

Cytogenetic studies in North American minnows (Cyprinidae). XXII. Chromosomal nucleolar organizer regions in the genus *Pimephales*

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Chromosomal nucleolar organizer region (NOR) phenotypes are documented for all four extant species in the North American cyprinid fish genus *Pimephales*. All four species (*P. notatus*, *P. promelas*, *P. tenellus*, and *P. vigilax*) possess $2n = 50$ chromosomes and a pair of NOR-bearing chromosomes with the NOR situated terminally on the short arm of a medium-sized to large submetacentric chromosome (NOR phenotype C). Trypsin G-banding demonstrated that the C NOR chromosome in all four species is homologous. Two of the species (*P. tenellus* and *P. promelas*) also possess a C' NOR chromosome, which is defined as an NOR situated terminally on the short arm of a large submetacentric chromosome that is also the largest chromosome in the complement. The C' NOR chromosome occurs infrequently in *P. promelas*, being found in only 8% or so of all metaphases examined. Trypsin G-banding demonstrated that the C' NOR chromosomes in the two *Pimephales* species are homologous to one another and to the C' NOR chromosomes found in the cyprinid genus *Cyprinella*. A presumed derivative of the C' NOR chromosome occurs in the monotypic cyprinid genus *Opsopoeodus*. The NOR chromosomal data support monophyly of the four extant species of *Pimephales*, and further suggest that the genus *Pimephales* belongs in a monophyletic assemblage with, among others, the cyprinid genera *Cyprinella* and *Opsopoeodus*. The data do not support the previous hypothesis that *Pimephales* is a basal clade outside of a larger assemblage of "Notropis"-like shiners.

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Les phénotypes chromosomiques de la région de l'organisateur du nucléole (NOR) sont connus chez les quatre espèces nord-américaines actuelles du genre *Pimephales*, (Cyprinidae). Les quatre espèces (*P. notatus*, *P. promelas*, *P. tenellus* et *P. vigilax*) possèdent $2n = 50$ chromosomes et une paire de chromosomes porteurs de NOR, celui-ci situé à l'extrémité du bras court d'un chromosome submetacentrique de taille moyenne ou grande (NOR phénotype C). La coloration au Giemsa après traitement à la trypsine a démontré que le chromosome NOR C est homologue chez les quatre espèces. Deux des espèces (*P. tenellus* et *P. promelas*) possèdent également un chromosome NOR C' dans lequel le NOR est situé sur le bras court d'un chromosome submetacentrique de grande taille qui est également le chromosome le plus grand du complément chromosomique. Le chromosome NOR C' se rencontre rarement chez *P. promelas*, puisqu'il n'a été retrouvé que dans environ 8% des métaphases examinées. La technique a également mis en lumière que les chromosomes NOR C' des deux espèces de *Pimephales* sont homologues l'un de l'autre et homologues des chromosomes NOR C' trouvés chez les cyprins du genre *Cyprinella*. Un chromosome probablement dérivé du chromosome NOR C' se retrouve chez le genre cyprinidé monotypique *Opsopoeodus*. Les données sur les chromosomes NOR confirment la relation monophylétique des quatre espèces actuelles de *Pimephales* et indiquent également que le genre *Pimephales* appartient à un groupement monophylétique qui comprend, entre autres, les genres de cyprinidés *Cyprinella* et *Opsopoeodus*. Les données n'appuient pas l'hypothèse proposée précédemment selon laquelle *Pimephales* représente un clade de base distinct d'un groupement plus grand de ménés de type *Notropis*.

[Traduit par la rédaction]

Introduction

Studies in our laboratory over the past decade have focused on the documentation of chromosomal nucleolar organizer regions (NORs) in North American cyprinid fishes (Amemiya and Gold 1990a; Amemiya *et al.* 1992). Since NORs represent the chromosomal sites of the 18S, 5.8S, and 28S ribosomal RNA genes (Ritossa and Spiegelman 1965; Howell 1982), identification of NOR-bearing chromosomes and comparison among species have proven useful in both cyprinid systematics and taxonomy (Amemiya and Gold 1988; Amemiya and Gold 1990a, 1990b). The recent development (Gold *et al.* 1990b; Gold and Li 1991) of procedures for serial or G-banding of cyprinid chromosomes has enhanced our studies to the extent of providing useful and necessary tests of homology of phenotypically similar NOR-bearing chromosomes in different species.

