organisms (Powell, 1971; Levinton, 1973; McDonald and Ayala, 1974). These results are like those of Gooch and Schopf (1972) and Somero and Soulé (1974) who demonstrated negative correlation between genetic variation of deep-sea organisms and environmental heterogeneity.

A variety of possible explanations might be proposed to explain the low hetero-

zygosity estimates in these species, but all are severely inadequate. All proposals must be made within the context of the considerable morphological diversity among the studied forms, and it is here that tenable explanations for the monomorphism are difficult. A typical Neo-Darwinian scenario of the evolution of these cichlids would involve the evolution of endemic forms from an ancestral species possessing moderate genic heterozygosity. If loci characterized in this study are indicative of largely homozygous genomes of existing species, then evolutionary events leading to homozygosity must have occurred after evolutionary divergence among the morphotypes. The Cuatro Cienegas forms conform nicely to this pattern in that each may have been exposed to constant and equivalent selection in the relatively invariant habitat of the desert lagunas. The homozygosity of *C. cyanoguttatum* is not, however, amenable to interpretation under this rationale, and we must therefore seek alternative explanations.

Many isoenzyme studies have described variable, but unexpectedly high, levels of genetic polymorphism for organisms inhabiting generally variable environments (see Selander et al., 1970 and Selander and Johnson, 1972, for general reviews). A few authors have reported extremely low levels of genetic polymorphism in some natural populations (Johnson and Selander, 1971; Serov, 1972; Webster et al., 1972). In low-variability populations, small effective population size may have led, either presently or in the past, to sampling errors such as genetic drift. For example, the low levels of observed variation in cave populations of the teleost *Astyanax* are probably due to drift (Avise and Selander, 1972). It is possible that many recent low variability observations are the results of historical sampling accidents (see Webster et al., 1972 for general discussion).

In the Cuatro Cienegas forms, population sizes are of an order where stochastic events could conceivably play a role. The lagunas they inhabit are generally small in size and unable to support large populations. From numerous underwater observations, it is our subjective impression that a thousand individuals would be a generous estimate of the inhabitants of an average laguna. However, stochastic processes cannot reasonably explain the observed *pattern* of homozygosity. If random drift within isolated subpopulations eroded variation during the differentiation of the two endemic cichlids, the presence of interspecific differences should have been noted. Assuming initial variability, if genetic drift were responsible for the observed monomorphism at most loci, it is improbable that both species would exhibit *identical* alleles at all loci.

Although population sizes of *C. cyanoguttatum* would appear to be quite large, thirty-eight specimens were tested and no empirical data on abundance or population structuring are available. It should be noted, however, that the specimens electrophoretically tested constitute a sample of fishes collected over a twenty mile range along the Rio Grande. If, on the other hand, we have gross misconceptions of the effective population sizes of *C. cyanoguttatum*, genetic drift might be an important evolutionary force in this species.

A final point must be considered as a tenable explanation for the observed patterns of homozygosity and is congruous with the evolutionary divergence that has occurred among these cichlids. It has been commonly assumed in studies of intraspecific genetic variability and interspecific genetic differentiation, that isoenzyme loci adequately reflect genetic events characteristic of the entire genome. However, loci studied here reflect neither patterns of environmental variability nor the morphological differentiation among the Cuatro Cienegas cichlids.

Interspecific Differentiation

Electrophoretic studies on genetic reorganization following speciation have been much debated (see Selander et al., 1969; Nei, 1971 for general discussion), but it

is generally felt that this technique provides a valid reflection of genetic differences between species (e.g., Hubby and Throckmorton, 1965, 1968; Selander et al., 1969; Johnson and Selander, 1971; Rockwood et al., 1971; Turner, 1972; Webster et al., 1972). Studies of genetic similarity between subspecies and sibling species have indicated an association between overall electrophoretic similarity and level of evolutionary divergence (Hubby and Throckmorton, 1965, 1968; Rockwood et al., 1971; Kornfield, 1976). The estimated genetic identity between *C. cyanoguttatum* and the Cuatro Cienegas cichlids (Coefficient of Jacqard = 0.44; Sneath and Sokal, 1973) is well within the range reported for sibling pairs. The virtual electrophoretic identity between the endemic algae-eating and snail-eating cichlids stands in sharp contrast to previous species comparisons and therefore leads one to question the specific status of these two morphotypes.

