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Silver-staining and Heteromorphism of Chromosomal Nucleolus Organizer Regions in North American Cyprinid Fishes

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Silver-stained karyotypes and patterns of variation in chromosomal nucleolus organizer regions (NORs) within and between six species of cyprinid fishes were studied. Interspecies heteromorphisms included the number of silver-stained NORs per genome and their chromosomal location. Intraspecies heteromorphisms included differences in sizes of silver-stained NORs and in apparent number of active NOR sites. In general, the patterns of silver-stained NORs in these fishes are no different from those found in other vertebrates, including the few fishes already studied. The interspecies differences in NORs may be of use in cyprinid phyletics or systematics, although confirmation must await sampling of additional species in concert with other types of chromosome banding. The intraspecific heteromorphisms in NORs were relatively frequent and raise the question of whether the variations are true genetic polymorphisms. Finally, the observed variations in chromosomal NORs among the six species suggest that major chromosome structural changes in North American cyprinid fishes may not be as infrequent as previously thought.

THE development of silver-staining techniques (Howell et al., 1975; Goodpasture and Bloom, 1975) to detect the metaphase chromosome sites of nucleolus organizer regions (NORs) has greatly facilitated comparative study of NOR variation within and between species. Thus far, the best studied group is the vertebrates where NOR patterns have been reported for over 150 species, primarily Mammalia (e.g., Tantravahi et al., 1976; Yosida, 1979; Sites et al., 1981), Amphibia (e.g., Schmid, 1978a, b; Macgregor and Sherwood, 1979) and Reptilia (Bickham and Rogers, pers. comm.). NOR patterns of one species of Aves (Biederman et al., 1980) and nine species of Pisces (see below) are also known. Because the NOR sites represent the chromosomal DNA sequences for the 18S and 28S ribosomal RNA genes (Henderson et al., 1972; Hsu et al., 1975; Goodpasture and Bloom, 1975), they are of interest both in terms of basic chromosomal organization and gene function. There also is the possibility that NOR site variations could prove useful as phyletic or systematic markers (Schmid, 1978a; Bickham and Rogers, pers. comm.).

Extensive interspecific and intraspecific differences or heteromorphisms in NORs have been documented in several groups. Most of the variations fall into one or more of the following categories: a) absolute number of NOR sites per genome, e.g., as in murid rodents or ranid frogs (Winking et al., 1980; Schmid, 1978b); b) position or chromosomal location of NOR sites, e.g., as in bufonid frogs or emydid turtles (Schmid, 1978a; Bickham and Rogers, pers. comm.); c) relative sizes of individual NOR sites (Ward, 1977; Macgregor et al., 1977); and d) number of active NOR sites per cell. Heteromorphisms in categories (a) and (b) are usually interspecific; those in categories (c) and (d) are generally intraspecific. The fourth category (active NOR sites per cell) is thought to reflect the preferential binding of silver only at NOR sites that were active at the preceding interphase (Miller et al., 1976a, b; Howell, 1977). There is evidence that both NOR size and activity polymorphisms are inherited germinally (Markovic et al., 1978; Lau et al., 1979). In species with multiple NOR sites per genome (i.e., more than one pair of NOR-bearing chromosomes), the observed number of silver-stained NOR sites varies from cell to cell around a mode that is usually less than the absolute number (Howell et al., 1975; Di Berardino et al., 1981).

Previous study of NOR chromosomal patterns in fishes is limited to observations on only nine species from widely separate groups. These include the Asian cyprinid *Carassius auratus* (Ojima and Yamano, 1980), the North American umbrid *Umbra limi* (Kligerman and Bloom, 1977a), the North American cyprinodontid *Fundulus diaphanus* (Howell and Black, 1979),

TABLE 1. SUMMARY OF SILVER-STAINED MATERIAL EXAMINED.

Taxon	Number of specimens examined	Number of metaphases examined	% metaphases with 2n = 50 chromosomes
Notropis lutrensis	20	170	85
Notropis venustus	29	234	83
Pimephales vigilax	9	79	86
Campostoma anomalum	6	43	86
Notemigonus crysoleucas	8	74	88
Notropis emiliae	1	16	94

the Old World cichlid *Sarotherodon galilaeus* (Kornfield et al., 1979), and five species from four genera of South American Gymnotiformes (Foresti et al., 1981). All nine species possessed only one pair of silver-stained NOR sites per genome. Heteromorphisms of the three other categories, viz., position, relative size, and apparent number of active NOR sites, were described in at least one instance. In this paper, the patterns of NOR silver-staining within and between six species of North American cyprinid fishes are described and discussed in relation to systematic and cytogenetic problems in these fishes.

