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Trypsin G-banding of North American Cyprinid Chromosomes: Phylogenetic Considerations, Implications for Fish Chromosome Structure, and Chromosomal Polymorphism

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Chromosome studies in fishes have been historically limited by the failure to successfully apply many of the serial or G-type banding procedures commonly used in other vertebrates (Gold 1979, Hartley and Horne 1985, Amemiya *et al.* 1991). Procedures for visualizing both chromosomal NORs (nucleolus organizer regions) and regions of constitutive heterochromatin (C-bands) on fish chromosomes are now well established (Gold 1984, Phillips and Ihssen 1985, Amemiya and Gold 1986, Gold *et al.* 1986, Phillips and Hartly 1988, Pleyte *et al.* 1989), and recently, Lloyd and Thorgaard (1988) and Cau *et al.* (1988) have published methods for producing restriction enzyme-induced bands. Serial bands produced by protein or heat denaturation treatments have been reported for only a few fishes (Wiberg 1983, Sola *et al.* 1984, Hartley and Horne 1985, Almeida Toledo *et al.* 1988, Amemiya and Gold 1990a), and with the exception of *Anguilla* species (see below), the resolution of individual chromosomes has been insufficient to unequivocally homologize most chromosomes within species. Alternatively, high quality serial bands using replication banding procedures have been reported for several fish species (Delany and Bloom 1984, Liu 1986, Giles *et al.* 1988, Almeida Toledo *et al.* 1988, Zhou *et al.* 1989). A limitation to replication banding, however, is that good cell synchronization is necessary to insure that the replication bands are homologus since both G- or R- (or both) bands can be produced even from the same culture.

Medrano *et al.* (1988) have suggested that the high quality G-bands produced on chromosomes of *Anguilla* species, but not other fishes, may be due to the DNA base pair heterogeneity and possible presence of GC-rich isochores in *Anguilla* genomes. In brief, the nuclear genomes of most fishes examined appear to exhibit considerable uniformity in nucleotide distributions and to lack DNA components that differ significantly in base pair composition from mainband DNA (Hudson *et al.* 1980, Karel and Gold 1987, unpubl., Gold and Karel 1988). Species in other major vertebrate groups, however, possess numerous, typically GC-rich components in addition to mainband DNA (Thiery *et al.* 1976, Guttman *et al.* 1977, Olmo 1981). These components have been termed "isochores" and represent fairly large (>200 kilobase) segments of GC-rich DNA (Cuny *et al.* 1981, Bernardi *et al.* 1985). The study of Medrano *et al.* (1988) demonstrated a parallelism between the occurrence of serial banding and genomic compartmentalization by base composition.

Studies in our laboratory over the last 7–8 years have centered on documenting chromosomal NORs in North American cyprinid fishes and using the chromosomal NOR phenotypes to infer phylogenetic relationships among various cyprinid species or species-groups (Amemiya and Gold 1988, 1990a, Amemiya *et al.* 1991). One limitation has been the difficulty in establishing homology of phenotypically similar NOR chromosomes across species. As an example, several North American cyprinids possess a submeta- or acro-/subtelocentric NOR chromosome which is the largest chromosome in the complement and where the NOR is located terminally on the short arm (Gold 1984, Amemiya and Gold 1988, 1990a). Homology

of this NOR chromosome among several species has been inferred largely on the basis of size, and in a few instances, on the basis of similar C-banding patterns (Gold and Amemiya 1986, Amemiya 1987, Amemiya and Gold 1988).

In this paper, a recently developed G-banding procedure using trypsin is used to test homology of NOR-bearing chromosomes from six cyprinid species and to demonstrate the occurrence of trypsin-induced G-bands on cyprinid chromosomes. The phylogenetic implications of the data are discussed as are the implications regarding cyprinid chromosome structure and the potential for detecting previously cryptic chromosomal polymorphisms.

Materials and methods

The specimens examined in this study were obtained by seine from natural populations. The species (collection localities) were as follows: *Cyprinella lutrensis*, *Cyprinella venusta*, *Notropis shumardi*, and *Opsopoeodus emiliae* (Little Brazos River, Brazos County, Texas; Brazos River, Burleson County, Texas); and *Cyprinella lepida* and *Notropis amabilis* (Nueces River, Real County, Texas). All specimens were returned live to the laboratory and maintained in aerated aquaria until sacrificed.

