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Chromosomal Heterochromatin Differentiation in North American Cyprinid Fishes*

J. R. Gold[1], C. T. Amemiya[1] and J. R. Ellison[2]

Departments of [1]Wildlife and Fisheries Sciences and
[2]Biochemistry and Biophysics, Texas A and M University,
College Station, Texas 77843, U.S.A.

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With the advent of chromosomal C-banding methodology (Arrighi and Hsu 1971, Yunis *et al.* 1971), constitutive heterochromatin differentiation on mitotic chromosomes of many animal and plant species has become fairly routine. Among animals, most of the comparative data are from higher vertebrates, chiefly mammals. This is due primarily to the superior methods of chromosome harvest available in these species (e.g., Lee and Elder 1980). Collectively, the vertebrate data have shown that variation in both heterochromatin amount and chromosomal position is or can be extensive both within and across phyletic groups (Árnason 1974, Yosida 1975, Mascarello and Mazrimas 1977, Pathak and Wurster-Hill 1977, Schmid 1978a, b, Mengden 1981, Haiduk *et al.* 1981, Baverstock *et al.* 1982, Patton and Sherwood 1982). Most species invariably possess C-bands at or around the centromeres (procentric), and frequently at the chromosome tips (telomeric). C-bands may also be found along chromosome arms (interstitial) and as entirely heterochromatic short arms of acrocentric (submetacentric or subtelocentric) chromosomes. The variation in such "short-arm" heterochromatin is particularly impressive in the rodent genera *Peromyscus* and *Onychomys* (Robbins and Baker 1981, Baker and Barnett 1981). The function(s) of constitutive heterochromatin are essentially unknown although several hypotheses have been forwarded, some of which are supported by empirical data (John and Miklos 1979). Because many if not most species appear to differ trenchantly in C-band amount and/or chromosomal distribution, one long-held hypothesis is that heterochromatin may function as a chromosomal reproductive isolation mechanism and hence in part underlie new species formation (Yunis and Yasmineh 1971, Hatch *et al.* 1976, Fry and Salser 1977).

Heterochromatin differentiation on the chromosomes of fish has lagged far behind studies in almost all other vertebrates, primarily for reasons which relate to the general difficulty in working with fish chromosomes (Gold 1979a). C-banded karyotypes are published for less than thirty fish species from several widely separated groups (Table 1). Briefly, most species possess relatively small amounts of heterochromatin, most of which is procentric. About one-fourth of the species possess no heterochromatin other than procentric, but with few exceptions the rest generally possess only one or two (haploid) C-bands. Four species, three in the salmonid genus *Salvelinus* and one in the gobiid genus *Gobius*, possess a considerable number of telomeric and other C-bands, and by far have the highest relative heterochromatin

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contents of the fishes thus far examined. The finding that heterochromatin amounts and variation in fish are generally small is somewhat surprising in view of the evolutionary heterogeneity of the group as a whole.

