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Chromosomal NORs as taxonomic and systematic characters in North American cyprinid fishes

C. T. Amemiya[1] & J. R. Gold[2]
Department of Wildlife and Fisheries Sciences, Texas A&M University, College Station, TX 77843, USA; [1]*Present address: Department of Molecular Genetics, Showa University Research Institute for Biomedicine, 10900 Roosevelt Boulevard, St. Petersburg, FL 33702, USA;* [2]*To whom reprint requests should be sent*

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Abstract

There is substantial interspecific variation in chromosomal NORs among North American cyprinid fishes. This variation is taxonomically informative. In addition, the chromosomal NOR database appears to provide a framework from which systematic or phylogenetic inferences can be made. Our present approach to making such inferences from chromosomal NOR data is discussed.

Introduction

The application of karyotypic data to taxonomy and systematics is based on the premise that the karyotype comprises a heritable character (or character set) of each species (Chiarelli & Capanna, 1973). In this sense, the karyotype should aid in the taxonomic differentiation of species (cytotaxonomy). Moreover, since karyotypes generally undergo specific patterns of chromosomal rearrangement within different evolutionary lineages (White, 1954; Chiarelli & Capanna, 1973), karyotypic differences between species should also be useful in systematic or phylogenetic inference (cytosystematics). Indeed, chromosomal phylogenies have been erected for numerous higher vertebrate groups, particularly mammals (e.g., Baker *et al.* 1982; Bickham, 1979; Dutrillaux *et al.*, 1982). The contribution of karyology to fish taxonomy and systematics, however, has been minimal. The limiting factors apparently are obtaining consistently good chromosome spreads from fish tissues and the problem that most fish complements contain a relatively large number of comparatively small chromosomes (Gold, 1979).

Studies in our laboratory over the past decade have focused on karyotypic differentiation among North American cyprinid fishes (Gold *et al.*, 1979, 1981; Gold, 1984; Gold & Amemiya, 1986). Cyprinids represent the dominant freshwater fish fauna in North America, comprising over 200 extant species in some 35–40 genera (Miller, 1959; Lee *et al.*, 1980). The group appears to have evolved recently and at heterogeneous rates (Miller, 1965; Gold, 1980; Smith, 1981). Cyprinids are extremely diverse in morphology, habitat, and behavior (Lee *et al.*, 1980), and as might be expected for such a large, diverse group, taxonomic and systematic problems abound. These problems are typified by the speciose genus *Notropis,* where many of the morphological characters generally used in cyprinid taxonomy and systematics apparently have been modified repeatedly during the evolution of the more than 100 extant species currently referred to this genus (Gilbert & Bailey, 1962; Swift, 1970; Lee *et al.*, 1980).

Standard karyotypes (chromosome and chromosome arm numbers) are known for over 90 North American cyprinid species, including nearly 40 species of *Notropis* (Gold *et al.*, 1980; Amemiya & Gold,

1987, unpublished). In contrast to morphological evolution, gross karyotypic change in cyprinids appears to have been minimal: most species (including all *Notropis* examined) possess diploid chromosome numbers of 50; estimated (diploid) chromosome arm numbers vary only from 92 to 100 (Gold *et al.*, 1980; Amemiya & Gold, 1987). Heteromorphic sex chromosomes have yet to be identified. As a result of this general uniformity, the utility of standard or gross karyotypes in cyprinid taxonomy and systematics has been severely limited.

Gold (1984) and Gold & Amemiya (1986) examined the variation of chromosomal nucleolus organizer regions or NORs among fourteen North American cyprinid species. Two of the major findings to emerge from these studies were that: (1) species-specific differences in chromosomal NOR phenotypes occur; and (2) the interspecific NOR differences are taxonomically and systematically informative. We have since assayed chromosomal NORs in ten additional cyprinid species. The results largely corroborate the above two findings. The purposes of this paper are to present these additional data and to discuss the utility of using chromosomal NORs as taxonomic and systematic characters in the North American Cyprinidae.