In several recent papers (Gold and Amemiya 1986; Amemiya and Gold 1988; Amemiya and Gold 1990b), we hypothesized that the monotypic cyprinid genus *Opsopoeodus* was related phylogenetically to the large cyprinid genus *Cyprinella* on the basis of similarities in the size and long-arm C-banding patterns

of the single pair of NOR-bearing chromosomes found in *Opsopoeodus emiliae* and four species of the large cyprinid genus *Cyprinella*. Subsequently, Gold and Li (1991) demonstrated homology of the long arms of the *O. emiliae* and *Cyprinella* NOR-bearing chromosomes by trypsin G-banding.

The above was of interest relative to two contrasting hypotheses of relationships of these two genera based primarily on morphological (including osteological) data. In brief, Cavender and Coburn (1986) and Coburn and Cavender (1992) hypothesized that *Opsopoeodus* was closely related to the cyprinid genus *Pimephales*, and that a clade including *O. emiliae* and *Pimephales* represented the sister-group to *Cyprinella*. Mayden (1989) hypothesized, alternatively, that *O. emiliae* belonged in the *Notropis volucellus* species-group (which Mayden considered as only distantly related to *Cyprinella*), and that the genus *Pimephales* represented a basal lineage outside of a major clade of "Notropis"-like shiners. The chromosomal NOR data supported the hypothesis of Cavender and Coburn (1986) and Coburn and Cavender (1992) of a close phylogenetic relationship between *Opsopoeodus* and *Cyprinella*.

At the time Amemiya and Gold's (1990b) paper was written, we had documented the NOR chromosome phenotypes of

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TABLE 1. Summary of NOR-stained material examined

	No. of specimens examined	No. of metaphases examined	No. of (haploid) NOR chromosomes	NOR chromosome phenotype
<i>P. notatus</i>	8	140	1	C
<i>P. promelas</i>	5	135	2	C, C'
<i>P. tenellus</i>	2	148	2	C, C'
<i>P. vigilax</i>	>25	>250	1	C

NOTE: The NOR chromosome phenotypes (after Gold and Amemiya 1986; Amemiya and Gold 1988) are as follows: C, NOR terminal on the short arm of a large submetacentric chromosome. A "prime" indicates that the chromosome is the largest in the complement. The C' NOR chromosome was found in only 8% of all metaphases examined in four of the five individuals of *P. promelas*; the fifth individual possessed only a pair of C NOR chromosomes (see text). Data for *P. notatus* and *P. vigilax* are, in part, from Amemiya (1987).

several individuals from two of the four extant species of *Pimephales* (*P. notatus* and *P. vigilax*) and from one individual of the species *P. promelas* (Gold 1984; Amemiya and Gold 1988, 1990b). Both *P. notatus* and *P. vigilax* (and the one individual of *P. promelas* examined) were found to possess a single pair of NOR-bearing chromosomes with the NOR situated terminally on the short arm of medium-sized to large submetacentric chromosome. The NOR chromosomes in the *Pimephales* species differed from those found in *Opsopoeodus* and *Cyprinella* in both size (the *Opsopoeodus* and *Cyprinella* NOR chromosomes are the largest in the complements) and C-banding pattern, with the net result that the NOR chromosome data did not support the inclusion of *Pimephales* within Cavender and Coburn's putative assemblage of *Opsopoeodus*, *Pimephales*, and *Cyprinella*.

In this study we examined the NOR karyotypes from additional individuals of *P. notatus*, *P. promelas*, and *P. vigilax*, and from individuals of the fourth extant species of *Pimephales*, *P. tenellus*. The NOR-bearing chromosomes in all four species were also tested for homology using trypsin G-banding. All four species were found to possess a homologous pair of NOR chromosomes with the NOR situated terminally on the short arm of a medium-sized to large submetacentric chromosome. However, two of the species, *P. promelas* and *P. tenellus*, were found to also possess a second pair of NOR chromosomes, which in size, centromere position, and long-arm G-banding pattern appear to be homologous to the NOR-bearing chromosomes in *Opsopoeodus* and *Cyprinella*. This appears to support the hypothesis of Cavender and Coburn (1986) and Coburn and Cavender (1992) that these three cyprinid genera are closely related phylogenetically.