An analogous taxonomic problem in cichlids from Lake Victoria caused us to carefully re-examine all evidence bearing on the question of specific designation. Greenwood (1959) described two subspecies of the African cichlid *Astatoreochromis alluaudi*. *A. a. alluaudi* is restricted to Lake Victoria and possesses an enlarged apophysis and massive pharyngeal teeth adapted to crushing gastropods. *A. a. occidentalis* occurs in lakes Albert and Edward and exhibits fairly fine pharyngeal teeth with a smaller apophysis. In a later study however, Greenwood (1965*b*) found that lab reared *A. a. alluaudi* fed exclusively soft diets developed dentition typical of *A. a. occidentalis*. In withdrawing the subspecific designation, Greenwood (1965*b*) concluded that the development of massive pharyngeal teeth and apophysis was dependent in part upon crushing action initiated at early ages. Laboratory reared specimens of the Mexican snail-eating endemic raised on soft food and specimens of the algae-eating endemic provided with snails however did not change in morphology (La Bounty, 1974). Additionally, unlike *A. alluaudi*, the *number* of teeth per pharyngeal bone is markedly different in the two endemic Mexican cichlids (see Fig. 3).

There are several lines of evidence supporting the specific status of the two Cuatro Cienegas endemics. First, in no-choice laboratory mating experiments, hybrids have not been observed between species, though progeny are produced in conspecific crosses (La Bounty, pers. comm.). Examination of over 2500 specimens of cichlids from Cuatro Cienegas revealed only 18 individuals with intermediate dentition (La Bounty, 1974). Fish hybrids typically exhibit intermediate morphology (Hubbs, 1955). Second, the cranial morphology of the two forms is differentiated in accordance with observed pharyngeal dentition. Differences in the head involve modification of the base of the neurocranial surfaces for muscle attachment and mill-skull articulation, modification of the anteriormost vertebrae, sizes and shapes of pharyngeal bones (Fig. 3) and the numbers of teeth (La Bounty, 1974). The length of the intestine of the algae-eating form is between two and four times greater than that of the snail-eating form. Additional morphological characters (as length of pectoral rays), though not significantly different between forms, are suggestive of morphological differentiation.

Third, in *all* the major African cichlid flocks, congeneric species exhibit patterns of morphological differentiation identical to that seen here.

It is, quite obviously, impossible to establish unequivocably by inferential argument the specific status of these two morphotypes, however persuasive the data may be. By current standards of fish taxonomy, these forms are dramatically different in morphology. The alternate hypothesis, that we are dealing with a single species polymorphic in genes determining the complex morphology of the entire head of these fishes, seems to us unlikely and without precedent.