Material and methods

Fish were collected by seine from natural populations in the southern United States. The ten cyprinid species examined in the study and their collection locales (drainages) are as follows. *Hemitremia flammea* (Jordan & Gilbert) – (1) Harrin Cr., Morgan Co., AL (Tennessee R.) and (2) Flint R., Madison Co., AL (Tennessee R.); *Nocomis leptocephalus* (Girard) – (1) Pushepatapa Cr., Washington Par., LA (Pearl R.) and (2) Bogue Chitto R., St. Tammany Par., LA (Bogue Chitto R.); *Notropis amabilis* (Girard) – (1) Miller Cr., Blanco Co., TX (Colorado R.) and (2) Guadalupe R., Comal Co., TX (Guadalupe R.); *Notorpis jemezanus* (Cope) and *Notropis proserpinus* (Girard) – Pecos R., Val Verde Co., TX (Rio Grande R.); *Notropis lepidus* (Baird & Girard) – (1) Nueces R., Real Co., TX (Nueces R.) and (2) Nueces R., Uvalde Co., TX (Nueces R.); *Notropis oxyrhynchus* (Hubbs & Bonham) – (1) Brazos R., Robertson Co., TX (Brazos R.) and (2) Brazos R., Brazos Co., TX (Brazos R.); *Notropis texanus* (Girard) – (1) San Gabriel R., Milam Co., TX (Brazos R.) and (2) Lake Conroe, Montgomery Co., TX (San Jacinto R.); and *Notropis umbratilis* (Girard) and *Pimephales notatus* (Rafinesque) – Blue R., Johnson Co., OK (Red River).

Metaphase chromosomes were prepared from either solid tissues (Gold, 1984) or cultured fibroblasts (Amemiya *et al.*, 1984). Specimens were individually tagged and deposited in our laboratory fish collection at Texas A&M University. AgNOR staining was essentially the controlled silver technique of Howell and Black (1980) as modified by Gold and Ellison (1983); chromomycin (CMA) NOR staining followed Amemiya and Gold (1986). The AgNOR method presumably differentiates only those NORs which were metabolically active during the preceding interphase (Howell, 1982; Hubbell, 1985); whereas CMA presumably differentiates NORs regardless of prior metabolic activity (Schmid, 1982; Amemiya & Gold, 1986). A minimum of 20 well spread NOR-stained metaphases were examined per specimen. Chromosomal C-banding followed Gold *et al.* (1986). Photomicrographs of AgNOR- and C-banded metaphases were taken on Kodak Technical Pan 2415 film (ASA 40) developed in Diafine (Acufine) or HC110 (Kodak), dilution D; photomicrographs of CMA-stained metaphases were taken on Kodak Panatomic-X FX402 film (ASA 125) developed in Diafine, D19 (Kodak) or HC110, dilution B.

Results and discussion

In Table 1, the NOR chromosome data from the 24 North American cyprinid species thus far examined in our laboratory are summarized. Data from 10 of these species are presented for the first time. Interspecies NOR differences include the number of NOR-bearing chromosomes per genome, the chromosomal position(s) of the NORs, and the type(s) of chromosomes upon which the NORs are located. The letter designations for NOR chromosome phenotypes shown in Table 1 are coded representations of actual chromosomal data. Assignment of these phenotypes was in most cases unequivocal.

Table 1. Summary of NOR-stained material examined.