Materials and methods

Most of the specimens examined in the study were obtained by seine from natural populations. The species, collection localities in parentheses, were as follows: *P. notatus* (Mill Creek, Kansas River drainage, Wabaunsee County, Kansas); *P. promelas* (pond at the Texas Parks and Wildlife Department Heart-of-the-Hills Research Facility near Ingram, Texas, Guadalupe River drainage, Kerr County, Texas, and Cottonwood Creek, Pecos River drainage, Eddy County, New Mexico); *P. tenellus* (Mercer Creek, Neosho River drainage, Chase County, Kansas); and *P. vigilax* (Little Brazos River, Brazos River drainage, Brazos County, Texas). Specimens of *P. promelas*, originating from a minnow bait farm in Arkansas, were also obtained from a local bait shop in Bryan, Texas. All individuals were returned live to the laboratory in College Station, Texas, and maintained in well-aerated aquaria until sacrificed.

Metaphase chromosomes were prepared either directly from solid tissues (Gold 1984) or from cultured fibroblasts (Amemiya *et al.* 1984; Gold *et al.* 1990b). Following standard hypotonic treatment (30 min in

7–8 mL of 0.56% KCl at 30°C), cells were fixed after Islam and Levan (1987). Microscope slides were prepared using the method of Kligerman and Bloom (1977) as modified by Rayburn and Gold (1982).

Silver staining (AgNOR-banding) was carried out via the controlled, one-step method of Howell and Black (1980) as modified by Gold and Ellison (1983). The AgNOR method presumably only differentiates those NORs that were transcriptionally active during the preceding interphase (Howell 1982). G-banding was carried out as described in Gold *et al.* (1990b) and Gold and Li (1991). To identify NOR chromosomes following G-banding, appropriately G-banded metaphases were first identified and photographed. The slides were then destained with freshly prepared 3:1 (methanol:acetic acid) fixative, rinsed with distilled water, air-dried, and silver stained as above. Bright-field microscopy of both G-banded and silver-stained preparations followed Gold and Amemiya (1986). Designation of NOR chromosome phenotypes followed Gold and Amemiya (1986) and Amemiya and Gold (1988).

Results and discussion

Summary data from the NOR-stained material are given in Table 1 and include data from Gold (1984) and Amemiya and Gold (1988). Difficulties in obtaining large numbers of individuals of both *P. promelas* and *P. tenellus*, and in obtaining high-quality G-banded metaphase preparations from those individuals examined, accounts for the lower number of specimens karyotyped in these two species. All individuals from all four species possessed $2n = 50$ chromosomes, as do most North American cyprinids (Gold *et al.* 1980; Amemiya and Gold 1990a). The chromosome number of *P. tenellus* is reported here for the first time.

Silver-stained metaphases from *P. promelas* and *P. tenellus* are shown in Fig. 1; silver-stained metaphases from *P. vigilax* and *P. notatus* may be found in Gold (1984) and Amemiya and Gold (1988), respectively. All four species possess a pair of NOR-bearing chromosomes with the NOR situated terminally on the short arm of a medium-sized to large submetacentric chromosome (NOR phenotype C), but which is not the largest chromosome in the complement (e.g., Fig. 1). G-band patterns (Fig. 2) indicate that the C NOR chromosome is homologous among all four species.

A second, nonhomologous NOR-bearing chromosome (NOR phenotype C', defined as a NOR situated terminally on the short arm of a large submetacentric chromosome, which is the largest chromosome in the complement) was found in four of the five individuals of *P. promelas* and in both individuals of *P. tenellus* (Table 1, Fig. 1). In size, shape, and long-arm G-band pattern, the C' NOR chromosome in the two species of *Pimephales* appears to be homologous to the single pair of C' NOR chromosomes found in *Cyprinella* and the single pair of E' NOR