MATERIALS AND METHODS

The six cyprinids examined in the study were collected by seining from the following localities in Texas: *Notropis lutrensis* (Little Brazos River, Brazos Co.), *Notropis venustus* (Bull Creek, Travis Co.), *Pimephales vigilax* (Little Brazos River, Brazos Co.), *Campostoma anomalum* (Blanco River, Hays Co.), *Notemigonus crysoleucas* (Camp Creek Lake, Robertson Co. and Brazos Utility Lake, Brazos Co.), and *Notropis* (=*Opsopoeodus*) *emiliae* (Mud Creek, Cherokee Co.). Specimens were returned live to College Station and maintained in well-aerated aquaria until karyotyped. Except for *N. emiliae*, chromosomal silver-staining patterns were examined from several individuals of each species (Table 1).

Metaphase chromosomes were prepared from kidney tissue following the methods of Kligerman and Bloom (1977b) with the following modifications: a) specimens were injected intraperitoneally with 0.8–1.0 micrograms colcemid (GIBCO) per gram body weight (range = 1–3 grams) and returned to aerated aquaria for 110–120 minutes before sacrifice; and b) chromosome spreading was carried out at 42–44 C using a 50 μl disposable accupette and rubber suction tube as described by Rayburn and Gold (1982). Slides prepared in this fashion were stored desiccated at room temperature for up to four weeks with no apparent adverse effects on chromosomes. Silver-staining of prepared slides essentially followed Howell and Black (1980), incorporating the suggestions of Gold and Ellison (1983). Counter-staining of chromosomes was for 1–2 minutes with 5% Giemsa in 10^{-2} M phosphate buffer at pH 6.8. Photomicrography of appropriate spreads was carried out using Kodak technical pan 2415 film (ASA 25–40) developed in Diafine.

RESULTS

All individuals from the six species examined had 2n = 50 chromosomes (Table 1) as expected for five of the species (*N. lutrensis*, *N. venustus*, *P. vigilax*, *N. crysoleucas*, and *C. anomalum*) based on earlier studies (Gold et al., 1980). The single individual of *N. emiliae* was found to have 2n = 50 chromosomes in 15 of 16 well-spread metaphases examined. Campos and Hubbs (1973) previously reported the chromosome number of *N. emiliae* to be 2n = 48 based on five specimens from Gibbons Creek, Nacogdoches Co., Texas.

Silver-stained karyotypes of the six species are shown in Figs. 1, 2. Five species, *N. lutrensis*, *N. venustus*, *N. emiliae*, *P. vigilax* and *N. crysoleucas*, possessed only one pair of silver-stained NORs; one species, *C. anomalum*, had up to six silver-stained NORs (three pair) and is the first reported fish species to possess multiple NOR chromosomes. The chromosomal positions of the NORs were as follows: a) terminal on the short arm of a large submetacentric (*N. lutrensis*, *N. venustus* and *P. vigilax*); b) terminal on one arm of a large metacentric (one pair in *C. anomalum*); c) subterminal on the short arm of a large submetacentric (*N. emiliae*); and d) terminal on the short arm of a medium-sized acrocentric (*N. crysoleucas* and two pair in *C. anomalum*). The NOR-bearing submetacentrics in *N. lutrensis*, *N. venustus* and *N. emiliae* were the largest chromosomes in each complement. The *P. vigilax* karyotype (Fig. 2a) displays a silver-stained NOR adjacent to the centromere on one homologue; however, in the eight other individuals of this species the NORs were clearly terminal (Fig. 3a,b). This indicates that the usu-

