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Allozymes encoded by 30 presumptive gene loci were used in phylogenetic analyses of the *Cyprinodon eximius* complex (Cyprinodontidae), a group of 12 species occurring primarily on the Mexican Plateau from the states of Zacatecas and Nuevo Leon north to the Rio Grande. The most parsimonious phylogenetic tree supported the following: (1) the *C. eximius* complex is paraphyletic, with a clade comprising *C. alvarezi* and *C. ceciliae* from the Potosí and Sandia basins being basal relative to the pupfishes examined (including five species from outside the *C. eximius* complex); (2) an early event separated the Río Conchos-Rio Grande-Old Río Nazas pupfishes from a previously identified clade of eight species (the "western pupfishes") that extends from the Guzmán Basin in northwestern Chihuahua to the Death Valley system of California; (3) the ancestral *C. meeki*, a species from the Pacific slope, was isolated from pupfishes in the Old Río Nazas by a Pleistocene stream capture; (4) *C. nazas*, a species from the headwaters of the Old Río Nazas is paraphyletic with respect to *C. atrorus*, one of two species from Cuatro Ciénegas (this supports one of the three suggested outlets of the Old Río Nazas to the Rio Grande, i.e., the Río Salado); (5) a clade of four species occupies the Río Conchos drainage in Mexico and contiguous waters of the Rio Grande drainage (this clade includes the relatively wide-ranging *C. eximius* and three local endemics from spring systems in Chihuahua, *C. macrolepis* and *C. pachycephalus*, and the Pecos River in Texas, *C. elegans*); and (6) the two pupfishes from Cuatro Ciénegas, *C. atrorus* and *C. bifasciatus*, represent divergent lineages, the latter apparently being a relatively early product of pupfish evolution on the Mexican Plateau.

ABOUT 20 species of *Cyprinodon* (Atherinomorpha: Cyprinodontidae) are endemic to remnants of more extensive, Pleistocene bodies of water in an area now dominated by the expansive Chihuahuan Desert of northcentral Mexico, southern New Mexico, and southwestern Texas (Miller, 1981; Lozano and Contreras, 1993). Twelve of these species belong to the *C. eximius* complex, a group of mostly allopatrically distributed, local endemics in springs and rivers from Zacatecas and Nuevo Leon in the south to the Rio Grande drainage of Texas in the north (Miller, 1976; Minckley and Minckley, 1986; Lozano and Contreras, 1993). Miller (1976) tentatively defined the complex with a combination of morphological traits, but none was individually diagnostic, and the monophyly of the group has not been tested. Our purpose was to use allozymes in a phylogenetic analysis of the *C. eximius* complex and to interpret results against the background of information on paleohydrology of surface waters in the region.

Miller (1976) originally included seven species in the *C. eximius* complex: (1) the relatively wide-ranging *C. eximius* from the Rio Grande Basin and the small, endorheic Río Sauz Basin in Chihuahua; (2) *C. macrolepis* from a spring-fed system in the Río Conchos drainage, Chihuahua; (3) *C. atrorus* in the Cuatro Ciénegas Basin, Coahuila; (4) *C. alvarezi* in an isolated spring in the Potosí Basin, Nuevo León; (5) *C. nazas* from endorheic drainages in the states of Coahuila, Durango, and Zacatecas; (6) *C. meeki* from a Pacific slope drainage, the upper Río Mezquital system in Durango; and (7) *C. latifasciatus* (extinct since at least the early 1950s; Miller, 1964) from the endorheic Parras Basin in Coahuila. Subsequently, an eighth member of the complex, *C. pachycephalus*, was described from a thermal spring in the Río Cónchos drainage (Minckley and Minckley, 1986), and Lozano and Contreras (1993) added four more species (*C. ceciliae, C. inmemoriam, C. longidorsalis*, and *C. veronicae*) from the Sandia Basin, a small, endorheic drainage in Nuevo Leon. Finally, an "apparently undescribed" species resembling *C. eximius* occurs in Ojo de Julimes, a spring in the Río Conchos drainage in Chihuahua (Contreras, 1991:193). For this study, we provisionally considered this form to be a population of *C. eximius*.

Smith and Miller (1980) suggested that *C. fontinalis*, from the Guzmán Basin in northwestern Chihuahua, and *C. macrolepis*, a member of the *C. eximius* complex, are closest relatives. However, analysis of allozymes and mitochondrial

(mt) DNA supported placement of *C. fontinalis* and two other species (both undescribed) from the Guzmán Basin in a group of eight genetically similar species (the "western pupfish" clade) that ranges from the Guzmán Basin west into Sonora and north to the Death Valley system of California (Echelle and Dowling, 1992; Echelle and Echelle, 1993a).

The overall view of the evolution of the *C. eximius* complex is that Pleistocene changes in surface hydrology of an ancestral Rio Grande system (Smith and Miller, 1986) allowed divergence from *C. eximius*, the wide-ranging member of the complex (Miller, 1968, 1981; Minckley and Minckley, 1986). Only one other species of pupfish, *C. bifasciatus*, occurs within the geographic range of the complex. This species, which is sympatric with *C. atrorus* in the Cuatro Ciénegas Basin (Miller, 1968), is one of the most distinctive members of the genus, and, largely on this basis, Miller (1968, 1981) suggested that it might have originated prior to most other species of *Cyprinodon* in the region.

Materials and Methods

We examined a total of 19 samples representing 14 species (Fig. 1). For the *C. eximius* complex, we examined multiple samples of the two relatively wide-ranging species, *C. eximius* [four samples, including one from Ojo de Julimes that may represent an undescribed species (Contreras, 1991) and *C. nazas* (three samples)], and single samples from each of six species restricted to single spring-systems (*C. alvarezi, C. ceciliae, C. macrolepis,* and *C. pachycephalus*) or relatively small areas (*C. atrorus* and *C. meeki*). These samples included all extant members of the complex except the two surviving members of the Sandia Basin group (*C. longidorsalis* and *C. veronicae*). Overpumping of groundwater has depleted or eliminated springflows in the basin, and, as a result, all four endemic species were extirpated from the wild in the past 15 years (Lozano and Contreras, 1993; L. Lozano, pers. comm.); *C. longidorsalis* and *C. veronicae* are being maintained as captive aquarium stocks (Echelle et al, 1995; L. Lozano and S. Contreras, pers. comm.). To represent this monophyletic group (Echelle et al., 1995), we used a sample of *C. ceciliae* collected before extinction of the species.