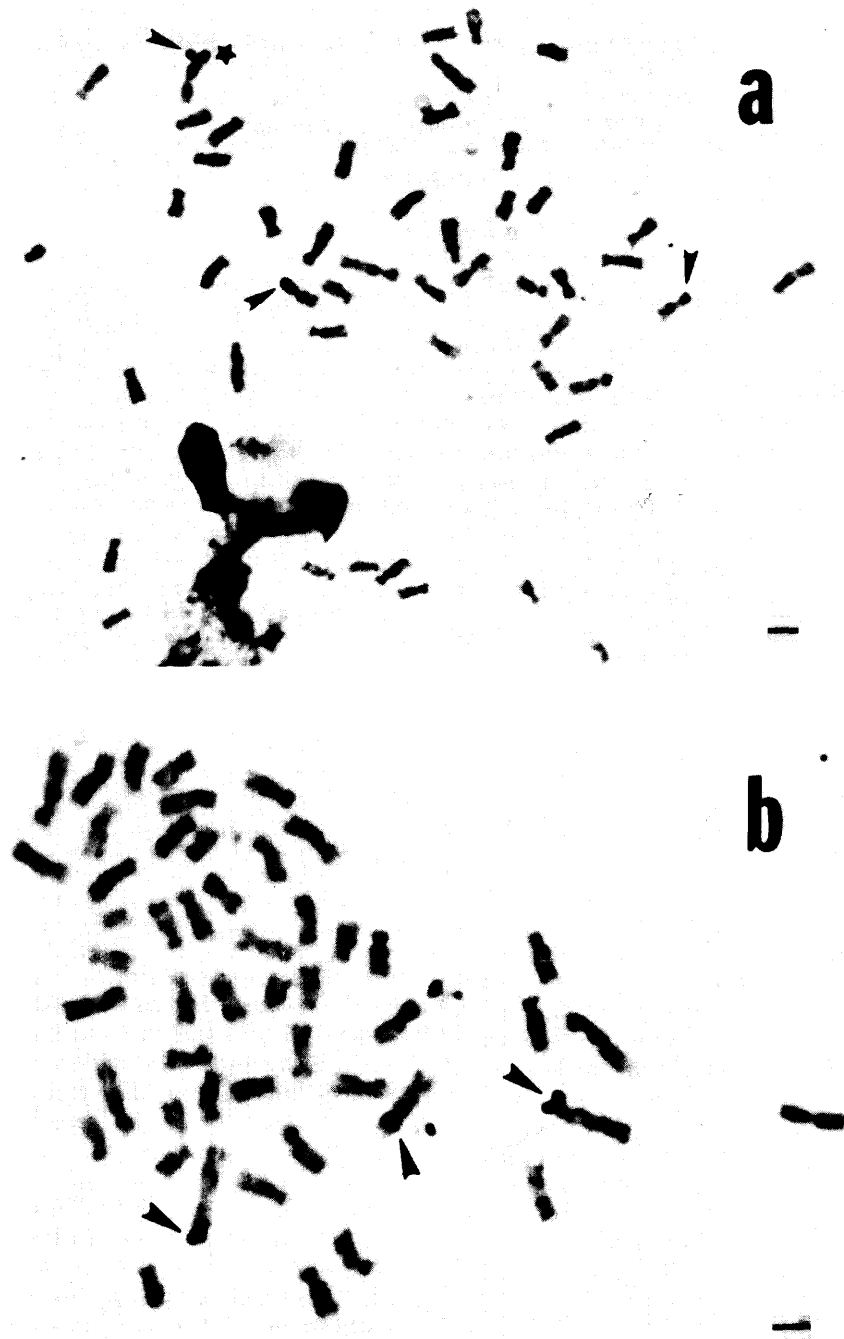


FIG. 1. Silver-stained metaphases of (a) *Pimephales promelas* and (b) *Pimephales tenellus*. The metaphase from *P. promelas* shows a silver-stained *C'* NOR chromosome (★). Chromosomal NORs are indicated by arrowheads. Scale bars = 5 μm .

chromosomes found in *Opsopoeodus* (Fig. 3). This conclusion regarding homology of the *C'* (and *E'*) NOR chromosomes is based in part on the observation that they are the largest chromosomes in the complements of the respective species in which they are found (Gold 1984; Gold and Amemiya 1986; Amemiya and Gold 1988; this paper). The G-band pattern of the short arm of the *C'* NOR chromosome in *Pimephales* also appears to be homologous to the short arm of the *C'* NOR chromosome in *Cyprinella* (Fig. 3). (Note: The NOR-bearing chromosome in *Opsopoeodus* differs from the others by a presumed paracentric inversion in the short arm (Gold and Amemiya 1986; Amemiya and Gold 1988).)