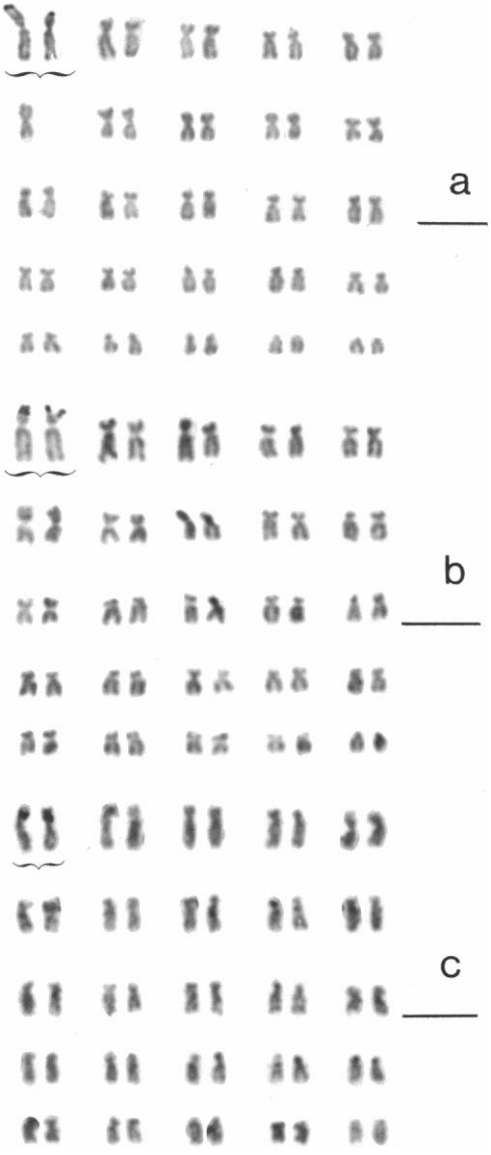

Fig. 1. Silver-stained karyotypes of (*a*) *Notropis lutrensis*, (*b*) *Notropis venustus*, and (*c*) *Notropis emiliae*. The *N. lutrensis* karyotype is short one chromosome. Horizontal parentheses underscore NOR chromosomes. Bars equal 5 μ.

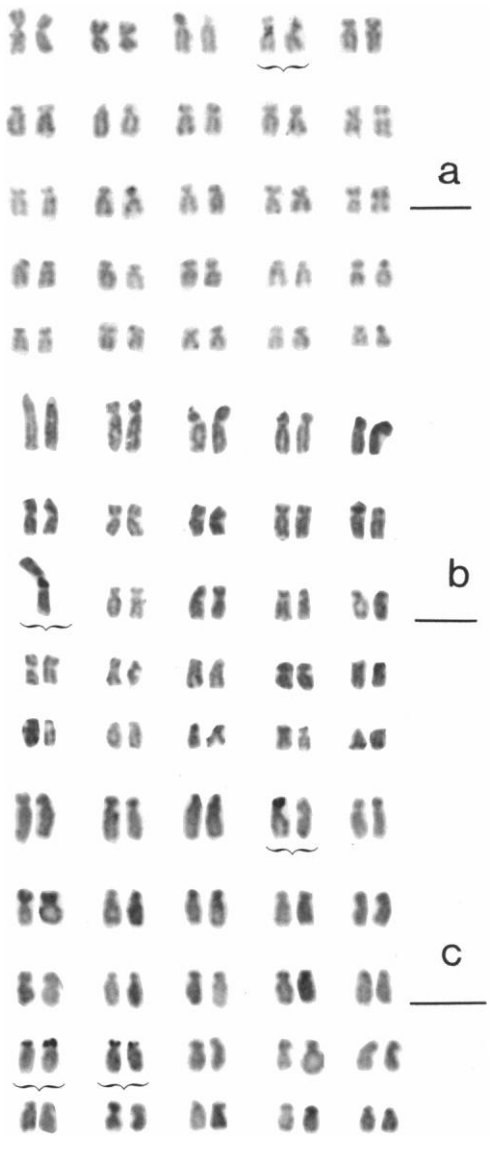

Fig. 2. Silver-stained karyotypes of (*a*) *Pimephales vigilax*, (*b*) *Notemigonus crysoleucas*, and (*c*) *Campostoma anomalum*. One NOR in *P. vigilax* is atypical (cf. text). Note the NOR association in the *N. crysoleucas* karyotype. Horizontal parentheses underscore NOR chromosomes. Bars equal 5 μ.

al NOR position in this species is terminal, and that the atypical individual is heterozygous for a chromosomal rearrangement (probably an inversion) involving the short arm of the NOR-bearing chromosome. In *C. anomalum*, the species with multiple NORs, none of six individuals examined displayed a consistent number of silver-stained NORs per cell. Over all individuals (43 cells), the number of silver-stained NORs varied from two to six around a weak mode of four (mean = 3.5); variation within in-

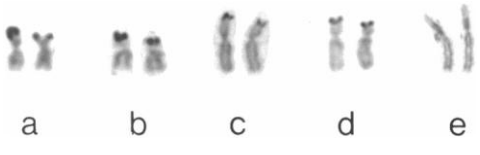

Fig. 3. Normal silver-staining patterns of (*a,b*) *Pimephales vigilax,* (*c*) *Notropis lutrensis,* and (*d*) *Notropis venustus.* (*e*) Conventional Giemsa staining showing terminal, achromatic secondary constrictions on the NOR chromosomes of *N. lutrensis.*

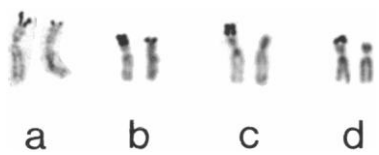

Fig. 4. Silver-stained size heteromorphisms in (*a*) *Notropis lutrensis* and (*b*) *Notropis venustus. (c,d.)* Silver-stained NOR activity heteromorphisms in *N. venustus.* Note that (*c*) is also a size heteromorphism.

dividuals followed approximately the same pattern. Similar data have been obtained from other vertebrate species with multiple NORs (Howell et al., 1975; Di Berardino et al., 1981).