To test monophyly of the *C. eximius* complex, we examined five additional species of *Cyprinodon* chosen to represent the diversity of *Cyprinodon* in North America. These included two of the most genetically divergent representatives (*C. macularius* and *C. nevadensis*) of the western

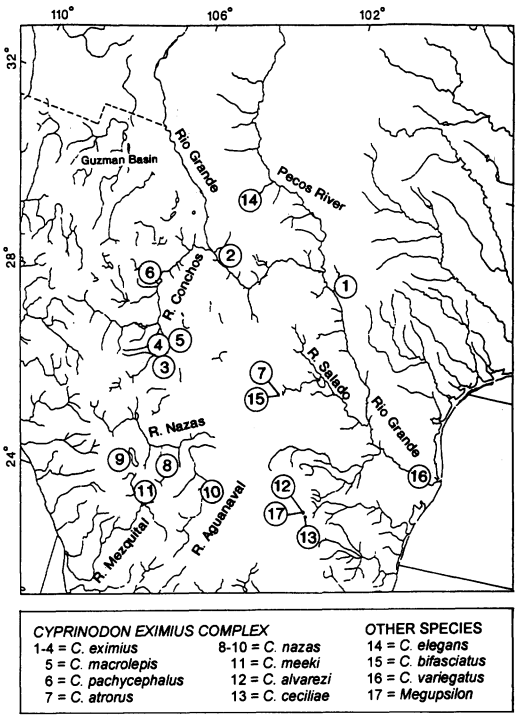

Fig. 1. Distribution of samples and drainages. Basins not labeled are Cuatro Ciénegas (site numbers 7 and 15), Laguna Santiaguillo (9), Potosí Basin (12 and 17), and Sandia Basin(13). Samples not represented are those for two species (*Cyprinodon nevadensis* and *C. macularius*) from outside the mapped area (see Material Examined).

pupfish clade (Echelle and Dowling, 1992; Echelle and Echelle, 1993a), one representative (*C. variegatus*) of a clade of species comprising *C. variegatus* and four genetically similar species from New Mexico, Texas, and Oklahoma (Echelle and Echelle, 1992), and two morphologically divergent species whose relationships have been considered particularly problematic (Miller, 1981); one of the latter pair (*C. bifasciatus*) occurs within the geographic range of the *C. eximius* complex, and the other (*C. elegans*) occurs in contiguous waters. As the outgroup for the phylogenetic analysis, we used *Megupsilon aporus* from a captive population maintained at the Universidad Autonoma de Nuevo Leon. A sister-group relationship between *Cyprinodon* and the monotypic genus *Megupsilon* is supported by allozymes and mtDNA data (Echelle and Echelle, 1993b; Parker and Kornfield, 1995).

Allozyme assays were performed on 10–26 specimens from each sample. Extracts of water-soluble proteins were obtained separately from liver, epaxial muscle, and combinations of eye and brain and stored at −60 C. Standard meth-

ods of horizontal starch-gel electrophoresis (Murphy et al., 1996) were used to examine products of 30 presumptive gene loci (Appendix 1). Locus nomenclature followed Buth (1983). Allele designations (Appendix 2) apply only to the present study. We used BIOSYS-1 (Swofford and Selander, 1981) to compute average heterozygosity per individual from allele frequencies and to perform an exact test, with Levene's correction for small samples, to determine whether genotypic frequencies were in agreement with Hardy-Weinberg expectations. Alpha level for these tests was adjusted by the sequential Bonferroni correction for a Type I error of 0.05 or less (Rice, 1989).

For phylogenetic analysis, we used an approach that resembles the stepmatrix method recommended by Mabee and Humphries (1993) but is based on allele frequencies rather than presence/absence of alleles (Berlocher and Swofford, 1997). Rogers (1984) and Swofford et al. (1996) effectively argued that allele frequencies are preferable to presence/absence information when estimating phylogenetic pattern from allozymes. In our analysis, the BIOSYS-1 datafile was converted to the format for FREQPARS, a program for analysis of allele frequency parsimony (Swofford and Berlocher, 1987), and imported into PAUP* (vers. 4d60; Swofford, 1991, unpubl.). PAUP* produced a matrix of pairwise Manhattan distances (MANOB metric; Swofford and Berlocher, 1987) and the associated distance-based stepmatrix. This stepmatrix was then subjected to the heuristic search, generalized parsimony algorithm in PAUP*, with *Megupsilon* as outgroup. We saved the 20 shortest trees derived with the simple addition-sequence option and used FREQPARS to test each one for allele-frequency parsimony. With FREQPARS, total tree length is the sum of branch lengths expressed in units of a Manhattan distance metric (MANAD) that is similar to MANOB but is constrained such that allele frequencies of hypothetical ancestors sum to 1.0. To assess efficiency of the simple addition-sequence option in obtaining the shortest MANOB trees, we also performed 100 replications of the heuristic search with the random addition-sequence option; the two methods gave identical results for the shortest MANOB tree. Rearrangements of the shortest MANAD tree were input into FREQPARS to examine alternative hypotheses of relationship.

RESULTS

Levels of heterozygosity ranged from zero in *C. eximius* from Devils River to 0.091 in *C. ceciliae* (Appendix 2). The captive population of *Megupsilon aporus* had a heterozygosity of 0.034, indicating that it retained at least some of the genetic diversity of the extinct wild population. Within *Cyprinodon*, two or more alleles were detected at 23 of the 30 gene loci examined; variation at two loci consisted only of autapomorphic alleles, leaving 21 phylogenetically informative loci. None of the tests of deviation from Hardy-Weinberg expectations indicated statistical significance.

MANAD lengths ranged from 158.0–158.9 for the 20 shortest trees detected in the heuristic search with PAUP. Most internal nodes of the shortest tree (Fig. 2) are supported by at least one synapomorphic allele. Such nodes were assigned capital letters (Fig. 2); the corresponding allele distributions are described in Appendix 3. The remaining nodes are supported only by frequencies of alleles that also occur in other clades. Collapsing Figure 2 to retain only the lettered nodes produced a tree that was identical to the strict consensus of the 20 shortest MANOB trees except that the latter retained the node separating *C. macrolepis* from *C. eximius*.