Table 1. Heterochromatin location and relative amount in various fish genomes

Species	Family	Hetero-chromatin location*	Hetero-chromatin amount (relative)	Reference
Anguilla anguilla	Anguillidae	1, 3, 4	Small	Wiberg (1983)
A. rostrata	〃	1, 3, 4	〃	Park and Grimm (1981)
Apteronotus albifrons	Apteronotidae	1, 3	〃	Almeida Toledo *et al.* (1981)
Salvelinus fontinalis	Salmonidae	1, 3	Moderate	Ueda and Ojima (1983)
S. leucomaenis	〃	1, 2	Large	Abe and Muramoto (1974)
S. malma	〃	1, 2	〃	〃
S. namaycush	〃	1, 2, 3	〃	Phillips and Zajicek (1982)
Salmo gairdneri	〃	1, 4	Small	Thorgaard (1976)
S. trutta	〃	1, 3, 4	〃	Zenzes and Voiculescu (1975)
Umbra limi	Umbridae	1, 2	〃	Kligerman and Bloom (1977)
Carassius auratus†	Cyprinidae	1, 4	〃	Ueda and Ojima (1978)
Cyprinus carpio	〃	1	〃	Ojima and Ueda (1978)
Notropis lutrensis	〃	1, 2, 3, 4	Large	This paper
N. venustus	〃	1, 2, 3, 4	〃	〃
Oryzias celebensis	Oryziatidae	1, 3, 4	Small	Uwa *et al.* (1981)
O. curvinotus	〃	1, 3	〃	Uwa *et al.* (1982)
O. latipes	〃	1	〃	Uwa and Ojima (1981)
Fundulus heteroclitus	Cyprinodontidae	1, 4	〃	Kornfield (1981)
F. parvipinnis	〃	1	〃	〃
Poecilia sphenops	Poeciliidae	1, 2	〃	Haaf and Schmid (1984)
Haemulon sciurus	Haemulidae	1	〃	Gregory *et al.* (1980)
Archosargus probatocephalus	Sparidae	1, 4	Moderate	〃
Bairdiella chrysoura	Sciaenidae	1, 3	Small	〃
Sarotherodon aurea	Cichlidae	1, 4	〃	Kornfield *et al.* (1979)
S. galilaeus	〃	1, 4	〃	〃
Tilapia zillii	〃	1, 4	〃	〃
Gobius fallax	Gobiidae	1, 2, 3	Large	Thode *et al.* (1983)

* 1=procentric; 2=telomeric; 3=interstitial; and 4=short-arm (see text for details).

† Includes 5–6 subspecies of *C. auratus*.

In this paper, we report the chromosomal heterochromatin distributions and amount in two closely related species of North American cyprinid fish. The study is part of our long term efforts to determine whether chromosomal mechanisms have contributed to the extensive and rapid speciation which characterizes North American Cyprinidae (Gold 1980, Gold *et al.* 1978, 1981), and to assess the cytotaxonomy of the species (Gold 1984). Herein we show that the two species examined possess an appreciable heterochromatic fraction in their genomes and also differ from one

another in both heterochromatin amount and chromosomal distribution. Implications of these results are discussed in terms of chromosomal evolution and cytotaxonomy in these fishes.

Materials and methods

The two cyprinids examined in the study were collected by seining from the following localities in Texas: *Notropis lutrensis* (Little Brazos River, Brazos County) and *Notropis venustus* (Bull Creek, Travis County). Specimens were returned live to College Station and maintained in well-aerated aquaria until karyotyped. Chromosome preparations were made from either fibroblast cultures as described by Amemiya *et al.* (1984) or directly from kidney tissue of colcemid-injected specimens as described by Gold (1984). Slides prepared by either method were stored desiccated at room temperature for three or more days prior to C-banding.

Heterochromatin differentiation or C-banding was carried out following standard methods. Briefly, slides (chromosomes) were hydrolysed 15–20 min in 0.2 N HCl at 37°C, denatured in a saturated $Ba(OH)_2$ solution at room temperature for 4–7 min, then incubated at 65°C for 60–90 min in humidity chambers (after Arrighi and Hsu 1974) using 2X SSC. We found it important after each of these steps to briefly rinse the slides for 1–2 min in distilled H_2O and then allow them to partially air dry for 10–15 min; we also found it useful to prepare (and prewarm) the solutions immediately before use, to keep the $Ba(OH)_2$ solution tightly capped with parafilm (even during slide treatment), to frequently remove the filmy residue (using a tissue) which invariably forms at the surface of a saturated $Ba(OH)_2$ solution, and to rinse the $Ba(OH)_2$-treated slides for 5–10 sec in 0.2 N HCl prior to the distilled H_2O rinse.