Intraspecific heteromorphisms involving silver-stained NORs were surprisingly common. Consistent differences between homologues in the size of silver-stained NORs were found in two of twenty individuals of *N. lutrensis* and three of twenty-seven individuals of *N. venustus.* Examples of typical amounts of silver deposited on NORs of these two species are shown in Fig. 3c, d. Four of the heteromorphic individuals had silver deposits on both homologues, but one homologue clearly had twice the silver deposit of the other (Fig. 4a, b); the remaining individual (from *N. venustus*) had twice the usual amount of silver on one homologue, but no silver deposit on the other (Fig. 4c). Examination of over twenty cells from each of these five individuals confirmed the consistency of the size differences.

Heteromorphisms involving apparent NOR activity (as defined by the absence of silver-stainability at known NOR sites) were found in two of eight *N. crysoleucas,* two of nine *P. vigilax,* five of 20 *N. lutrensis* and six of 29 *N. venustus.* In each case, all the cells (range = 9–17) examined from a given heteromorphic individual had only one of two homologues silver-stained (Fig. 4c, d), and also displayed only one (instead of two) silver-stained nucleolus in interphase cells. No individuals in any of the four species above were found that had both NORs silver-stained in some cells, and only one in others. The consistency of these observations suggests that the patterns are real, and that this type of heteromorphism may be inherited, at least mitotically.

Two additional heteromorphisms involving the NOR chromosome were found in the Little Brazos River *N. lutrensis* population, unfortunately prior to the development of the silver-staining technique. In one individual, the entire short arm of the NOR submetacentric was deleted (Fig. 5a). Eleven complete cells were karyotyped and all showed the same heteromorphism, suggesting the individual was heterozygous for a chromosomal deletion which included the NOR. In the other individual, the terminal, achromatic secondary constriction frequently observed in conventionally stained preparations (Fig. 3e) was extended nearly sevenfold (Fig. 5b). The heteromorphism was consistent in all cells examined from this individual suggesting structural heterozygosity for an extensive amplification of the NOR. The latter is based on the assumption that achromatic secondary constrictions generally define NOR genetic material (Hsu et al., 1975; Lau et al., 1979). Such unusual size heteromorphism in a NOR also has been observed in a gymnotiform fish of the genus *Eigenmannia* (Foresti et al., 1981).

Discussion

From the above it would appear that patterns of NOR variation in cyprinid fishes are generally similar to those in other vertebrates, including the few fishes already studied (references in Introduction). Interspecies heteromorphisms detected in this study included the number of silver-stained NORs per genome and their chromosomal location. Intraspecies heteromorphisms detected included differences in the sizes of silver-stained NORs and in the apparent number of active NOR sites. No interspecies heteromorphisms in NOR size as reported in the gymnotiform fish genus *Eigenmannia* (Foresti et al., 1982) were detected.

The interspecies differences in NORs may be of use in cyprinid phyletics and systematics. In *N. lutrensis* and *N. venustus,* the NOR is located at identical sites on what appears to be the same chromosome in both species. These two species

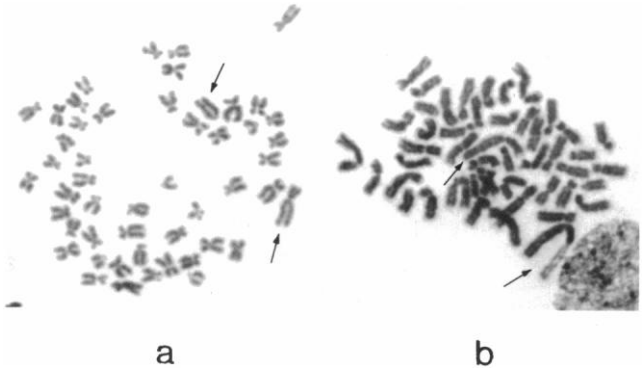

Fig. 5. Metaphases from conventional Giemsa staining of the structural changes in the NOR-bearing chromosomes of *Notropis lutrensis*: (*a*) short-arm deletion, and (*b*) extensive NOR amplification. Arrows indicate heteromorphic chromosomes and their normal homologues.

