Cytogenetic Studies in North American Minnows (Cyprinidae) XVIII. Chromosomal NOR variation among eight species

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Studies in our laboratory over the last five or six years have been focused on the localization of nucleolus organizer regions or NORs on the chromosomes of North American cyprinid fishes (Gold 1984, Gold and Amemiya 1986, Amemiya 1987, Amemiya and Gold 1988, 1990, Gold *et al.* 1988). A total of 50 North American cyprinid species have been studied, and substantial NOR variation has been documented both within and among species. In addition, we also have demonstrated (Amemiya and Gold 1988, 1990) that chromosomal NORs can often serve as useful characters for testing or inferring hypotheses of systematic or phylogenetic relationships. In brief, twelve different NOR chromosome phenotypes (defined by the position of a NOR on a chromosome and by the centromere position and relative size of the NOR-bearing chromosome) have been identified and used to define nineteen discrete NOR conditions or character states. The latter have been used in phylogenetic analysis using both outgroup comparison and the commonality method (Watrous and Wheeler 1981).

In this paper, we document the chromosomal NOR phenotypes of eight North American cyprinids, including six species from the diverse, putatively monophyletic lineage now referred to as “*Notropis*”-like shiners (Mayden 1989) or shiners (Coburn and Cavender 1990). This lineage contains most of the species formerly referred to the “unnatural” genus *Notropis* and contains well over 100 living representatives (Miller 1965, Mayden 1989, Coburn and Cavender 1990). Systematic problems within the lineage, however, remain. The other two species examined in this study include one species from the so-called “chub” lineage (*sensu* Mayden 1989) and one species which is currently not placed in either the shiner or chub assemblages. The overall purposes of the study were to continue our sampling of chromosomal NOR phenotypes among North American cyprinids and to examine the proposed relationships of the species examined which have been based primarily on morphological criteria.

Materials and methods

Seven of the cyprinid species examined in this study were collected by seine from natural populations. The species (collection localities) were as follows: *Cyprinella gibbsi* (unnamed tributary of the Tallapoosa River, Randolph County, Alabama), *Notropis boops* (Blue River, Johnston County, Oklahoma), *Notropis dorsalis* (unnamed tributary of the Mississippi River, Union County, Illinois), *Notropis petersoni* (Hatch Creek, Alachua County, Florida), *Notropis sabinae* (Sabine River, Newton County, Texas and Bundick Lake, Beauregard Parish, Louisiana), *Phenacobius mirabilis* (Dutch Creek, Union County, Illinois), and *Semotilus atromaculatus* (Obatoma Creek, Covington County, Mississippi and Henderson Creek, Mercer County, Illinois). Specimens of *Cyprinella formosa* were obtained from the Dexter National Hatchery in Dexter, New Mexico. Specimens obtained in Texas and New Mexico were

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returned live to the laboratory and maintained in aerated aquaria until sacrificed. For the remaining specimens, cell cultures were seeded in the field using clipped pieces of the caudal fin. The exact procedures for the latter are described elsewhere (Gold *et al.* 1990).

Metaphase chromosome preparations were made either directly from kidney tissues of colcemid- or colchicine-injected specimens (following Gold 1984) or from fibroblast cultures (following Amemiya *et al.* 1984). NOR-banding was carried out by the controlled silver-staining procedure of Howell and Black (1980) as modified by Gold and Ellison (1983). Photomicrographs of silver-stained metaphases were taken using Kodak Technical Pan 2415 film (ASA 25–40) and developed in Diafine (Acufine)). Determinations of NOR-band position(s) and of centromere position and relative size of NOR-bearing and other chromosomes were made from positive prints using a digitizer, a small laboratory computer, and the BANDSCAN program described in Gold and Amemiya (1986). The letter designations used for NOR chromosome phenotypes follow Gold and Amemiya (1986) and Amemiya and Gold (1988) and are based on the position of the NOR on the chromosome (terminal, subterminal, etc.), the centromere position of that chromosome (median, submedian, etc.), and the relative size of the chromosome within the complement.

We have used the nomenclatorial revisions of Mayden (1989), with two exceptions. The changes from previous taxonomic concepts (relative to taxa discussed in this paper) include the elevation of the *Notropis* subgenera *Cyprinella* and *Lythrurus* to generic status and the use of feminine (instead of masculine) endings for several species in *Cyprinella* (e. g., *formosa*, *lepida*, etc., in place of *formosus*, *lepidus*, etc.). The exceptions are: (i) the retention of *N. dorsalis*, *N. sabinae*, and two species of *N. longirostris* in *Notropis* as opposed to placement in *Hybopsis*, and (ii) the placement of *Notropis emiliae* into the monotypic genus *Opsopoeodus*. The reason for the former is that Coburn and Cavender (1990) have evidence that *N. dorsalis*, *N. sabinae*, and the two species of *N. longirostris* may properly belong in *Notropis*, suggesting that a conservative approach is warranted. The reasons for the latter are given in Amemiya and Gold (1990).