Metaphase chromosomes were prepared from cultured fibroblasts following the methods of Amemiya *et al.* (1984) as modified by Gold *et al.* (1990). The modifications include the use of 2.5 ml of 1X antibiotic/antimycotic (Gibco) solution per 500 ml of growth media and the use of 1X Leibovitz's L-15 growth media with L-glutamine (Gibco) instead of Medium 199. Following standard hypotonic treatment (30 min in 7–8 ml of 0.56% KCl at 30°C), cells were fixed following a procedure outlined in Islam and Levan (1987) and Gold *et al.* (1990). Microscope slides were prepared using the method of Kligerman and Bloom (1977) as modified by Rayburn and Gold (1982).

For G-banding, prepared slides were stored in a 60°C oven for 3–5 days or at room temperature for 7–10 days. Slides were then incubated in 2X SSC for 2 hrs at 60°C, rinsed individually with 0.9% NaCl, partially air dried, and treated/stained in a trypsin/Giemsa solution for 15 min at room temperature. The trypsin/Giemsa solution contained 600 μl of a trypsin stock solution and 1.5 ml Giemsa stain in 45 ml 0.01 M phosphate buffer at pH 7.2. The trypsin stock solution contained 0.1 ml of 2.5% trypsin (Gibco) in 2.4 ml distilled water and was made just prior to use. Following treatment/staining, slides were individually rinsed twice in distilled water and air dried. Appropriately G-banded metaphases were identified and photographed. Slides were then destained with freshly prepared 3: 1 (methanol: acetic acid) fixative, rinsed with distilled water, air dried, and silver-stained (after Howell and Black 1980) to resolve chromosomal NORs. Bright field photomicroscopy of both G-banded and silver-stained preparations followed procedures outlined in Gold and Amemiya (1986).

Fluorescent staining of metaphases was carried out using both quinacrine dihydrochloride and DAPI (4, 6-diamidino-2-phenylindole). For quinacrine staining, prepared slides were stained 7–10 min in 0.5% quinacrine dihydrochloride (0.2 g quinacrine/4 ml distilled H_2O), rinsed 5 min in distilled H_2O, and then coverslipped. For DAPI staining, prepared slides were rinsed 1–2 min in 95% ethanol, stained with 2–4 drops of a stock DAPI solution for 3–5 min, rinsed 5 min in distilled H_2O, and then coverslipped. The DAPI stock solution consisted of 1 mg DAPI in 30 ml absolute ethanol stored at −4°C. For fluorescence microscopy, an XBO 75W/AC lamp and the following filter systems were employed: (i) quinacrine —BP 495 exciter filter, FT 510 chromatic beam splitter, and LP 530 barrier filter; and (ii) DAPI— UG-1 exciter filter, FT 420 chromatic beam splitter, and LP 395 barrier filter. Fluorescent photomicroscopy followed procedures outlined in Amemiya and Gold (1986).

Results and discussion

The NOR chromosome phenotypes of the six species were known from previous studies (Gold 1984, unpubl., Amemiya and Gold 1988): *C. lepida*, *C. lutrensis*, and *C. venusta* each possess a single pair of NOR chromosomes of the *C'* phenotype defined as a NOR terminal on the short arm of a submetacentric chromosome which is the largest chromosome in the complement; *N. amabilis* and *N. shumardi* each possess a single pair of NOR chromosomes of the *F'* phenotype defined as a NOR terminal on the short arm of a subtelo-/acrocentric chromosome which is the largest chromosome in the complement; and *O. emiliae* possesses a single pair of NOR chromosomes of the *E'* phenotype defined as a NOR subterminal (interstital) on the short arm of a submetacentric chromosome which is the largest chromosome in the complement. Based on the similarities in size and the presence of an unusually large, long-arm C-band, Gold and Amemiya (1986) and Amemiya and Gold (1988) suggested that the *C'* NOR chromosomes of the *Cyprinella* species were homologous and that the *E'* NOR