Taxon	Number of (haploid) NOR chromosomes	NOR chromosome phenotype(s)*
Family Cyprinidae		
Subfamily Abramidinae		
1. *Notemigonus crysoleucas*† (12)	1	A
Subfamily Leuciscinae		
2. *Campostoma anomalum*† (6)	3	A A G
3. *Hemitremia flammea* (4)	1	B
4. *Hybognathus placitus*§ (1)	1	B
5. *Hybopsis aestivalis*§ (7)	2	B F
6. *Nocomis leptocephalus* (3)	2	B B
7. *Pimephales notatus* (4)	1	C
8. *Pimephales vigilax*† (15)	1	C
Genus *Notropis*		
Subgenus *Alburnops*		
9. *N. longirostris*§ (5)	1	D
10. *N. potteri*§ (3)	1	C′
11. *N.* sp. cf. *longirostris*§▾ (4)	1	D
12. *N. texanus* (6)	1	D
Subgenus *Cyprinella*		
13. *N. lepidus* (7)	1	C′
14. *N. lutrensis*† (40)	1	C′
15. *N. proserpinus* (6)	1	H
16. *N. venustus*† (36)	1	C′
Subgenus *Lythrurus*		
17. *N. ardens*§ (6)	2	F′C
18. *N. umbratilis* (2)	2	F′H
Subgenus *Notropis*		
19. *N. amabilis* (6)	1	F′
20. *N. jemezanus* (6)	1	I′
21. *N. oxyrhynchus* (7)	1	J
22. *N. shumardi*§ (6)	1	F′
Subgenera unknown		
23. *N. braytoni*§	1	C′
24. *N. emiliae*† (7)	1	E′

Classification of taxa folows Lee *et al.* (1980). Numbers in parentheses represent individuals examined.

*NOR chromosome phenotypes: A = Terminal on short arm of a medium-sized acro-/subtelocentric; B = Terminal on short arm of a small-sized acro-/subtelocentric; C = Terminal on short arm of a large-sized submetacentric; D = Terminal on short arm of a medium-sized submetacentric; E = Interstitial (subterminal) on short arm of a large-sized submetacentric; F = Terminal on short arm of a large-sized acro-/subtelocentric; G = Terminal on one arm of a large-sized metacentric; H = Terminal on one arm of a medium-sized metacentric; I = Terminal on long arm of a large-sized acro-/subtelocentric; J = Terminal on short arm of a small-sized submetacentric.
A prime symbol (′) indicates the chromosome is the largest in the complement.

† Data in part form Gold (1984).

§ Data in part from Gold & Amemiya (1986).

▾ An undescribed species from the Mobile Bay drainage (Heins *et al.*, 1980).

Some subjectivity was unavoidable, however, especially when differentiating the relative size of NOR-bearing chromosomes in *Pimephales* and the *Notropis* species with *D* NOR chromosome phenotypes. For example, the single NOR-bearing chromosome in *Pimephales* (*cf.* Fig. 2a in Gold, 1984) might easily be called a medium-sized submetacentric (*D* phenotype) instead of a large-sized submetacentric (*C* phenotype). Likewise, the acro-/subtelocentric NOR chromosomes in *N. amabilis* and *N. shumardi* (scored as *F'* phenotypes) are on the border of being submetacentric (*C'* phenotypes). Gold and Amemiya (1986) in fact originally described the NOR chromosome in *N. shumardi* as being a *C'* phenotype. Examination of additional specimens (e.g., Fig. 1C) now convinces us that the *N. shumardi* NOR chromosome is acro-/subtelocentric and not submetacentric. Finally, intraspecific heteromorphisms in NOR chromosomes were observed in roughly 10% of all specimens examined regardless of species. These heteromorphisms, however, were of a qualitatively different nature than the NOR variants found between species. More specific details regarding the nature of intraspecific NOR chromosome heteromorphisms observed in cyprinids may be found in Gold (1984) and Gold and Amemiya (1986).