Results and discussion

Summary data of the silver-stained material from the eight species are shown in Table 1; representative silver-stained metaphases are shown in Figs. 1 and 2. All individuals from all eight species possessed $2n=50$ chromosomes as do most North American cyprinids (Gold *et al.* 1980, 1988, Amemiya and Gold 1987). The chromosome numbers of *C. formosa*, *C. gibbsi*, *N. dorsalis* and *N. petersoni* are reported here for the first time.

Four of the species examined were found to possess a single pair of NOR chromosomes (Table 1). In two of these (*N. boops* and *N. dorsalis*), the NOR was located terminally on the short arm of a medium-sized submetacentric chromosome (NOR phenotype D). In *C. formosa*, the single NOR was located terminally on the short arm of a large submetacentric chromosome which also was the largest chromosome in the complement (NOR phenotype C'); whereas in *C. gibbsi*, the single NOR was located subterminally (interstitially) on one arm of a large-sized metacentric chromosome (NOR phenotype K). The latter NOR phenotype has not been observed previously among North American Cyprinidae. The remaining four species examined were found to possess multiple NORs. *Notropis petersoni* and *N. sabinae* each possess one pair of NOR chromosomes with a D phenotype and one pair with an F phenotype (NOR terminal on the short arm of a large-sized acro-/subtelocentric chromosome). *Semotilus atromaculatus* possesses two pair of NOR chromosomes of the D NOR phenotype. *Phenacobius mirabilis* possesses three pairs of NOR chromosomes: two pair are of the D NOR phenotype, whereas one pair has a NOR located terminally on one arm of a large-sized met-

acentric chromosome (NOR phenotype G). Haploid idiograms based on computer-assisted measurements of silver-stained metaphases of the eight species are shown in Fig. 3. These idiograms are intended only to demonstrate the NOR position and the centromere position(s) and relative sizes of the NOR-bearing chromosomes within the complements of each species.

The chromosomal NOR phenotypes of the eight cyprinid species documented in this paper bring the total number of North American cyprinids assayed for chromosomal NORs to 58 (Amemiya 1987, Amemiya and Gold 1988, 1990, Gold *et al.* 1988). Forty-one species (71%) possess only a single NOR pair, 14 species (24%) possess two NOR pairs, and three species (5%) possess three NOR pairs. Most of the cyprinid chromosomal NORs are found

Fig. 1. Silver-stained metaphases of (A) *Cyprinella formosa*, (B) *Notropis boops*, (C) *Notropis sabinae* and (D) *Notropis dorsalis*. Chromosomal NORs are indicated by arrowheads. Bars equal 5 microns.

terminally on the short arms of submetacentric or acro-/subtelocentric chromosomes. The exceptions are NORs found (i) on metacentric chromosomes in four species of the genus *Cyprinella* (Gold *et al.* 1988), three species of the genus *Lythrurus* (Gold and Amemiya 1986, Amemiya 1987) and *Campostoma anomalum* (Gold 1984), (ii) the subterminal (interstitial) NORs found in *Opsopoeodus emiliae* and *C. gibbsi* (Gold 1984, this paper), and (iii) the NOR which is found terminally on the long arm of a large-sized acro-/subtelocentric chromosome in *Notropis jemezianus* (Amemiya and Gold 1988). Collectively, differences in the number and positions of chromosomal NORs observed thus far among North American cyprinids are similar to those observed in Asian cyprinids and in other fish (Galetti *et al.* 1984, Moreira-Filho *et al.* 1984, Takai and Ojima 1986, Mayr *et al.* 1987). In the following, the

cytosystematic implications of the chromosomal NORs of the eight species examined in this study are briefly discussed.