The results indicate paraphyly for the *C. eximius* complex. Two species from the complex (*C. alvarezi* and *C. ceciliae* from the closely associated Potosí and Sandia basins, respectively) fell outside of a large clade that contained the remaining members of the *C. eximius* complex and five other species. To further examine placement of the *C. alvarezi/C. ceciliae* clade, we computed FREQPARS length for a tree in which the *C. eximius* complex (plus *C. elegans*) was monophyletic with a *C. alvarezi/C. ceciliae* basal clade. Except for placement of *C. alvarezi/C. ceciliae*, the topology was identical to Figure 2. This resulted in notably increased tree length (164.1), as did moving the *C. alvarezi/C. ceciliae* clade to a basal position relative to all species except *C. variegatus* and *C. bifasciatus* (162.7).

The FREQPARS topology (Fig. 2) supports the following conclusions from previous phylogenetic studies of *Cyprinodon*: (1) species from the closely associated Sandia and Potosí basins (*C. ceciliae* and *C. alvarezi* in this analysis) represent a monophyletic group (Echelle et al., 1995); (2) *C. macularius* and *C. nevadensis*, two representatives of a previously recognized clade of eight "western species" (Echelle and Dowling, 1992; Echelle and Echelle, 1993a), also form a clade in our analysis; and (3) a previously suggested (Echelle and Echelle, 1992) close relationship between *C. elegans* and *C. eximius* is supported by inclusion of these two spe-

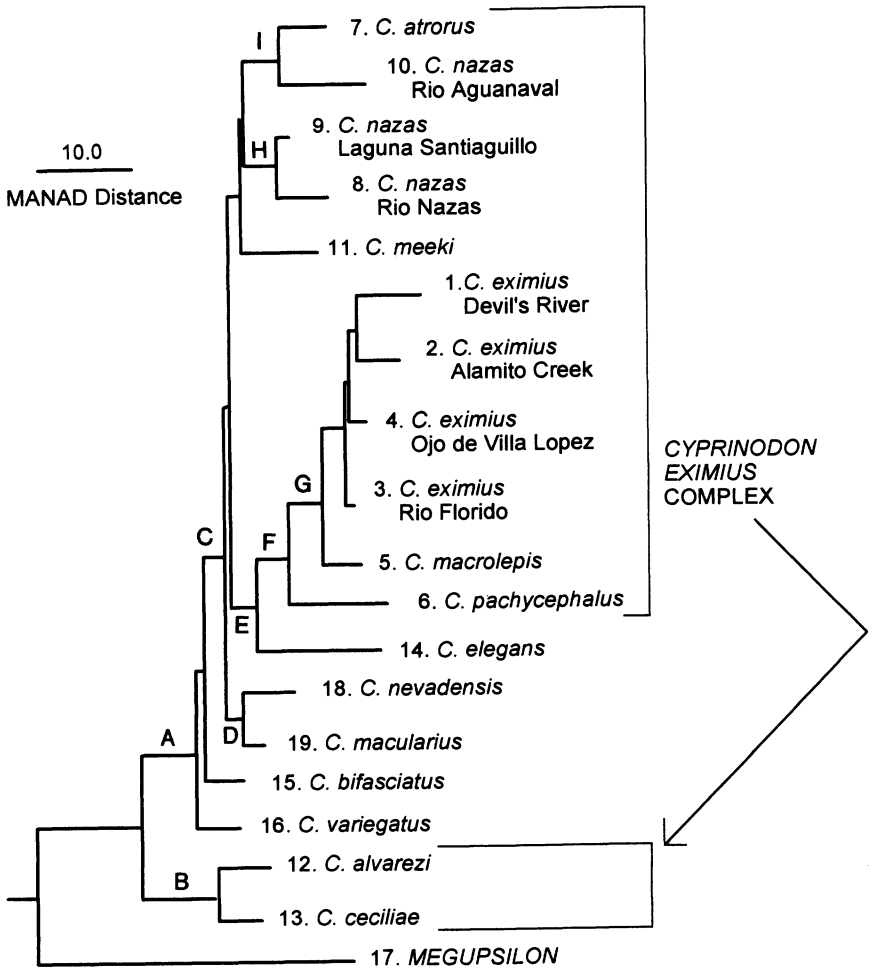

Fig. 2. The shortest FREQPARS tree for the samples examined. Numbers correspond to those given in Figure 1. Letters on the tree signify nodes supported by at least one synapomorphic allele; remaining nodes supported only by frequencies of alleles that also occurred in other clades (see text and Appendix 1). Note paraphyly for *Cyprinodon nazas*; on this basis, the Río Aguanaval pupfish is treated as a separate species (*C.* sp.) in Results and Discussion.

cies in a small clade that excluded all other species except *C. macrolepis* and *C. pachycephalus*.

Allele frequencies provided support for monophyly of *C. eximius*, one of the two relatively wide-ranging species we examined (Fig. 2). However, there was no synapomorpic allele that united two or more samples of *C. eximius*. Allele sharing of *C. eximius* with *C. macrolepis* was particularly striking; there was no fixed difference between these two species, whereas all other species pairs in our analysis exhibited at least one fixed allele difference.

The other wide-ranging species, *C. nazas*, appears paraphyletic (Fig. 2), with the Río Aguanaval population being more closely related to *C. atrorus* than to the other two populations of *C. nazas* (Río Nazas and Laguna Santiaguillo). To further assess this relationship, we derived the MANAD length of a tree identical to Figure 2, except that *C. nazas* was monophyletic with a basal Río Aguanaval population. The resulting tree was notably longer than the shortest MANAD tree (160.8 vs 158.0). Correspondingly, four loci (Fbp-C, G3pdh-A, Mpi-A, and Pep-D) were fixed for different alleles between the Río Aguanaval pupfish (*C.* sp. hereafter) and the Río Nazas/Laguna Santiaguillo samples of *C. nazas*.