are very closely related within the diverse genus *Notropis* (Gibbs, 1957), and it is possible that the shared NOR sites represent an apomorphic character. The NOR site of *N. emiliae* is of interest since there is debate on the taxonomy of this species (Gilbert and Bailey, 1972). Campos and Hubbs (1973) placed the species in monotypic *Opsopoeodus* largely because their five specimens had 2n = 48 chromosomes instead of 2n = 50 which is characteristic of most North American cyprinids including all examined *Notropis* (Gold et al., 1980). The single *N. emiliae* examined here had 2n = 50 chromosomes, and the NOR site was located on the short arm of a chromosome very similar in relative size and centromere position to the one in the two other *Notropis* species. The difference in NOR position (subterminal in *N. emiliae* and terminal in *N. lutrensis* and *N. venustus*) could be accounted for by a small inversion. In the remaining two species with a single pair of NOR sites (*P. vigilax* and *N. crysoleucas*), the NORs were located on dissimilar chromosomes. The NOR chromosome of *N. crysoleucas* will be of future interest as this species is possibly the only North American representative of the Old World cyprinid subfamily Abramidinae (Miller, 1959). The multiple NORs of *C. anomalum* may be of significance if this feature (multiple vs single NORs) is in fact a derived rather than primitive state (Hsu et al., 1975; Schmid, 1978b). In summary, the interspecies differences in NORs appear to correspond to the established taxonomy of the six species. This suggests that NOR phenotypes may prove useful as systematic markers in cyprinids, although sampling of additional species is obviously necessary to more thoroughly test this possibility. It also will be important to develop other chromosome banding methodologies (e.g., G- or C-bands) in order to fully demonstrate homologies between NOR- bearing chromosomes of different species (e.g., *N. lutrensis* vs *N. emiliae*).

The relatively frequent intraspecific heteromorphisms in NOR size and apparent activity raise questions regarding both the nature and extent of the variations and whether they are true genetic polymorphisms. Extensive amplifications of NOR sites have been reported in individuals of several vertebrate species including fishes (Goodpasture and Bloom, 1975; Ward, 1977; Macgregor et al., 1977; Foresti et al., 1981), and NOR size and activity heteromorphisms apparently can be inherited germinally in humans (Markovic et al., 1978; Lau et al., 1979). The consistency of the silver-stained NOR heteromorphisms within individual fish observed here suggests first, that the variations are real and at least somatically inherited, and second, that considerable variation in the number and/or activity of ribosomal RNA genes exists in natural populations of these fishes. A limitation to these interpretations, however, is that the specificity of chromosomal silver-staining apparently lies only in NOR sequences that were actively transcribed at the preceding interphase, and not in the sequences themselves (Miller et al., 1976a, b; Howell, 1977). Thus,

an important question to answer in the future is whether the NOR heteromorphisms represent loss (or gain) of transcriptional activity or loss (or gain) of NOR sequences themselves. This question can be directly tested by in situ autoradiography of chromosomes using radioactively labeled ribosomal RNA or complementary DNA sequences. It also will be important in the future to document frequencies of NOR heteromorphism in different populations of the same species.

The observed variations in chromosomal NORs among these six cyprinid species appear somewhat at odds with previous conclusions based on standard karyotypes that chromosomal evolution in North American Cyprinidae has been highly conserved (Gold et al., 1978, 1979, 1981). Obviously, if the variations in NOR number and position are derived from classical chromosomal rearrangement (as in Elder, 1980), then one or more rearrangement events separate all the species examined excepting *N. lutrensis* and *N. venustus*. In addition, two of the individuals sampled in this study were apparently heterozygous for a classical chromosomal rearrangement (viz., the putative inversion in *P. vigilax* and the short-arm deletion in *N. lutrensis*). Thus, it may be that major chromosomal changes occur more frequently in these fishes than previously thought.

Alternatively, it is possible that NORs could be moved from chromosome to chromosome or site to site by mechanisms not involving major chromosomal rearrangement (e.g., as transposition units). In species with terminal NORs (e.g., *N. lutrensis*, *N. venustus*, *P. vigilax* and *N. crysoleucas*), such mechanisms might not necessarily disrupt internal chromosome arrangement nor promote fertility or other problems usually associated with major chromosome structural changes such as inversions. Further study using other chromosome banding methodology (e.g., G- or C-bands) will help resolve the issue. It is important to note at this point that there is no a priori reason to assume that changes in NOR number and position are invariably concomitant or concordant with major types of chromosomal change.

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IX in the series "Cytogenetic studies in North American minnows (Cyprinidae)."

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