Two of the species examined were from the large cyprinid genus *Cyprinella* which contains 27 extant species (Mayden 1989). The total number of *Cyprinella* species now examined for chromosomal NORs is eleven (Amemiya 1987, Gold *et al.* 1988, this paper). Of these, five species (*C. formosa*, *C. lepida*, *C. lutrensis*, *C. spiloptera* and *C. venusta*) possess a single pair of NOR chromosomes of the C' phenotype (cf Table 1), four species (*C. camura*, *C. galactura*, *C. proserpina* and *C. whipplei*) possess a single pair of NOR chromosomes of the H phenotype which is defined as a NOR situated terminally on one arm of a medium-sized meta-

Fig. 2. Silver-stained metaphases of (A) *Phenacobius mirabilis*, (B) *Semotilus atromaculatus*, (C) *Notropis petersoni* and (D) *Cyprinella gibbsi*. Chromosomal NORs are indicated by arrowheads. Bars equal 5 microns.

centric chromosome, one species (*C. trichroistia*) possesses a single pair of NOR chromosomes of the J phenotype which is defined as a NOR situated terminally on the short arm of a small-sized submetacentric chromosome, and one species (*C. gibbsi*) possesses a single pair of NOR chromosomes of the K phenotype (cf Table 1).

A phylogenetic hypothesis of the 11 *Cyprinella* species based on chromosomal NORs and on DNA melting rate profiles (see below) is shown in Fig. 4A. The hypothesis assumes that *Cyprinella* is monophyletic (Mayden 1989). Briefly, the C' NOR state is hypothesized to be plesiomorphic for *Cyprinella* for reasons discussed elsewhere (Gold *et al.* 1988, Amemiya and Gold 1990). The J NOR state in *C. trichroistia* and the K NOR state in *C. gibbsi* are hypothesized to represent autapomorphies, whereas the H NOR state is hypothesized to represent a

synapomorphy uniting *C. camura*, *C. galactura*, *C. proserpina* and *C. whipplei*. It should be noted that the H NOR chromosomes in these four species have not been tested for homology using alternative banding methods. Amemiya (1987) and Amemiya and Gold (1988), however, have demonstrated by C-banding that the C' NOR chromosomes in *C. lepida*, *C. lutrensis*, *C. spiloptera* and *C. ventusta* are homologous, and that the H NOR chromosome in

Table 1. Summary of NOR-stained material examined

Taxon	Number of specimens examined	Number of metaphases examined	Number of (haploid) NOR chromosomes	NOR chromosome phenotypes*
<i>Cyprinella formosa</i>	3	31	1	C'
<i>Cyprinella gibbsi</i>	4	59	1	K
<i>Notropis boops</i>	7	42	1	D
<i>Notropis dorsalis</i>	3	43	1	D
<i>Notropis petersoni</i>	3	18	2	D, F
<i>Notropis sabinae</i>	18	>200	2	D, F
<i>Phenacobius mirabilis</i>	4	55	3	D, D, G
<i>Semotilus atromaculatus</i>	5	51	2	D, D

* NOR chromosome phenotypes: C, terminal on the short arm of a large-sized submetacentric; D, terminal on the short arm of a medium-sized submetacentric; F, terminal on the short arm of a large-sized acro-/subtelocentric; G, terminal on one arm of a large-sized metacentric; and K, subterminal (interstitial) on one arm of a large-sized metacentric. A prime (') symbol indicates that the chromosome is the largest in the complement.

Fig. 3. Haploid idiograms of NOR karyotypes of the eight species. Chromosomes are arranged into rows in each karyotype on the basis of centromere position (after Levan *et al.* 1964): metacentric chromosomes are in the top row, submetacentric chromosomes are in the middle row, and acrocentric (subtelocentric) chromosomes are in the bottom row. Within rows, chromosomes are arranged according to relative size. NORs are indicated by darkened areas. (A) *Phenacobius mirabilis*, (B) *Semotilus atromaculatus*, (C) *Notropis petersoni*, (D) *Cyprinella gibbsi*, (E) *Cyprinella formosa*, (F) *Notropis boops*, (G) *Notropis sabinae*, and (H) *Notropis dorsalis*.

C. proserpina could easily be derived from a C' NOR chromosome by a non-Robertsonian whole arm translocation. The presumed synapomorphy uniting *C. formosa*, *C. lepida*, *C. lutrensis*, *C. spiloptera* and *C. venusta* is the presence in the genomes of all five species of a unique, GC-base pair rich DNA melting component not present in the genomes of nearly 70 other North American cyprinids including the other *Cyprinella* species shown in Fig. 4A

(Karel and Gold 1987, unpublished). For comparison, a phylogenetic hypothesis of the 11 species based on morphology (Mayden 1989) is shown in Fig. 4B. The two phylogenetic hypotheses shown in Fig. 4 differ trenchantly in several respects, viz., the placements of *C. proserpina*, *C. spiloptera* and *C. venusta*. Additional work, including tests of homology of the H NOR chromosomes plus sampling of other *Cyprinella* species, is obviously required. It is interesting to note, however, that a recent hypothesis (M. M. Coburn, personal communication) of relationships in *Cyprinella*, based on morphology, agrees with ours relative to the inclusion of *C. spiloptera* and *C. venusta* into a putatively monophyletic lineage with *C. lepida*