Discussion

Beginning with Meek (1904), and based primarily on fish distributions and surface topography, various workers have postulated that most of the isolated surface waters of the Chi-

huahuan Desert region and adjacent areas of northcentral Mexico were parts of the ancestral Rio Grande system during late Pliocene to early Pleistocene (Conant, 1963; Miller, 1981; Smith and Miller, 1986). Correspondingly, phylogenetic analyses have revealed two cyprinid clades that are endemic to remnant waters of the ancestral Rio Grande: one includes seven species of the *Dionda episcopa* complex (Mayden et al., 1992), whereas the other includes five to six species in the *Cyprinella lepida* complex (Mayden, 1989; Contreras and Lozano, 1994). Our analysis indicates the possibility of a clade of eight species of *Cyprinodon* that is endemic to the ancestral Rio Grande system. This clade includes four species from the present Rio Grande drainage (*C. elegans, C. eximius, C. macrolepis,* and *C. pachycephalus*) and four species from the now-isolated waters of the ancestral system (*C. meeki, C. nazas,* and *C.* sp. in endorheic streams draining the western highlands region of Durango and Zacatecas, and *C. atrorus,* one of the two species from the Cuatro Ciénegas Basin). Support for this clade needs further corroboration (Fig. 2). It is worth noting, however, that the indicated sister-group relationship between the two clades is congruent with Mayden's (1989) hypothesis from morphology for the *C. lepida* complex. The most-parsimonious trees from both analyses support an early vicariant event separating the Guzmán Basin fauna from that of the remainder of the ancestral Rio Grande system in north-central Mexico and Texas. Although we did not survey samples from the Guzmán Basin, *C. macularius* and *C. nevadensis* are divergent members of the western pupfishes, a clade that includes the three Guzmán Basin pupfishes (Echelle and Dowling, 1992; Echelle and Echelle, 1993a).

The possible sister relationship between the western pupfishes and the clade of seven species from the remainder of the ancestral Rio Grande system, together with Miller's (1981) argument from the fossil record that pupfish must have had access to the Death Valley system by late Pliocene, coincides with the possibility of a Plio-Pleistocene connection (Smith and Miller, 1986) between the lower Rio Grande and Lake Cabeza de Vaca. The latter was a system of interconnected interior basins centered in the area of the present Guzmán Basin and covering large parts of northwestern Chihuahua and southwestern New Mexico. Any connection between Lake Cabeza de Vaca and the lower Rio Grande would have been severed by at least mid-Pleistocene when the upper Rio Grande, previously a major tributary of the lake, was diverted eastward to join the lower Rio Grande (Smith and Miller, 1986). A vicariant event considerably earlier than mid-Pleistocene is suggested by the mtDNA sequence divergence values (Echelle and Dowling, 1992) between members of the western clade and three species from the east (*C. eximius,* 5.6–8.2%; $\bar{x}$ = 7.1%; *C. meeki,* 6.8–8.2%, $\bar{x}$ = 7.5%; and *C. variegatus,* 6.1–9.5%; $\bar{x}$ = 7.5%) compared to a maximum divergence of 3.9% among the eight western species. Estimates of divergence times from mtDNA sequences must be viewed with caution because the evolutionary rate for the molecule varies both within and among groups of organisms (Dowling and Brown, 1989; Avise et al., 1992). However, the maximum rate estimated for fishes appears to be around 2% per million years (Brown and Chapman, 1991; Martin and Palumbi, 1993). Thus, we hypothesize that isolation of the ancestral western pupfish from eastern forms probably occurred by at least late Pliocene.

Our phylogenetic analysis supported a sister relationship between two forms on opposite sides of the Mexican Plateau: *C. atrorus* of the Cuatro Ciénegas Basin in the east and the Río Aguanaval pupfish (*C.* sp.) in the west. This sister pair is part of a clade that includes *C. nazas* and *C. meeki. Cyprinodon* sp., *C. nazas,* and *C. meeki* occupy headwater portions of a proposed Plio-Pleistocene system ("Old Río Nazas"; Conant, 1963) that flowed eastward off the Sierra Madre Occidental and connected with the Rio Grande downstream of the Río Conchos (Meek, 1904; Arellano, 1951; Smith and Miller, 1986). Stream capture from the Old Río Nazas (Albritton, 1958) would have brought ancestral *C. meeki* into a Pacific slope drainage (Río Mezquital), allowing divergence from the pupfish in the Old Río Nazas.

One of the three hypothesized connections between the Old Río Nazas and the Rio Grande (Meek, 1904; Arellano, 1951) conforms especially well with the sister relationship between *C. atrorus* and the Río Aguanaval pupfish. According to this hypothesis, the Old Río Nazas flowed to the Rio Grande via the Cuatro Ciénegas Basin and its outlet, the Río Salado (Arellano, 1951, Conant, 1963; but see Minckley, 1969). The Río Aguanaval pupfish is not morphologically divergent from *C. nazas* (Miller, 1976), but the allozymes of these two species indicate a long history of isolation. This is somewhat problematic because the Ríos Nazas and Aguanaval empty into the closely associated Lagos de Mayrán and Viesca, respectively. These lakes are now dry because of agricultural diversion of streamflows, but they may have been connected historically (Conant, 1963) and

probably were parts of an extensive lake system that persisted throughout the Pleistocene (Miller, 1981; Smith and Miller, 1986). Regardless of mechanism, however, *C. nazas* and the Río Aguanaval pupfish apparently diverged prior to divergence of *C. atrorus* in Cuatro Ciénegas. Conant (1963) suggested that uplifting on the east side of the Mexican Plateau would have isolated Cuatro Ciénegas from the headwaters of the Old Río Nazas; this would have allowed divergence of ancestral *C. atrorus* from the Río Aguanaval pupfish.

Although not analyzed phylogenetically, the biogeography of the watersnake *Nerodia* (= *Natrix*) *erythrogaster* also is supportive of a connection between the Old Río Nazas and Cuatro Ciénegas (Conant, 1977). Two divergent forms (endemic subspecies) of *N. erythrogaster* occur in the headwaters of the Old Río Nazas, one each in the Ríos Nazas and Aguanaval (Conant, 1969), supporting the conclusion from pupfish that the faunas of these two drainages were long isolated. The geographically nearest population of this rather wide-ranging species is a divergent but not taxonomically recognized form in Cuatro Ciénegas (Conant, 1969).