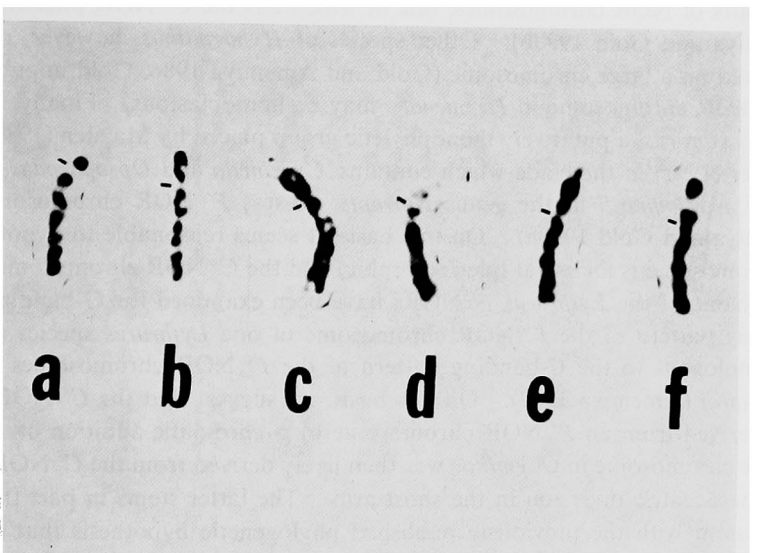

Fig. 1. Trypsin G-banded NOR chromosomes of: a) *Notropis amabilis*; b) *Notropis shumardi*; c) *Cyprinella lepida*; d) *Cyprinella lutrensis*; e) *Cyprinella venusta*; and f) *Opsopoeodus emiliae*. Bars indicate positions of centromeres.

chromosome in *O. emiliae* differed from the *C'* NOR chromosome by a small paracentric inversion in the short arm. G-banded preparations of the NOR chromosomes of the six species are shown in Figure 1. The NOR chromosomes of all six species possess at least six dark G-bands on the long arm of each chromosome, indicating (i) homology of the long arms among the six species, and (ii) chromosomal rearrangements among the six species have all involved the short arm.

Phylogenetic considerations

Mayden (1989, pers. comm.) and Coburn and Cavender (1991) proposed hypotheses of species relationships for the majority of eastern North American cyprinids based primarily on morphological (including osteological) characters. While salient differences in the placement of several species or species-groups occur between the two studies, both are in agreement that the majority of "*Notropis*"-like shiners (which includes the six species examined in this

study) belong to a putatively monophyletic assemblage. Both studies also are in agreement that the "*Notropis*"-like shiner assemblage is comprised of two major clades, one of which contains (among other genera) *Cyprinella* and *Opsopoeodus*, while the other clade contains (among others) the genus *Notropis* (*sensu* Mayden 1989).

Our finding that the long arms of the NOR chromosomes of the three species of *Cyprinella*, of *O. emiliae*, and of the two species of *Notropis* are homologus in G-banding pattern suggests that this chromosome with a NOR situated on the short arm is the plesiomorphic NOR character state for the "*Notropis*"-like shiner assemblage, i. e., all other NOR character states within "*Notropis*"-like shiners are apomorphic. Given that all but one of the North American cyprinids outside of the "*Notropis*"-like shiners examined to date for chromosomal NORs do not posses a NOR on the largest chromosome in the complement (Amemiya and Gold 1990a, Amemiya *et al.* 1991), the phylogenetic inference is that this chromosome may also represent a synapomorphy for the "*Notropis*"-like shiner assemblage. The one exception is *Hybognathus nuchalis*, a member of the "chub" (*sensu* Mayden 1989) assemblage, which possesses two pairs of NOR chromosomes, one of which has the *C'* NOR phenotype (Amemiya 1987, Amemiya and Gold 1990b). Other species of *Hybognathus*, however, do not possess a NOR located on a large chromosome (Gold and Amemiya 1986, Gold unpubl.), suggesting that the *C'* NOR chromosome in *H. nuchalis* may be homoplasious. Finally, several species in the genus *Lythrurus*, a putatively monophyletic group placed by Mayden (1989) and Coburn and Cavender (1991) in the clade which contains *Cyprinella* and *Opsopoeodus*, as well as *N. amabilis* and *N. shumardi* of the genus *Notropis*, possess *F'* NOR chromosomes (Amemiya 1987, Amemiya and Gold 1990a). On this basis, it seems reasonable to hypothesize that an *F'* NOR chromosome is ancestral (plesiomorphic) and the *C'* NOR chromosome derived (apomorphic). None of the *Lythrurus F'* NORs have been examined for G-banding pattern, but the C-banding pattern of the *F'* NOR chromosome of one *Lythrurus* species (*L. roseipinnis*) appears homologous to the C-banding pattern of the *F'* NOR chromosomes in *N. amabilis* and *N. shumardi* (Amemiya 1987). On this basis, we suggest that the *C'* NOR chromosome was likely derived from an *F'* NOR chromosome by a chromatic addition on the short arm. The *E'* NOR chromosome in *O. emiliae* was then likely derived from the *C'* NOR chromosome by a small paracentric inversion in the short arm. The latter stems in part from parsimony and is consistent with the previously published phylogenetic hypothesis that *Cyprinella* and *Opsopoeodus* are closely related phylogenetically (Cavender and Coburn 1986, Amemiya and Gold 1990b).