Interestingly, the frequency of occurrence of the silver-stained *C'* NOR chromosome in metaphases examined from the four specimens of *P. promelas* that possessed this NOR chromosome was low (mean 7.7%; range 6.5–9.0%) (Table 1), and in each case, only a single silver-stained NOR chromosome (instead of two) was observed. Normally, one would expect both of a homologous pair of NOR chromosomes to stain with silver, at least in some metaphases, even though not all NOR-bearing chromosomes will show silver staining in species (including cyprinids) with more than a single pair of NOR-bearing chromosomes (Gold and Amemiya 1986; Amemiya and Gold 1988). Alternatively, all the metaphases examined from the two

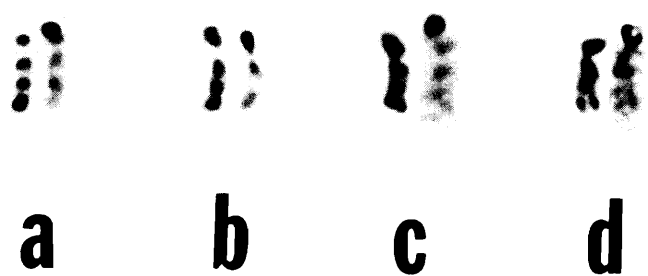


FIG. 2. Trypsin G-banded *C* NOR chromosomes from (a) *Pimephales vigilax*, (b) *Pimephales notatus*, (c) *Pimephales promelas*, and (d) *Pimephales tenellus*. For each pair, the chromosome on the left is from a G-banded metaphase and the chromosome on the right is from sequential silver staining (to identify NOR chromosomes) of the same metaphase.

specimens of *P. tenellus* exhibited silver staining on both *C'* NOR chromosomes. This suggests that the *C'* NOR chromosome is fixed in *P. tenellus* but represents an ancestral polymorphism in *P. promelas*. It is worth noting that this putative "ancestral polymorphism" is not localized, since *C'* NOR chromosomes were found in *P. promelas* from both Texas and New Mexico.

The finding of a *C'* NOR chromosome in *Pimephales*, which is homologous in G-band pattern to the *C'* NOR chromosome in *Cyprinella* (and its derivative, the *E'* NOR chromosome in *Opsopoeodus*), strongly supports the hypothesis of Cavender and Coburn (1986) and Coburn and Cavender (1992) that *Pimephales* belongs in a monophyletic assemblage with these two cyprinid genera. Mayden (1989), however, hypothesized that *Pimephales* belonged in a basal clade outside of the "Notropis"-like shiners, and that *O. emiliae* was a member of the *N. volu-*

cellus species-group. To date, we have documented NOR chromosome phenotypes from nearly 90 North American cyprinid species, including over 50 species from the "Notropis"-like shiners (sensu Mayden 1989). Thus far, the *C'* NOR chromosome (or its presumed derivative, the *E'* NOR chromosome) has been found in only eight species: five species from the genus *Cyprinella*, *O. emiliae*, *Notropis braytoni* (a species whose phylogenetic affinities are essentially unknown), and *Hybognathus nuchalis*, a putative member of the "chub" (sensu Mayden 1989) assemblage (Gold 1984; Gold and Amemiya 1986; Amemiya and Gold 1988, 1990a, 1990b; Gold *et al.* 1990a). Except for *H. nuchalis*, all the North American cyprinids examined to date outside of the "Notropis"-like shiner assemblage do not possess an NOR on the largest chromosome in the complement, whereas within the "Notropis"-like shiners, only the taxa listed above possess a *C'* NOR chromosome. Alternatively, several species in the "Notropis"-like shiner genera *Notropis* and *Lythrurus* possess an *F'* NOR chromosome (defined as an NOR situated terminally on the short arm of an acrocentric or subtelocentric chromosome, which is also the largest chromosome in the complement). For reasons outlined in Gold and Li (1991), a single pair of *F'* NOR chromosomes has been hypothesized as being plesiomorphic for the "Notropis"-like shiners. This suggests that the *C'* NOR chromosome is derived (apomorphic) and represents a chromosomal synapomorphy uniting the genera *Pimephales*, *Cyprinella*, and *Opsopoeodus*, among others. At present, the occurrence of a *C'* NOR chromosome in *H. nuchalis* remains problematic, although as suggested by Gold and Li (1991), it may represent a homoplasy.