Additional phylogenetic studies are needed to further examine the possibility of a past connection between headwaters of the Old Río Nazas and the Cuatro Ciénegas/Río Salado. Of particular interest are relationships of *Cyprinella alvarezdelvillari* (Contreras and Lozano, 1994), a member of the *C. lepida* group that is endemic to the Río Nazas but was not included in Mayden's (1989) phylogenetic analysis of the genus. The prediction from the results for *Cyprinodon* is that *C. alvarezdelvillari* is part of a clade that otherwise includes only *C. xanthicara* from Cuatro Ciénegas and *C. rutila* from the Río Salado and a more downstream tributary of the Rio Grande, the Río San Juan. *Dionda* also is of interest in this respect because Mayden et al. (1992) found that the sister group to a clade of two species confined to headwaters of the Old Río Nazas (Río del Tunal) is sister to *D. diaboli*, a species from Rio Grande tributaries downstream of the Pecos River, including the Río Salado, a Pleistocene outlet for the Cuatro Ciénegas Basin. As in our results, more distant relatives of this sister-group pair occur in the Río Conchos and other waters in the Río Grande Basin upstream of the mouth of the Río Salado. However, Mayden et al. (1992) suggested that the Río Conchos might harbor cryptic species not examined in their analysis. Inclusion of such taxa might alter inferred biogeographic history.

Our phylogenetic and biogeographic conclusions are compromised to an unknown degree by hybridization and introgression following secondary contact between previously isolated, genetically divergent lineages. Morphologically divergent pupfishes often exhibit high levels of reproductive compatibility in "no-choice" experiments in the laboratory (Turner and Liu, 1977; Villwock, 1982), and artificial introductions of one species, *C. variegatus*, into the ranges of two of its close relatives (*C. pecosensis* and *C. bovinus*) demonstrated the potential for extensive introgression in relatively short periods (Childs et al., 1996; Echelle and Echelle, 1996). However, some species pairs of *Cyprinodon* exhibit evidence of genetic incompatibilities (deformed embryos and sex-ratio biases among hybrids) in the laboratory (Turner and Liu, 1977; Villwock, 1982), and in one seminatural situation, there is little evidence of genetic introgression despite more than 20 years of contact between *C. elegans* and an introduced population of *C. variegatus* (Echelle and Echelle, 1994).

Among the species we examined, three pairs of genetically distinct species either are (*C. eximius–C. pachycephalus* and *C. atrorus–C. bifasciatus*) or undoubtedly were (*C. eximius* and *C. macrolepis*) in contact in the recent past. Although *C. macrolepis* and *C. eximius* exhibited no fixed allele differences, their color patterns and other morphological features are divergent (Miller, 1976). Their allozyme similarities may be a result of hybridization. At some time in the relatively recent past, the now-isolated spring supporting *C. macrolepis* was in contact with the Río Florido (Miller, 1976), a tributary of the Río Conchos. Correspondingly, at the highly conservative *Ak-A* locus, *C. macrolepis* is fixed for an allele (*Ak-A*^{b}) otherwise absent in our survey except for its low frequency (0.05) in the Río Florido sample of *C. eximius*. All other samples, including *Megupsilon*, are fixed for *Ak-A*^{a}. Another allele, *sAat-A*^{e}, also is restricted to these two populations (frequency = 0.10–0.33), possibly as a result of hybridization.

The other two instances of contact between species show no evidence of genetic introgression. *Cyprinodon pachycephalus* and *C. eximius* exhibit marked genetic differences despite morphological evidence of hybridization (Minckley and Minckley, 1986) in downstream reaches of the spring outflow where the species are in contact. The samples of *C. atrorus* and *C. bifasciatus* were from the headwaters of the small Río Churince and its tailwaters, respectively, at Laguna Grande, approximately 3 km downstream. These two small samples (n = 10) exhibited fixed allele differences at two loci and differed significantly ($P < 0.02$ to $P < 0.00001$) at eight

additional loci, despite the persistent occurrence, at least in the 1970s (Minckley, 1977), of a hybrid swarm in intervening water contiguous with our two sample sites. Evidence of a general lack of introgression between *C. atrorus* and *C. bifasciatus* is that 10 alleles were restricted to this pair of species (frequencies = 0.15–1.0), and none was shared between the two.

There are two situations in the ancestral Rio Grande system where representatives of divergent lineages occur in the same region, suggesting multiple colonizations. First, as suggested by Miller (1968), the two Cuatro Ciénegas pupfishes, *C. atrorus* and *C. bifasciatus*, are rather distantly related, with *C. bifasciatus* being basal to most pupfish lineages on the Mexican Plateau. The second situation is the Pecos River drainage in Texas, where there are three allopatric species representing two divergent lineages: *Cyprinodon elegans* is closely related to *C. eximius*, whereas the other two species in the Pecos River drainage, *C. pecosensis* and *C. bovinus*, represent one of several lineages referred to as the "inland members of the *C. variegatus* complex" (Echelle and Echelle, 1992).

On the basis of present distributions and a phylogenetic analysis, Echelle and Echelle (1992) suggested that different lineages among the inland members of the *C. variegatus* complex from New Mexico, Texas, and Oklahoma might have evolved as peripheral isolates of the widespread coastal species, *C. variegatus*. This hypothesis can be extended to include deeper nodes of the phylogenetic tree for *Cyprinodon*. It is conceivable that a hardy, euryhaline coastal form like *C. variegatus* might have extended from coastal areas into the ancestral Rio Grande and associated waters early in the history of pupfish evolution in the interior of North America. Echelle and Dowling (1992) found that mtDNA of *C. meeki* was more closely related to that of the two specimens they examined from *C. variegatus* than it was to those of *C. eximius* and the eight species of the western pupfish clade represented in this study by *C. macularius* and *C. nevadensis*. This might reflect lineage sorting from a polymorphic coastal species that was ancestral to *C. meeki* and other lineages among interior pupfishes. Speciation followed by subsequent invasions by the coastal form might have resulted in the situations now seen in Cuatro Ciénegas and the Pecos River, where there are divergent forms of pupfish, one closer to *C. variegatus* than the other. This would explain the paraphyly of the interior pupfishes with respect to *C. variegatus*, the present form in coastal North America.