Fig. 4. Phylogenetic hypothesis of relationships among 11 species of *Cyprinella*. A) Hypothesis based on chromosomal NORs and DNA melting rate profiles. Cross-hatched bar defines the presence of a unique, GC-base pair rich DNA component. Presumed chromosomal NOR state changes are (1) $C' \rightarrow J$, (2) $C' \rightarrow K$, and (3) $C' \rightarrow H$. The transition $C' \rightarrow K$ requires a minimum to two chromosomal alterations (cf text). B) Hypothesis of Mayden (1989) based on morphology.

and *C. lutrensis*. One last point to note regards the chromosomal NOR states of *C. trichroistia* and *C. gibbsi*. These two species are now considered to be sister species (Howell and Williams 1971, Mayden 1989), although Gibbs (1955, cited from Lee *et al.* 1980) originally regarded *C. gibbsi* as an allopatric population of *C. trichroistia*. Our finding that the two differ in NOR chromosome state confirms the species status of *C. gibbsi*. However, the NOR chromosome states found in the two species (i. e., J vs K) differ by at least two presumed chromosomal rearrangements, one of which (given the subterminal or interstitial NOR in *C. gibbsi*) was most likely an inversion. This is unusual to the extent that most closely related cyprinid species

typically have the same NOR chromosome phenotype or differ by only one presumed chromosomal alteration (Gold and Amemiya 1986, Amemiya 1987, Amemiya and Gold 1988, 1990, Gold *et al.* 1988).

Four of the species (*N. boops*, *N. dorsalis*, *N. petersoni* and *N. sabinae*) examined in this study were provisionally placed (by us) in the recently revised genus *Notropis*, largely for convenience. The species *N. boops* and *N. petersoni* were placed by Mayden (1989) in the *N. texanus* species-group of *Notropis* along with six other species. Coburn (1982) placed *N. petersoni* in the *N. texanus* species-group and *N. boops* in the "black chin" species-group, although clearly he believed the two species groups to be closely related. Mayden (1989), however, placed *N. dorsalis* and *N. sabinae* in his revised genus *Hybopsis* and in a lineage which included, among others, the monotypic genus *Ericymba* (*bucatta*). Mayden (1989) placed this lineage outside of a larger assemblage (referred to as "*Notropis*"-like shiners) which included the genus *Notropis*. Coburn and Cavender (1990, personal communication), alternatively, placed *N. dorsalis* and *N. sabinae* in the genus *Notropis* along with a number of species-groups including the *N. texanus* species-group and *Ericymba bucatta*. Both Mayden (1989) and Coburn and Cavender (1990, personal communication) agree on morphological grounds that *N. dorsalis* and *N. sabinae* belong in a putatively monophyletic assemblage with *Notropis longirostris* and one undescribed species referred to as *Notropis* sp. cf *longirostris* (Heins *et al.* 1980), and that this lineage is closely related to *Ericymba bucatta*.

We have now examined chromosomal NOR phenotypes of four of the eight species belonging to the *N. texanus* species-group. These include *Notropis chalybaeus* and *Notropis texanus*, both of which possess a single pair of NOR chromosomes of the D phenotype (Gold and Amemiya 1986, Amemiya 1987), and the two species (*N. boops* and *N. petersoni*) examined in this study (Table 1). All four species possess a pair of D NOR chromosomes, and *N. petersoni* possesses a second pair with an F phenotype. Based on both outgroup comparison and commonality (Watrous and Wheeler 1981), and assuming the *N. texanus* species-group is monophyletic (Mayden 1989), a D NOR chromosome state is hypothesized to be plesiomorphic for the species-group, with the transition to a D, F NOR state in *N. petersoni* representing an autapomorphy. The evidence is based largely on our finding that 17 of the 18 species now examined from *Notropis* (*sensu* Mayden 1989) possess only a single pair of NOR chromosomes, and of these, 12 are of the D NOR phenotype (Gold and Amemiya 1986, Amemiya 1987, Amemiya and Gold 1988, Gold *et al.* 1988, this paper). Our hypothesis also assumes that the D NOR chromosomes in the *N. texanus* species-group are homologous.