Cytotaxonomy

As shown in Table 1, there is considerable interspecific variation in chromosomal NORs among North American Cyprinidae. This variation, in some cases, is taxonomically informative. For example, the four species examined from the subgenus *Notropis* of *Notropis,* all have a single pair of NORs (Table 1 and Fig. 1), but differ with respect to NOR chromosome phenotypes: *N. shumardi* and *N. amabilis* have NORs located terminally on the short arm of a large acro-/subtelocentric chromosome, which is the largest chromosome in the complement; *N. jemezanus* has a terminal NOR on the long arm of a large acro-/subtelocentric chromosome (also the largest in the complement); and *N. oxyrhynchus* has a terminal NOR on the short arm of a small submetacentric chromosome. This example serves to demonstrate that NORs can aid in the taxonomic identification of morphologically similar forms. Indeed, in the above example, several individuals from the Pecos River in southwest Texas were initially thought to be *N. amabilis.* All were found to possess a different NOR chromosome phenotype than previously observed in *N. amabilis* from the Guadalupe River. A morphological reexamination of the Pecos River specimens revealed that all were actually *N. jemezanus* rather than *N. amabilis* (*cf.* Fig. 1). NOR karyotypes also can be particularly useful where morphologically similar forms (differing in NOR phenotypes) are found sympatrically. Examples include *N. lutrensis* and *N. proserpinus, N. oxyrhynchus* and *N. shumardi,* and *N. amabilis* and *N. jemezanus* (Table 1). Finally, our NOR chromosome database from 24 taxa reveals 15 species-specific NOR states. This means that we can unequivocally identify 15 of the 24 taxa solely on the basis of chromosomal NOR phenotypes.

Cytosystematics

Chromosomal NORs represent the sites for the tandemly arranged 18S and 28S ribosomal RNA genes (Ritossa & Spiegelman, 1965; Wallace & Birnstiel, 1966), and their importance to cellular and organismic function can be understood in view of their intimate relationship with protein synthesis (Howell, 1982; Schwarzacher & Wachtler, 1983). In addition, studies at the molecular level have shown striking evolutionary conservatism and intraspecific homogenization of the 18S and 28S rRNA coding sequences (Brown *et al.,* 1972; Gourse & Gerbi, 1980; Coen *et al.,* 1982). Collectively, these observations suggest that NORs are under strong functional and organizational constraint, and that interspecific rearrangements involving NORs may be systematically or phylogenetically informative.

We have examined the latter prediction by assaying the extent and pattern of chromosomal NOR variation among North American Cyprinidae. Interrelationships among North American cyprinids are not especially well known, although discrete species-groups or subgenera within *Notropis* have been defined and provide a foundation to test the validity