Material Examined

Arranged by numbers (in parentheses) indicated for each locality in Figure 1; brackets give catalog numbers for vouchers at the Oklahoma State University Collection of Vertebrates (OSUS): *Cyprinodon eximius*, (1) Devils River at Pafford's crossing, Val Verde Co., Texas, (2) Alamito Creek, 5 km SE of Presidio Co., Texas, (3) Río Florido, 1 km SE Villa Lopez, Chihuahua [18240], (4) Ojo de Villa López at Villa López, Chihuahua [18229]. *Cyprinodon macrolepis*, (5) Ojo de Hacienda Dolores, 11.2 km S Jimenez, Chihuahua. *Cyprinodon pachycephalus*, (6) Ojo de San Diego, 57 km SE Chihuahua, Chihuahua. *Cyprinodon atrorus*, (7) Laguna Grande at inlet from Río Churince, 15.5 km SE Cuatro Ciénegas, Coahuila [18231]. *Cyprinodon nazas*, (8) Outflow of Ojo de la Concha, 9 km W of Peñon Blanco, Durango [18235], (9) tributary of Laguna Santiaguillo, the Río Guatimape at Guatimape, Durango [18244], (10) Río Aguanaval at Rancho Grande, Zacatecas [18232]. *Cyprinodon meeki*, (11) tributary of Río del Tunal, 40 km NE of Durango, Durango [18224]. *Cyprinodon alvarezi*, (12) irrigation canal from Ojo del Potosí at Ejido Catarino Rodriquez, Nuevo León [18243]. *Cyprinodon ceciliae*, (13) La Presa, a spring at San Juan de Avilés, Nuevo León [18226]. *Cyprinodon elegans*, (14) Giffin Spring canal at Toyahvale, Reeves Co., Texas [18246]. *Cyprinodon bifasciatus*, (15) Pozo Churince, 16 km SE Cuatro Ciénegas, Coahuila [18227]. *Cyprinodon variegatus*, (16) Edinburg Water Supply Canal, 1 km N Edinburg, Hidalgo Co., Texas [18317]. *Megupsilon aporus*, (17) captive stock from outdoor tanks at Universidad de Autonoma de Nuevo León, Monterrey, Mexico—wild population (now extinct) endemic to the spring at locality 12 (see above). *Cyprinodon nevadensis mionectes*, (18) Point of Rocks Spring, Ash Meadows National Wildlife Refuge, Nye Co., Nevada. *Cyprinodon macularius eremus*, (19) Quitobaquito Spring, Organ Pipe Cactus National Monument, Pima Co., Arizona.

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APPENDIX 1. PROTEINS, PRESUMPTIVE LOCI, TISSUE SOURCES, AND BUFFER SYSTEMS USED.

Protein	Locus	Tissue	Analytical system[a]
Aconitase hydratase (EC 4.2.1.3)	*mAcoh-A*	eye-brain	2
	sAcoh-A	eye-brain	2
Adenylate kinase (EC 2.7.4.3)	*Ak-A*	eye-brain	2
Alcohol dehydrogenase (EC 1.1.1.1)	*Adh-A*	liver	2
Aspartate transaminase (EC 2.6.1.1)	*sAat-A*	muscle	1
Calcium binding protein[b]	Cbp-1	muscle	1
	Cbp-2	muscle	1
Creatine kinase (EC 2.7.3.2)	*Ck-A*	eye-brain	2
	Ck-B	eye-brain	2
	Ck-C	eye-brain	2
Fructose biphosphatase (EC 3.1.3.11)	*Fbp-C*	eye-brain	1
Fumarate hydratase (EC 4.2.1.2)	*Fh-A*	muscle	1
Glyceraldehyde-3-phosphate dehydrogenase *EC 1.2.1.12)*	*Gapdh-A*	muscle	1[c]
	Gapdh-C	muscle	1[c]
Glycerol-3-phosphate dehydrogenase (EC 1.1.1.8)	*G3pdh-A*	muscle	2
Glucose-6-phosphate isomerase (EC 5.3.1.9)	*Gpi-A*	eye-brain	2
	Gpi-B	muscle	1
Isocitrate dehydrogenase (EC 1.1.1.42)	*mIdhp-A*	muscle	2
	sIdhp-A	liver	2
L-Lactate dehydrogenase (EC 1.1.1.27)	*Ldh-A*	eye-brain	1
	Ldh-B	eye-brain	1
	Ldh-C	eye-brain	1
Malate dehydrogenase (EC 1.1.1.37)	*mMdh-A*	eye-brain	3
	sMdh-A	eye-brain	3
Malate dehydrogenase ($NADP^+$) (EC 1.1.1.40)	*mMdhp-A*	eye-brain	3[d]
Mannose-6-phosphate isomerase (EC 5.3.1.8)	*Mpi-A*	muscle	2[d]
Proline dipeptidase (EC 3.4.13.9)	*Pep-D*	eye-brain	3
Phosphogluconate dehydrogenase (EC 1.1.1.44)	*Pgdh-A*	eye-brain	3[d]
Phosphoglucomutase (EC 5.4.2.2)	*Pgm-A*	eye-brain	2
Superoxide dismutase (EC 1.15.1.1)	*Sod-A*	liver	2

[a] Analytical systems as follows: (1) after Turner (1983)—stock solution: 0.9 M tris-hydroxymethylaminomethane (= "Tris"), 0.5 M boric acid, 0.1 M disodium EDTA, pH 8.6; electrode buffer: 1 vol stock solution + 6.9 vols H_2O; gel buffer: 1 vol stock solution + 24 vols H_2O; (2) after Stein et al. (1985) except adjust pH with 10 N NaOH—electrode buffer: 0.1 M Tris, 0.03 M citric acid, pH 7.5; gel buffer: 1 vol electrode solution + 6 vols H_2O; (3) after Shaw and Prasad (1970)—electrode buffer: 0.69 M Tris, 0.16 M citric acid, pH 8.0; gel buffer: 0.02 M Tris, 0.005 M citric acid, pH 8.0.

[b] Scored from gels stained for general proteins (Buth, 1982).

[c] 20 Mg NAD added to starch-gel solution after boiling.

[d] 20 mg NADP added to starch-gel solution after boiling.