It has been commonly assumed in studies

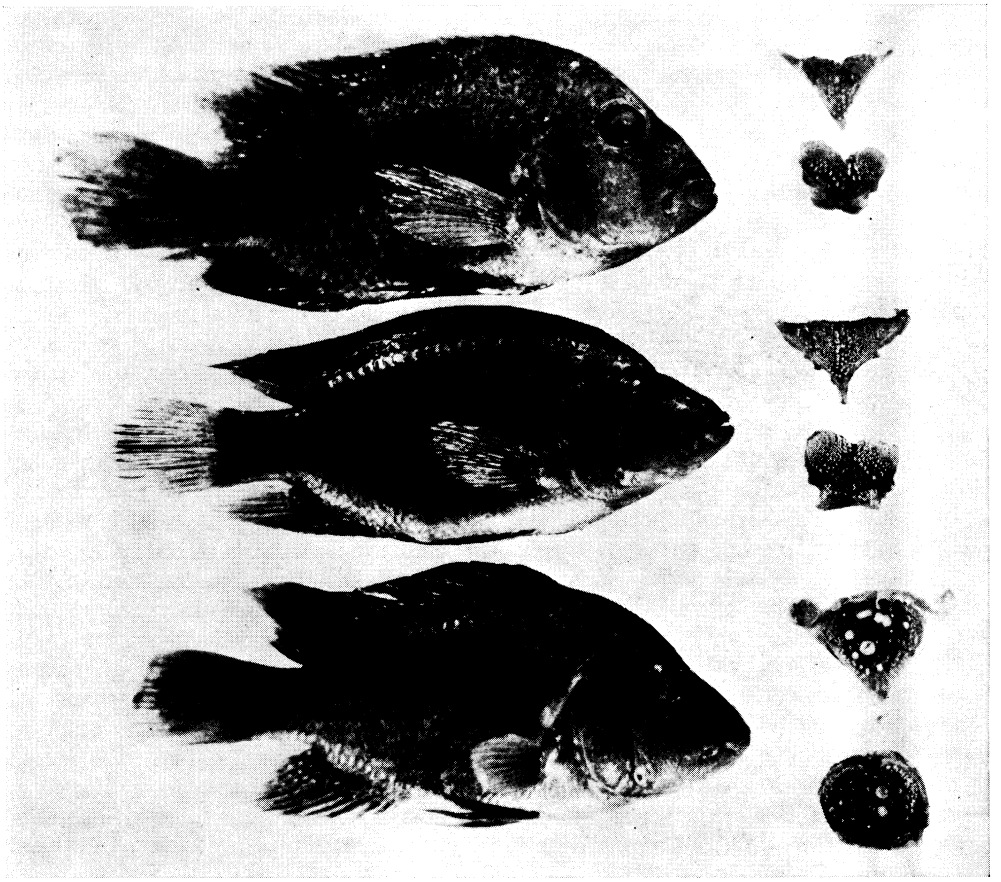

FIG. 3. External morphology and pharyngeal bones of *Cichlasoma cyanoguttatum* (top), endemic algae-feeding Cuatro Cienegas cichlid (middle), endemic snail-feeding Cuatro Cienegas cichlid (bottom). Infrapharyngeal (below) and superpharyngeal bones (above) are at the right of each form.

of intraspecific genetic variability and inter-specific genetic differentiation, that isoenzyme loci adequately reflect genetic events characteristic of the entire genome. The loci studied here, however, do not reflect the genic differentiation that must have accompanied speciation of the Cuatro Cienegas cichlids. In other words, speciation in this case apparently did not involve random genetic reorganization, but rather reorganization of some restricted portion(s) of the genome (for example probably those concerned with mating behavior).

The low genic heterozygosity of these cichlid species, covering a broad range of patterns of environmental heterogeneity and population sizes, is extraordinarily perplexing in view of the many correlations with genetic heterozygosity reported by other investigators in a diversity of organisms. Whatever the cause of this dilemma, these data indicate that isoenzyme loci may not always accurately reflect evolutionary events involving the total genome.

SUMMARY

Genetic variability was examined in two endemic cichlid fishes from Cuatro Cienegas, Coahuila, Mexico, and in *Cichlasoma cyanoguttatum* from the Rio Grande in Texas. The Mexican cichlids exhibit

morphological divergence, endemism, and sympatry that is strikingly similar to that observed in cichlids of the African lake species-flocks.

The endemic species occur in restricted waters that are relatively thermally constant. *C. cyanoguttatum* is widespread and can be regarded as occurring in a relatively variable environment. A comparison of the levels of genetic variability among species was proposed as a test of the generally recognized relationship between environmental heterogeneity and genetic variability.

A minimum of thirteen electrophoretic loci were studied in all species. *C. cyanoguttatum* was monomorphic at all loci in a sample of 38 individuals. The endemic detritus-eating cichlid ($N = 79$) and endemic snail-eating cichlid ($N = 39$) possessed 3.63 and 3.43% heterozygous loci per individual, respectively. These levels of heterozygosity are low relative to other vertebrates, and are not consistent with interpretations of inbreeding or founder effects. Moreover, the occurrence of monomorphism in a 'variable' environment causes us to question the generality of a relation between environmental heterogeneity and genetic variability.

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APPENDIX

Description of sampling localities in Mexico—Numbers refer to locations on Figure 1. Sample sizes are in parentheses.

1. El Mojarral East. Irregular border. Approximately 50–70 meters long, 15–20 meters wide. Average depth 0.3–0.6 meters. Sampling done on northwest corner ($N = 32$). 2. Pond approximately ¾ kilometer northeast of Rancho Orozco. Almost circular in outline, diameter approximately 25 meters. Maximum depth 5 meters. A small stream flows out the southeast corner ($N = 90$). 3. Pond approximately ½ kilometer northeast of Laguna Tio Candido. There was a series of small ponds in this area roughly in a line. This is the first pond encountered coming from Tio Candido. Roughly circular in outline, the diameter is approximately 5 meters. The pond is drained by a small channel on the north bank ($N = 29$). 4. Pond approximately 3 kilometers southeast down road from Rancho Orozco, then northeast approximately ½ kilometer. A very small pond approximately 4 meters diameter. Depth less than one meter ($N = 7$).