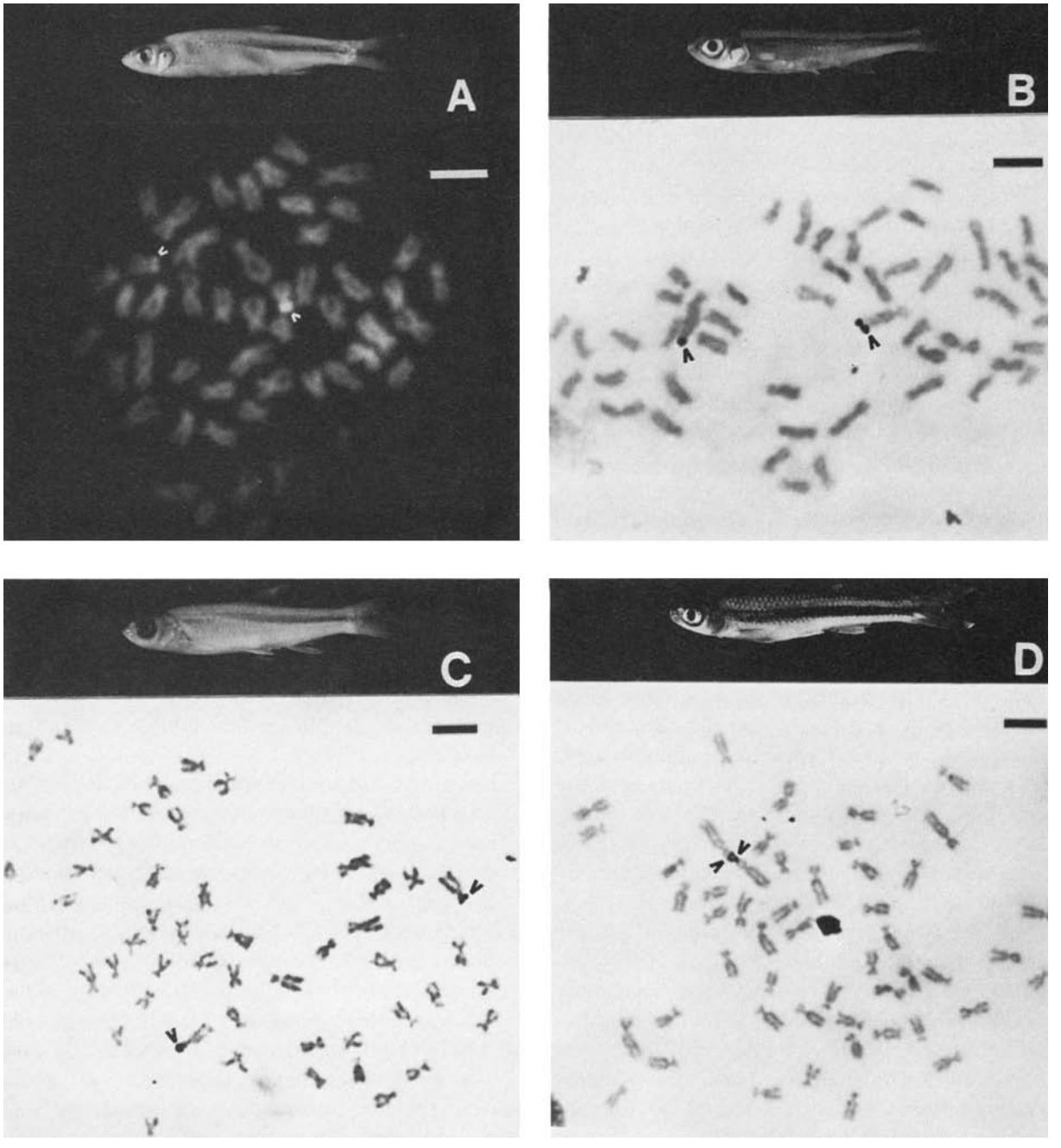

Fig. 1. Specimens and NOR-banded karyotypes of four species of the subgenus *Notropis* (genus *Notropis*): (A) *N. oxyrhynchus*; (B) *N. jemezanus*; (C) *N. shumardi*; (D) *N. amabilis*. NORs are indicated by arrowheads. The chromosomes in (B)–(C) were stained with Ag; the chromosomes in (A) were stained with CMA. Bars equal 5 μm.

of chromosomal NORs as systematic characters. Inspection of the data in Table 1 reveals several phenetic similarities in NOR chromosome phenotypes, which corroborate present concepts of North American cyprinid relationships. Briefly, these include the two *Pimephales* species with *C* NOR chromosomes; the two *Notropis* (*Notropis*) species with *F'* NOR chromosomes; the two *Notropis* (*Lythru-*

rus) species with multiple NOR chromosomes (i.e., more than one pair); and the three *Notropis* (*Alburnops*) and three *Notropis* (*Cyprinella*) species with *D* and *C'* NOR chromosomes, respectively. These observations indicate that chromosomal NORs are systematically informative.

With regard to the absolute number of NORs per species, most North American cyprinids possess a single pair of NOR chromosomes (Table 1). By the 'commonality principle' (Watrous & Wheeler, 1981), Gold and Amemiya (1986) suggested that a single NOR chromosome pair is plesiomorphic or primitive for the group and that the multiple NOR condition is apomorphic or derived. This is in agreement with the suggestions of Hsu *et al.* (1975) and Schmid (1978) that a single homologous pair of NORs is primitive for most vertebrates. This hypothesis has not been fully substantiated in cyprinids by outgroup comparison (Watrous & Wheeler, 1981). However, we have discovered single NOR pairs in a catostomid (*Carpiodes carpio*) and characid (*Astyanax mexicanus*) (Amemiya & Gold, 1986, unpublished), and three of four ictalurid genera (Amemiya *et al.*, 1986). This tentatively suggests that a single (haploid) NOR is primitive for the Otophysians (Cypriniformes + Chariciformes-Siluriformes) (Fink & Fink, 1981). The single NOR chromosome pair in *C. carpio* is notable in that the family Catostomidae is hypothesized to be of tetraploid origin (Uyeno & Smith, 1972; Ferris, 1984). This may indicate that the single NOR pair (instead of two) found in *C. carpio* may be the result of genome diploidization (*sensu* Ohno, 1970).