APPENDIX 2. GENOTYPE ARRAYS FOR 24 POLYMORPHIC GENE LOCI IN 18 SAMPLES OF *Cyprinodon* AND ONE SAMPLE OF *Megupsilon aporus*. Sample numbers correspond with those in Figure 1, except for two species, *C. nevadensis* (#18) and *C. macularius* (#19) not represented in the figure. Letters represent alleles in order of decreasing mobility starting with "a." Parentheses show numbers of specimens for each genotype. *H* represents heterozygosity.

	Sample number									
Locus	1	2	3	4	5	6	7	8	9	10
sAat-A	dd(21)	dd(15)	dd (8) de (2)	dd(10)	dd (9) de (9) ee (2)	dd(10)	dd(10)	bb (9) bd (1)	ab (1) bb (9)	dd(10)
mAcoh-A	cc(21)	cc(15)	cc(10)	cc(10)	cc(19) cd (1)	ce (1) ee (9)	cc(10)	cd (3) dd (7)	cd (6) dd (4)	cc(10)
sAcoh-A	bb(21)	aa (2) ab (8) bb (5)	ac (1) bb (9)	bb(10)	bb (1) cc(10) bc (9)	ab (2) ac (1) bb (2) bc (4) cc (1)	bb (7) bc (3)	bb(10)	bb(10)	bb(10)
Adh-A	dd(21)	dd(15)	dd(10)	dd(10)	ad (5) dd(15)	dd(10)	dd(10)	dd(10)	ad (5) dd (5)	dd (8)
Ak-A	aa(21)	aa(15)	aa (9) ab (1)	aa(10)	bb(20)	aa(10)	aa(10)	aa(10)	aa(10)	aa(10)
Ck-A	cc(21)	cc(15)	cc(10)	cc(10)	cc(20)	aa (6) ac (2) cc (2)	cc(10)	cc(10)	cc(10)	cc(10)
Ck-B	bb(21)	bb(15)	bb(10)	bb(10)	bb(20)	bb(10)	aa (7) ab (3)	bb (4) bc (4) cc (2)	bb(10)	aa (9) ab (1)
Ck-C	cc(21)	cc(15)	cc(10)	cc(10)	cc(20)	cc(10)	bc (5) cc (5)	cc(10)	cc(10)	cc(10)
Fbp-C	dd(21)	ad(10) dd (5)	dd(10)	dd (9) de (1)	dd(20)	dd(10)	dd(10)	dd(10)	dd(10)	gg(10)
Fh-A	cc(21)	cc(15)	cc(10)	cc(10)	cc(20)	cc(10)	cc (3) cf (4) cg (2) fg (1)	bc (6) cc (4)	cc(10)	cc(10)
G3pdh-A	cc(21)	cc (6) ce (9)	cc(10)	cc(10)	cc(20)	cc (1) cf (1) ff (8)	cc(10)	cc (9) cd (1)	cc(10)	bb(10)
Gpi-A	dd(21)	dd(15)	bd (1) dd (9)	dd(10)	bd (5) dd(12) dg (3)	bb (3) bc (6) cc (1)	be (1) ee (9)	bb(10)	bb(10)	bb(10)
Gpi-B	ee(21)	ee(15)	ee(10)	ee(10)	ee(20)	ee(10)	ee(10)	ee(10)	ee(10)	ee(10)
sldhp-A	gg(21)	df (7) ff (8)	dd (1) df (1) ff (8)	df (2) ff (8)	dd (1) df (9) ff(10)	ff(10)	dd(10)	dd (8) dh (2)	dd(10)	bb(10)
Ldh-A	bb(21)	bb(15)	bb(10)	bb(10)	bb(20)	bb(10)	bb(10)	bb(10)	bb(10)	bb(10)
Ldh-B	bb(21)	bb(15)	bb(10)	bb(10)	bb(20)	bb(10)	bb(10)	bb(10)	bb(10)	bb(10)
Ldh-C^{a}	bb(21)	bb(15)	bb(10)	bb(10)	bb(20)	bb(10)	bb(10)	bb(10)	bb(10)	bb(10)
mMdh-A	cc(21)	bb(15)	bb(10)	bb(10)	bb(20)	bb(10)	aa(10)	aa(10)	aa(10)	aa(10)
sMdh-A	bb(21)	bb(15)	bb(10)	bb(10)	bb(20)	bb(10)	bb(10)	bb(10)	bb(10)	bb(10)

APPENDIX 2. CONTINUED.

Sample number								
11	12	13	14	15	16	17	18	19
dd(15)	bd (1) dd (9)	dd(16)	dd(10)	dd(10)	cc (7) cd (5) dd(14)	bb(11)	dd(16)	dd(15)
ac (1) cc(14)	cc(10)	aa(11) ac (5)	cc(10)	cc(10)	cc(26)	ac (7) cc (4)	bb (3) bc(10) cc (3)	bc (8) cc (7)
ab (1) bb(14)	bb(10)	bb(10) bc (6)	dd(10)	bc (7) cc (3)	ee (5) ef (8) ff(10) fg (2)	ee (5)	cc(16)	cc(15)
dd(15)	ee(10)	bb (1) bd (7) dd (7) de (1)	cc (1) cd (5) dd (4)	dd(10)	bd (2) dd(24)	ee(11)	dd (9) de (5) ee (2)	dd(15)
aa(15)	aa(10)	aa(16)	aa(10)	aa(10)	aa(26)	aa(11)	aa(16)	aa(15)
cc(15)	bb(10)	bb(16)	cc(10)	cc(10)	cc(26)	aa(11)	cc(16)	cc(15)
bb(14) bd (1)	bb (6) bd (3) dd (1)	bb(16)	bd (1) dd (9)	bb(10)	bb(26)	bb(11)	bb(16)	bb(15)
cc(15)	aa (1) dd (9)	cc(15) cd (1)	cc(10)	cc(10)	cc(26)	ff(11)	cc(15) ce (1)	ee(15)
cd (1) dd(14)	ff(10)	ff(16)	dd(10)	bb(10)	dd(26)	ff(11)	dd(16)	dd(15)
bb (5) bc (3) cd (3) dd (4)	ac (4) cc (6)	ac (1) cc(13) ce (2)	cc(10)	cc(10)	cc(26)	cc(11)	cc(16)	bc (1) cc(14)
cc(15)	cc (7) cg (2) gg (1)	cc(16)	cc(10)	cc(10)	cc(26)	aa(11)	cc(16)	cc(15)
bb(10) bf (5)	dd(10)	dd (6) df (2) dg (6) gg (2)	bb(10)	bb(10)	ab (1) bb(22) bc (3)	dd(11)	bb(14) be (2)	bb(15)
ee(12) eg (3)	ee(10)	ee(14) ef (2)	dd (1) ee (9)	ee(10)	be (1) ee(25)	ac (2) cc (9)	ee(14) eg (2)	ee(15)
cc(14) ci (1)	ad (1) dd (9)	dd(16)	df (5) ff (5)	dd (6) de (3) ee (1)	dd(26)	bb(10) bd (1)	dd(16)	dd(15)
bb(15)	bb(10)	bb(16)	bb(10)	bb(10)	bb(26)	aa(11)	bb(16)	bb(15)
bb(15)	bb(10)	bb(16)	bb(10)	ab (2) bb (8)	bb(26)	cc(11)	bb(16)	bb(15)
bb(14) bd (1)	bb(10)	bb(16)	fg(10)	aa (4) ab (4) bb (2)	bb(26)	ee(11)	bb(16)	bb(15)
aa(15)	aa (9) ad (1)	aa(16)	aa(10)	aa(10)	aa(26)	aa(11)	aa(14) ae (2)	aa(15)
bb(15)	aa(10)	aa(16)	bb(10)	bb(10)	bb(26)	cc(11)	bb(16)	bb(15)