Implications for Fish Chromosome Structure

Our ability to resolve G-bands on cyprinid chromosomes using trypsin raises a question regarding the hypothesis of Medrano *et al.* (1988) that G- or R-banding of chromosomes is correlated with genomic compartmentalization by base composition. In brief, Medrano *et al.* (1988), based in part on an earlier suggestion by Cuny *et al.* (1981), proposed that the occurrence of G- or R-bands was related to the presence of DNA segments called "isochores" which are enriched in GC base pairs and which are found in the genomes of most higher vertebrates. As evidence, Medrano *et al.* (1988) showed that better quality G- and R-bands were resolved on chromosomes of *Anguilla anguilla*, a species with considerable DNA base composition asymmetry biased towards GC-rich DNA, as compared to two fish species (*Labeo bicolor*, a cyprinid, and *Epinephelus guttatus*, a serranid) in which G- and R-bands were not resolved and which were less heterogeneous in DNA base composition.

Thus far, we have been able to produce trypsin G-bands on nearly every cyprinid species examined, including several species whose genomic DNA appears remarkably uniform in DNA base composition (Karel and Gold 1987, Gold and Karel 1988, unpubl.). Although

technological difficulties remain in terms of resolving trypsin-induced G-bands on all chromosomes of a given metaphase, completely G-banded metaphases have been obtained (Fig. 2), indicating that G-bands exist on all chromosomes within cyprinid complements. Taken together, these results suggest that G-bands do occur on chromosomes of fish species with homogeneous DNA base compositions.

Alternatively, we have been unable to resolve serial (G- or R-) bands on cyprinid chromosomes using DNA binding fluorochromes. Both quinacrine and DAPI, fluorochromes which are known to resolve (presumably AT-rich) G-bands in higher vertebrates (Benn and Perle 1986, Bickmore and Sumner 1989), yield fairly uniform fluorescence on cyprinid chromosomes except for quenched regions corresponding to the chromosomal NORs and to a number of centromeres. Both of the latter are presumed to be enriched in GC base pairs, and in the case of the centromeric regions, to be comprised largely of highly repetitive, satellite type DNA sequences (John and Miklos 1979).

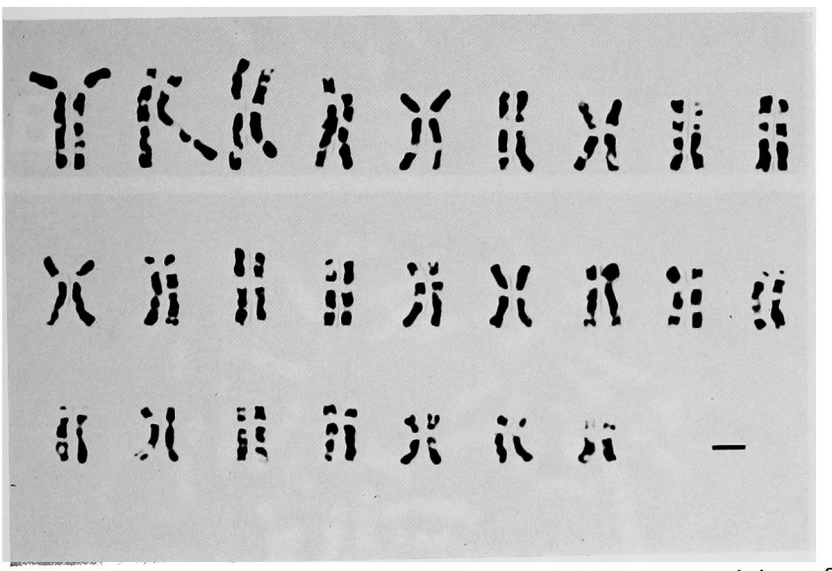

Fig. 2. Trypsin G-banded karyogram from the cyprinid fish *Plagopterus argentissimus*. Specimens were obtained from the Dexter National Fish Hatchery in Dexter, New Mexico. Bar equals 5 μm.