Gold and Amemiya (1986) also suggested that the plesiomorphic NOR condition in North American Cyprinidae was for the single (haploid) NOR to be situated terminally on the short arm of an acrocentric chromosome. This hypothesis was based on the commonality principle and the observation that all of the cyprinid genera examined except for *Notropis* and *Pimephales* possessed acrocentric NOR chromosomes. The submetacentric NOR chromosomes in *Notropis* and *Pimephales* were hypothesized to represent a synapomorphy, which corroborated the close relationship between these two genera suggested by Hubbs and Black (1947) and Cavender and Coburn (1985) on morphological evidence. The above chromosomal hypotheses are not contradicted, with one exception, by the inclusion of the ten cyprinids whose NOR chromosome phenotypes are reported in this paper. Both *Hemitremia flammea* and *Nocomis leptocephalus* possess *B* NOR chromosomes (supporting the proposed plesiomorphy); whereas *Pimephales notatus* and five of the seven *Notropis* species examined possessed NORs on biarmed chromosomes. The exception was the single NORs (*F'* and *I'*) found in *Notropis amabilis* and *Notropis jemezanus*. In both, however, the single acrocentric NOR chromosomes are the largest in their respective complements and are clearly differentiated in size from the much smaller NOR-bearing acrocentrics found elsewhere in the family (Table 1). The *F'* NOR chromosome is also found in *N. ardens, N. umbratilis,* and *N. shumardi,* and in size is evidently related to the *C'* NOR chromosome. On the basis of commonality (*cf.* Table 1), it is tempting to speculate that a single NOR, located terminally on the short arm of the largest chromosome in the genome represents a plesiomorphic condition for the genus *Notropis*.

The central problem in using chromosomal NORs for systematic or phylogenetic inference stems from the difficulty in establishing chromosomal homologies among taxa. More specifically, NOR banding does not provide the resolution necessary for determining whether a given NOR phenotype found in different species represents the same, homologous character. For example, *Pimephales* and *Notropis ardens* both have a *C* NOR chromosome. Whether these two *C* NOR chromosomes are homologous cannot be ascertained from NOR banding alone. Likewise, *Campostoma anomalum* has two pairs of *A* NOR chromosomes, each of which, by definition, must be nonhomologous. These examples demonstrate the need for unequivocally identifying NOR chromosomes. Consequently, we cannot confidently treat all NOR phenotypes as character states. We discuss below how we are addressing this problem with the aid of C-banding. A truncated data set from the genus *Notropis* is used for this demonstration.

The *C'* NOR chromosome phenotype is found in a wide array of taxa: in the *Notropis* subgenera *Alburnops* and *Cyprinella,* and in *Notropis braytoni* (Table 1). This condition is when the NOR is termi-

nal on the short arm of a large submetacentric chromosome which is the largest chromosome in the complement (Fig. 2A). The frequency of the *C′* NOR chromosome within *Notropis,* but its absence elsewhere, tentatively suggests that it may represent a synapomorphy within the genus. Homology of *C′* NOR chromosomes in three *N.* (*Cyprinella*) species (*N. lepidus, N. lutrensis* and *N. venustus*) and *N. braytoni* has been established by C-banding (Fig. 2B–E). In addition, the *H* and *E′* NOR chromosomes in *Notropis proserpinus* and *Notropis emiliae,* respectively, appear to be derived from a *C′* NOR chromosome by single chromosomal rearrangements. The NOR- and C-band patterns of *N. proserpinus* are shown in Figure 3A and B, respectively; those for *N. emiliae* may be found in Gold (1984) and Gold and Amemiya (1986). The C-band patterns for the NOR chromosomes of *N. proserpinus* (Fig. 3B) and *N. emiliae* (Fig. 6 in Gold & Amemiya, 1986) support the hypothesis that the *H* NOR chromosome in *N. proserpinus* arose from a *C′* NOR chromosome by a non-Robertsonian whole arm translocation; whereas the *E′* NOR chromosome in *N. emiliae* arose from a *C′* NOR chromosome by a paracentric inversion (Fig. 3C). Especially diagnostic are the two C-bands which flank the centromere of the *C′* chromosome, viz. a small band on the short arm and a large, conspicuous band on the long arm (Fig. 2B–E).