APPENDIX 2. CONTINUED.

Locus	Sample number									
	1	2	3	4	5	6	7	8	9	10
sMep-A	aa(21)	dd(15)	dd (9)	dd(10)	dd(20)	dd(10)	dd(10)	dd(10)	dd(10)	dd(10)
Mpi-A	cc(21)	bb(11) bc (4)	cc(10)	cc(10)	cc(18) cf (1) ff (1)	ff(10)	cc (8) ce (2)	aa (6) ac (4)	cc(10)	dd(10)
Pep-D	bb(21)	bb(15)	ab (4) bb (6)	ab (3) bb (7)	bb (1) bd(11) dd (8)	de (1) ee (9)	dd (3) de (5) ee (2)	ee(10)	ee(10)	dd(10)
Pgdh-A	cc(21)	cc(15)	cc(10)	cc(10)	cc(20)	cc(10)	ce (1) ee (9)	bb (6) bc (4)	cc(10)	cc(10)
Pgm-A	ee(21)	ee(15)	ee(10)	ee (9) ef (1)	ee(20)	eh (1) hh (9)	ee(10)	ee(10)	ee(10)	ce (1) ee (9)
H	0.000	0.067	0.040	0.022	0.083	0.072	0.081	0.076	0.031	0.007

[a] *Cyprinodon elegans* (sample 14) is a fixed heterozygote at this locus, presumably as a result of tandem gene duplication (Echelle and Echelle, 1991).

APPENDIX 3. DISTRIBUTION OF SYNAPOMOPRHIC ALLELES SUPPORTING THE LETTERED NODES IN FIGURE 2. Based on the FREQPARS output of allele frequencies for hypothetical ancestors. Losses of alleles are not considered, and descriptions of allele occurrences refer only to the node in question; unless otherwise noted, the alleles are absent outside the clade.

Node A: *Gpi-A*^{c}, moderate (0.13) to high (1.0) frequencies in all members except *C. eximius*, in which it was absent except for rare (0.05) occurrence in one of four samples. **Node B**: *Ck-A*^{b}, *Fbp-C*^{f}, and *sMdh-A*^{a}, fixed in both species (*Fbp-C*^{f}) also present in *Megupsilon*—treated as a homoplasy by FREQPARS). **Node C**: *Fbp-C*^{d}, high frequencies (0.68–1.0) except absent in Río Aguanaval *C. nazas*; *Pgdh-A*^{d}, fixed in all except *C. atrorus* and Río Aguanaval *C. nazas*, in which it occurred at frequencies of 0.05 and 0.20, respectively. **Node D**: *mAcoh*^{c}, moderate frequencies (0.27–0.50) in both species; *Ck-C*^{e}, fixed in *C. macularius*, rare in *C. nevadensis* (0.03). **Node E**: *sIdhp-A*^{f}, high frequencies (0.72–1.00) except absent in the sample of *C. eximius* from Devils River, which is fixed for an autapomorphy (*sIdhp-A*^{g}). **Node F**: *mMdh-A*^{c}, fixed in all samples of this clade except Devils River *C. eximius*, which is fixed for an autapomorphy at this locus (*mMdh-A*^{d}). **Node G**: *Pep-D*^{b}, present at frequencies of 0.33 in *C. macrolepis* and 0.80–1.00 in *C. eximius*, otherwise present only in *Megupsilon* (treated as a homoplasy by FREQPARS). **Node H**: *mAcoh*^{e}, high frequency in both samples (0.70 and 0.85); *sAat-A*^{c}, high frequency (0.95) in both samples—FREQPARS treated this occurrence as a homoplasy because it also occurred in *Megupsilon* (frequency = 1.0) and *C. alvarezi* (0.05). **Node I**: *Ck-B*^{a}, high frequency (0.85 and 0.95) in both samples.

APPENDIX 2. CONTINUED.

Sample number								
11	12	13	14	15	16	17	18	19
cc(15)	dd(10)	dd(16)	ee(10)	dd(10)	dd(26)	bb(11)	df (1) ef (3) ff(12)	bb(15) dd(15)
cc(14) cf (1)	cc(10)	ac (1) cc(12) cg (3)	hh(10)	cc(10)	ac (1) cc(23) cf (1) ff (1)	cf (4) ff (7)	aa(11) af (4) ff (1)	bb (3) bc (6) cc (6)
de (1) ee(14)	ee(10)	ee(13) ef (3)	cc(10)	ee(10)	dd(26)	bb(11)	ee(16)	ee(15)
cc(15)	dd(10)	ff(16)	cc(10)	dd(10)	dd(17) dg (8) gg (1)	aa(10)	cc(16)	cc(15)
bb (1) be (6) ee (8)	ab (2) bb (8)	ab (8) bb (8)	ae (3) ee (7)	ee(10)	ee(24) eg (2)	ee(11)	de (1) ee(15)	ee(15)
0.069	0.026	0.091	0.065	0.052	0.060	0.034	0.066	0.032