Assuming *Pimephales*, *Cyprinella*, and *Opsopoeodus* consti-



FIG. 3. Trypsin G-banded *C'* (*Pimephales* and *Cyprinella*) and *E'* (*Opsopoeodus*) NOR chromosomes: (a) *P. tenellus*; (b) *P. promelas*; (c) *C. lutrensis*; (d) *C. lepida*; and (e) *O. emiliae*. For a and b, the chromosome on the left is from a G-banded metaphase and the chromosome on the right is from sequential silver staining of the same metaphase.

tute a monophyletic assemblage, the homologous pair of *C* NOR chromosomes in the four species of *Pimephales* is hypothesized to represent a chromosomal synapomorphy for the genus, since none of the other 11 species examined from the *Pimephales*–*Cyprinella*–*Opsopoeodus* clade possess a *C* NOR chromosome (Gold *et al.* 1988, 1990a; Amemiya *et al.* 1992). *C* NOR chromosomes have been documented among other species of "Notropis"-like shiners: these include *L. ardens* and *L. roseipinnis* of the genus *Lythrurus* (Amemiya and Gold 1990a), and six species or subspecies from the genus *Luxilus* (Powers and Gold 1992). For reasons outlined elsewhere (Amemiya *et al.* 1992), the *C* NOR chromosomes in the two species of *Lythrurus* are likely derived in that lineage, and hence are interpreted as being either nonhomologous or homoplastic relative to the *C* NOR chromosomes in *Pimephales*. The *C* NOR chromosomes in the species of *Luxilus*, although homologous by serial banding to one another (Powers and Gold 1992), are probably nonhomologous to the *C* NOR chromosomes in *Pimephales*. The evidence for the latter is based primarily on relative chromosome size. The *C* NOR chromosome in the species of *Luxilus* is the second largest chromosome in the complement (Powers and Gold 1992), and initially was considered, on the basis of relative size alone, to possibly be a *C'* NOR chromosome (Amemiya and Gold 1990b). The *C* NOR chromosome in *Pimephales*, alternatively, is much smaller (Fig. 1), and morphometrically is close to the border that delineates the *C* and *D* NOR chromosomes (Amemiya 1987). (Note: *D* NOR chromosomes are defined (Gold and Amemiya 1986; Amemiya and Gold 1988) as terminal NORs on the short arm of a medium-sized submetacentric chromosome.)

Assuming that the *C'* NOR chromosome is ancestral in *Pimephales*, the plesiomorphic NOR state for the genus is then hypothesized to be *C, C'*. On the surface, this would appear to

suggest that the loss of a *C'* NOR chromosome could represent a chromosomal synapomorphy uniting *P. notatus* and *P. vigilax* into a sister-group, both *P. promelas* and *P. tenellus* retaining the ancestral *C, C'* NOR state. This hypothesis is not supported by morphological evidence. On the basis of several osteological characters, including the shape of the pharyngeal pad, Mayden (1987), hypothesized that *P. notatus* and *P. promelas* are sister-groups to one another, and both are sister-groups to a clade composed of *P. tenellus* and *P. vigilax*. The caveat to the chromosome hypothesis is the observed low frequency of the *C'* NOR chromosome in *P. promelas* and the possibility that its presence in *P. promelas* may represent an ancestral polymorphism rather than a fixed NOR character state, i.e., the "true" NOR state in *P. promelas* is *C* rather than *C, C'*. Given our hypothesis that the *C, C'* state is plesiomorphic for *Pimephales*, this could suggest that *P. tenellus* is the sister-species to the other members of the genus, and that *P. notatus*, *P. promelas*, and *P. vigilax* represent a derived (but unresolved) clade. This latter hypothesis is supported by morphological evidence (M. Coburn, personal communication). Of importance in the future will be further documentation of the frequency of occurrence of the *C'* NOR chromosome in *P. promelas*.

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