A simple cladogram depicting relationships among the six cyprinid species whose NOR chromosomes have been putatively homologized by C-banding is shown in Figure 4. On the basis of commonality, the *C′* NOR chromosome is assumed to be plesiomorphic for the six species assemblage which means that both the *H* and *E′* NOR chromosomes represent chromosomal autapomorphies. Four of the species (*N. lepidus, N. lutrensis, N. proserpinus* and *N. venustus*) are currently placed in the presumably monophyletic *Notropis* subgenus *Cyprinella* (Gibbs, 1957; Mayden, 1985). The placement of *N. emiliae* within this assemblage is of interest given the confusion regarding the taxonomic status of this species. In 1972, Gilbert and Bailey reallocated the monotypic *Opsopoeodus emiliae* to the genus *Notropis.* Campos and Hubbs (1973) questioned this on the basis of their finding that five specimens had 2n = 48 somatic chromosomes instead of the 2n = 50 which is characteristic of most North American cyprinids including all examined *Notropis* (Gold *et al.,* 1980). Thus far, we have examined seven specimens from this taxon (from four different collection locales) and all possessed 2n = 50 chromosomes. This, coincident with the phylogenetic considerations above, strongly indicates a direct relationship of *N. emiliae* with other members of the genus *Notropis,* and specifically with members of the subgenus *Cyprinella.* A close relationship between *N. emiliae* and *Cyprinella* has been suggested recently by Coburn and Cavender (1985) and Dimmick (1987) on the basis of morphological and protein electrophoretic data, respectively. Finally, the placement of *N. braytoni* into this assemblage is of interest in view of the paucity of systematic information on this poorly known species. At the very least, the possession of a *C′* NOR

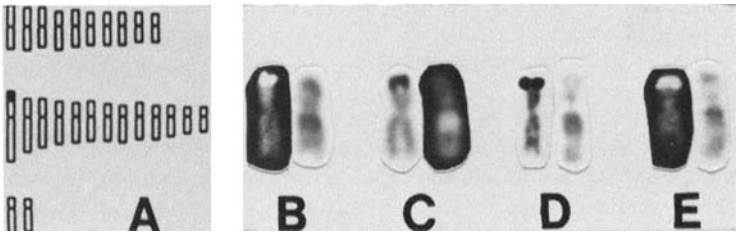

Fig. 2. The *C′* NOR phenotype: (A) Computer generated haploid idiogram (see Gold & Amemiya, 1986) of a *Notropis lutrensis* karyotype which exemplifies a typical *C′* NOR chromosomal phenotype; (B–E) NOR-banded (left) and C-banded *C′* chromosomes from (B) *N. braytoni,* (C) *N. lepidus,* (D) *N. lutrensis,* (E) *N. venustus.* NOR-banding in (C) and (D) employed Ag; NOR-banding in (B) and (E) employed CMA. C-banded chromosomes in (B), (D) and (E) were stained with Giemsa; the C-banded chromosome in (C) was stained with DAPI.