The finding that cyprinid chromosomes possess trypsin-induced G-bands which cannot be differentiated with quinacrine or DAPI in part supports the hypothesis of Holmquist *et al.* (1982) that the organization of eukaryotic chromosomes into compartments preceded the evolution of AT- and GC-rich G- and R-band regions. Their hypothesis related to temporal clusters of units of DNA replication (replicons) which presumably represented the "ancient basic pattern" which then evolved into the differentially AT- and GC-base pair rich G- and R-bands. Since both replication bands (using BrdU substitution) and trypsin-induced G-bands are present on cyprinid chromosomes (Gold *et al.* 1990, this paper), the apparent absence of fluorochrome-resolved serial bands may suggest that the evolution of at least trypsin-induced G-bands also preceded the evolution of differential AT-/GC-richness. This suggestion should be considered tentative, however, given the frequent difficulty in producing quinacrine- or DAPI-resolved G-bands on the chromosomes of certain mammals known to have heterogeneous DNA base compositions (J. W. Bickham, pers. comm.).

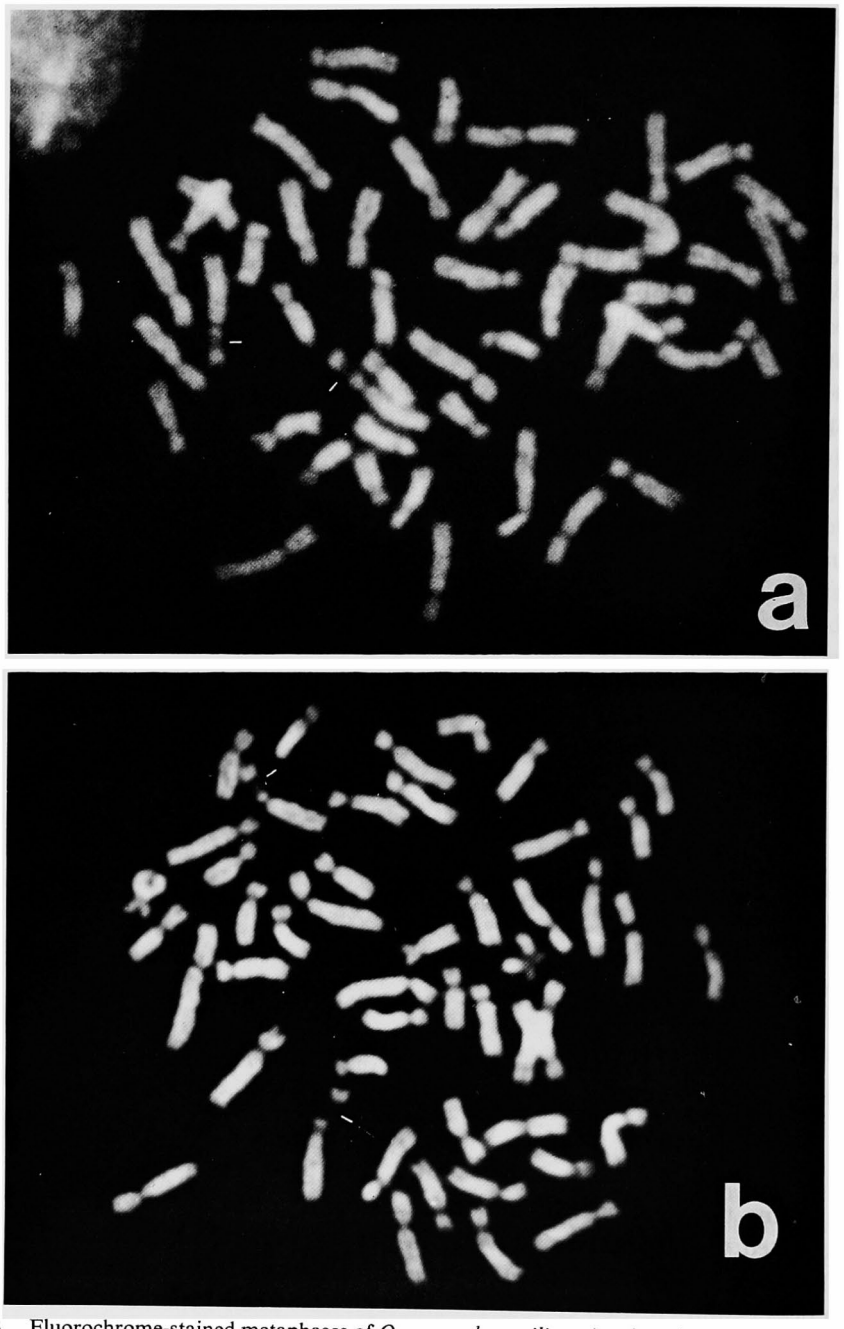

Fig. 3. Fluorochrome-stained metaphases of *Opsopoeodus emiliae*: a) quinacrine dihydrochloride; and b) DAPI. Bars indicate chromosomal NORs.