A similar situation exists in the putatively monophyletic lineage comprised of *N. dorsalis*, *N. longirostris*, *N. sp. cf longirostris* and *N. sabinae* (referred to hereafter as the *N. dorsalis* species-group). Three of the species (*N. dorsalis* and the two *N. longirostris* species) have a single pair of NOR chromosomes of the D phenotype (Gold and Amemiya 1986, this paper), whereas *N. sabinae* possesses both a D and an F NOR pair (this paper). By commonality, a D NOR state would be hypothesized as plesiomorphic for this lineage, with transition to a D, F NOR state in *N. sabinae* representing an autapomorphy. The closest outgroup to this lineage would appear to be *E. bucatta* (Mayden 1989, Coburn and Cavender 1990), which previously (Amemiya 1987) was found to possess a single pair of NOR chromosomes of the B phenotype. The B NOR phenotype is defined as a NOR situated terminally on the short arm of a small-sized acro-/subtelocentric chromosome, and has been hypothesized to represent the plesiomorphic NOR state for a large, putatively monophyletic assemblage of eastern North American cyprinids defined by an opening in the posterior myodome (Gold and Amemiya 1986, Amemiya 1987). These findings suggest either that (i) *Ericymba* is misplaced and does not belong in a putatively monophyletic assemblage with the *N. dorsalis* species-group, (ii) the B NOR chromosome in *Ericymba* represents a homoplasy or is non-homologous with B

NOR chromosomes found elsewhere in North American Cyprinidae (cf Gold and Amemiya 1986, Amemiya 1987), or (iii) the D NOR state in the *N. dorsalis* species-group represents a chromosomal synapomorphy uniting the four species into a monophyletic assemblage. The last possibility assumes first, that *Ericymba* is the sister to the *N. dorsalis* species-group (as proposed by Coburn and Cavender (1990)), and secondly, that the D NOR chromosomes in the *N. dorsalis* species-group are homologous. Experiments to distinguish among these alternatives using other chromosome banding methodologies to test NOR chromosome homologies are now in progress.

The last two species examined in this study were *Phenacobius mirabilis* and *Semotilus atromaculatus*. Mayden (1989) and Coburn and Cavender (1990) placed *Phenacobius* in a putatively monophyletic assemblage (referred to as the "chubs" by Mayden (1989)) which contains approximately 13 North American cyprinid genera. To date, we have examined chromosomal NORs from only eight species of chubs (Amemiya 1987, Amemiya and Gold 1988, this paper), and hence do not have sufficient data from which to infer or test phylogenetic hypotheses. It is of interest to note, however, that six of the eight chub species examined (including *P. mirabilis*) possess multiple NORs, i. e., more than one pair of NOR chromosomes. As noted previously, we have hypothesized (Gold and Amemiya 1986, Amemiya 1987) that a single pair of NOR chromosomes is the plesiomorphic NOR state for an assemblage of eastern North American cyprinids (including the chubs) which possess an opening in the posterior myodome. This hypothesis was based in part on commonality (the majority of species belonging to this large assemblage possess a single pair of NOR chromosomes), and in part on outgroup comparison with two North American cyprinids (*Hemitremia flammea* and *Notemigonus crysoleucas*) which are outside of this assemblage (Mayden 1989) and which possess only a single pair of NOR chromosomes (Gold 1984, Amemiya and Gold 1988). Our finding that six of the eight chub species examined possess multiple NORs suggests by commonality that multiple NORs may be plesiomorphic, at least for the chubs. Further confounding the issue is our finding that *S. atromaculatus* also possesses multiple NORs (Table 1). Mayden (1989, personal communication) places *Semotilus* outside of the assemblage defined by the open posterior myodome, further suggesting (via outgroup comparison) that multiple NORs may be plesiomorphic rather than apomorphic for this assemblage. One final possibility is that the assemblage may be artificial and that the opening in the posterior myodome is not a reliable morphological character in North American cyprinid taxonomy and systematics. This possibility was suggested recently by Coburn and Cavender (1990) and indicates that we are still far from a consensus regarding the systematics of this diverse and complex group.

Summary

The karyotypes and chromosomal nucleolus organizer region or NOR phenotypes of eight species of North American cyprinid fishes are documented. All eight species possessed $2n=50$ chromosomes. Four species (*Cyprinella formosa*, *Cyprinella gibbsi*, *Notropis boops*, and *Notropis dorsalis*) possessed only a single pair of NOR-bearing chromosomes, whereas four species (*Notropis petersoni*, *Notropis sabinae*, *Phenacobius mirabilis*, and *Semotilus atromaculatus*) possessed multiple NORs, i. e., more than one pair. The phylogenetic implications of the chromosomal NOR data in the eight species are discussed relative to current concepts of North American cyprinid systematics based largely on morphological data. A phylogenetic hypothesis based on chromosomal NOR and other data is presented for 11 species of the cyprinid genus *Cyprinella*, and our previous hypothesis that a single pair of NOR chromosomes is the plesiomorphic NOR state for a large eastern North American cyprinid lineage is questioned.

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