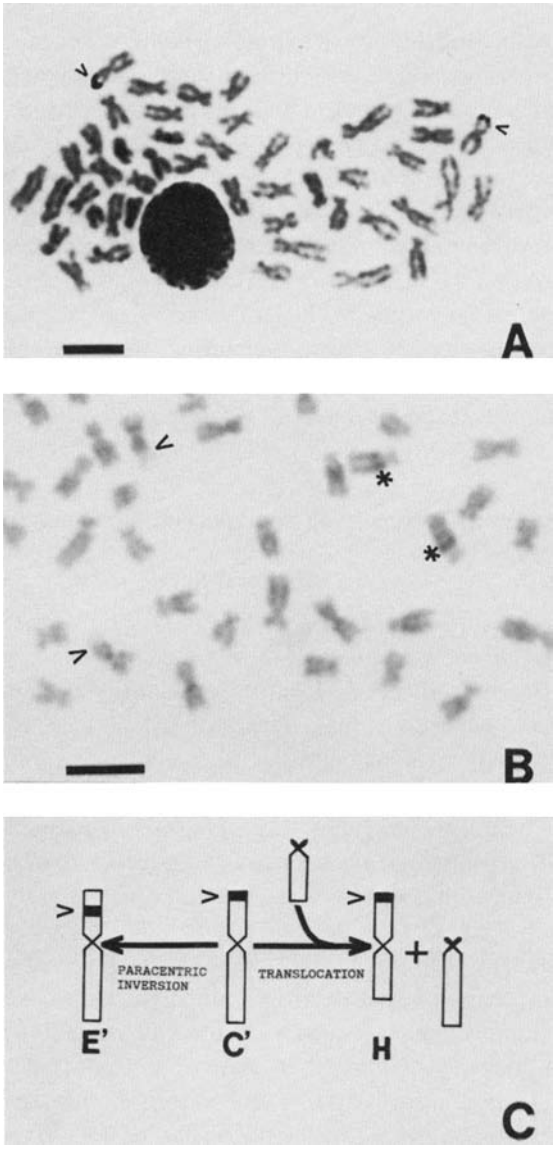

Fig. 3. (A) AgNOR-banded metaphase of *Notropis proserpinus.* (NORs are indicated by arrowheads): (B) Partial C-banded karyotype of *N. proserpinus.* (NORs are seen as achromatic regions and are indicated by arrowheads); asterisks (*) indicate the putative homolog to the *C'* long arm, identified on the basis of size and position of the highly conspicuous sub-centromeric C-band on the *C'* chromosome, *cf.* Fig. 2B – E); (C) Proposed scheme for the derivation of *E'* (*N. emiliae*) and *H* (*N. proserpinus*) NOR chromosomes from a *C'* chromosome. The NOR sites are indicated by arrowheads. Bars in (A) and (B) equal 5 μm.

chromosome with a C-band pattern similar to that found in the *Cyprinella* species may indicate a relationship between *N. braytoni* and that subgenus.

In summary, chromosomal NORs appear to be

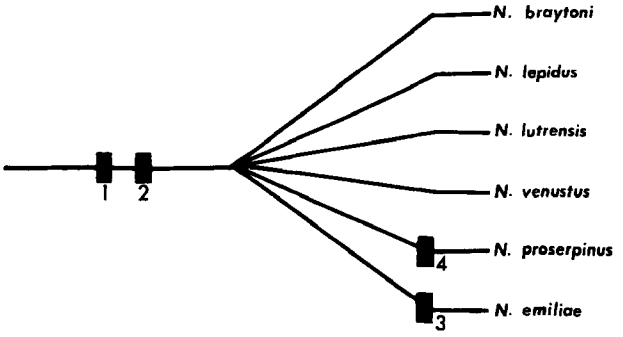

Fig. 4. Proposed assemblage within North American Cyprinidae. Synapomorphies or autapomorphies are as follows: 1 – NORs found terminally on the short arms of very large submeta- or acro-/subtelocentric chromosomes; 2 – *C'* NOR chromosome; 3 – *C'* → *E'* NOR chromosome transformation; 4 – *C'* → *H* NOR chromosome transformation.

useful in both cytotaxonomy and cytosystematics of North American cyprinids. With regard to phylogenetic considerations, assessment of NOR chromosome homologies and character states is enhanced when additional banding methods, such as C-banding, are employed. Future study of North American cyprinid systematics and phylogeny using chromosomal NORs will need to focus on homologization of NOR chromosomes among species.

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