Chromosomal Polymorphism

In several of our early studies (Gold *et al.* 1978, 1979), the apparent conservatism of gross chromosomal evolution (viz., differences in chromosome and chromosome arm numbers) in North American cyprinids was considered noteworthy in view of the relatively rapid rate of organismal speciation in the group and the correlations found in other organisms between chromosomal evolution and organismal evolution. We noted, however, that the possibility

of cryptic chromosomal rearrangement (undetectable in gross or standard karyotypes) represented a major caveat. NOR-banding of cyprinid complements has provided evidence of several chromosomal differences between species (Amemiya and Gold 1990a, Amemiya *et al.* 1991), and, in a few instances, evidence of chromosomal polymorphism within species (Gold and Zoch 1990). In Figure 4, a G-banded metaphase from an individual of *C. lutrensis* clearly shows two chromosomal polymorphisms. One of these, involving a large chromosome pair, appears to be an extensive chromatic addition; whereas the other, involving a small chromosome pair, appears to be a pericentric inversion. Further study of cyprinids using G-banding may well document other previously cryptic chromosomal rearrangements both within and among species and force a reconsideration of the hypothesis that cyprinids are chromosomally conservative.

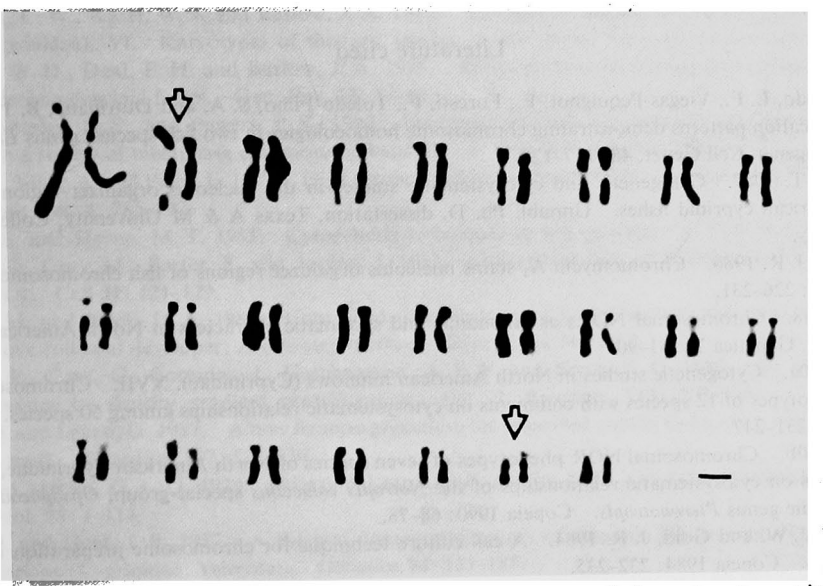

Fig. 4. Trypsin G-banded karyogram from an individual of *Cyprinella lutrensis*. Arrows indicate putative heteromorphic chromosomes. The second largest pair of chromosomes appears heteromorphic in the length of the short arm. The second smallest pair of chromosomes appears heteromorphic for a pericentric inversion.

Summary

Trypsin G-banding of chromosomes from North American cyprinid fishes was used to address phylogenetic problems within the group and to demonstrate the occurrence of G-bands which are not differentially rich in AT DNA base pairs. G-band homology of the long arm of the F', C', and E' NOR chromosomes found among six North American cyprinid species, in concert with a hypothesis of species relationships based on morphology, suggests that a NOR situated terminally on the largest chromosome in the complement may represent the plesiomorphic NOR character state within the large "*Notropis*"-like shiner assemblage. Outgroup comparison suggests that this chromosome may also represent a synapomorphy for the same lineage. Evidence exists which suggests that the F' NOR is ancestral and that the C' and E' NORs are derived. The occurrence in cyprinids of trypsin-induced G-bands which are not differentially rich in AT-/GC-DNA base pairs may indicate that the evolution of trypsin-induced G-bands preceded the evolution of differential AT-/GC-richness. The use of trypsin G-banding in cyprinids is expected to permit the identification of previously cryptic chro-

mosomal rearrangements both within and among species.

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