Chromosome staining was carried out on fully air dried slides using either Giemsa or the fluorochrome acridine orange (AO). Giemsa staining was for 10–15 min using a 5% solution (v/v) in 10^{-2} M phosphate buffer at pH 6.8–7.2. Slides were stained at room temperature, rinsed 1–2 min in distilled H_2O, air-dried, cleared in xylenes for 10 min, and mounted in Permount. AO staining was for 5 min at room temperature using a 0.1% solution (w/v) in 0.1 M NaCl. Slides were stained, washed twice for 30 sec *each* in 0.1 M NaCl, rinsed 20–30 sec in distilled H_2O, and mounted in 0.1 M NaCl by sealing the coverslip edges with fingernail polish. We found it important to prepare both the AO and NaCl solutions immediately before use. Chromosome preparations stained with Giemsa were photographed in bright-field using technical pan 2415 film at ASA 25–40; those stained with AO were photographed using epifluorescence and panatomic X film at ASA 100–160. Both films were developed in Diafine.

Quantitative determinations of heterochromatin amounts and chromosomal positions were made off positive prints using a digitizer, a small laboratory computer, and a program (BANDSCAN) written by one of us (JRE). The program essentially i) sorts the chromosomes by length, centromere position, and band size and position, ii) arranges the chromosomes into homologous pairs and then the pairs on the basis of chromosome length, and iii) provides both hard copy data and an idealized karyogram of each measured spread. The program is written in BASIC and is available from JRE.

Results and discussion

C-banded metaphases stained with AO or Giemsa are shown in Fig. 1; idealized, computer-assisted karyograms averaged from separate measurements of the same and different metaphase spreads from both species are shown in Fig. 2. The latter are particularly impressive and demonstrate the potential use of computer-assisted measurement of fish karyotypes. Some caution, however, is advisable, particularly as regards homologization of chromosomes within or between the species. Several of the chromosomal "pairs" shown in the idealized karyograms are very similar in total length, centromere position, and C-band size and location. As examples, consider the second and third pair in *N. lutrensis* or the third and fourth pair in *N. venustus*. Other examples include pairs five, seven, and nine in *N. lutrensis*, and pairs eight through thirteen in *N. venustus*. In each case, differential chromosome

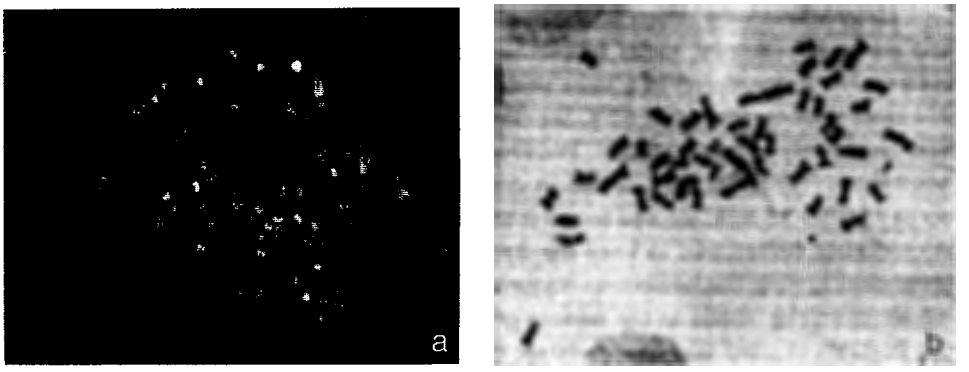

Fig. 1. C-banded metaphases from *N. lutrensis* stained with (a) acridine orange and (b) Giemsa.

contraction during mitosis as well as differential effects of the mitotic inhibitors on chromosome condensation could easily affect both chromosome arm and C-band lengths, and hence render homologization somewhat artificial. Another problem is that both Giemsa and AO are non-specific chromatin or DNA stains and do not differentiate qualitative variation (e.g., AT- or GC- base pair content) in chromosomal heterochromatin. Both problems are aggravated in part by the relatively large amounts of heterochromatin found in the two species (see below). There are, however, more than a few chromosomes whose homologies within species can be inferred from obvious differences in chromosome size, centromere position, or C-banding. Examples include pairs one, four, and ten in *N. lutrensis*, and pairs one, two, and six in *N. venustus*. Conservatively, we estimate that we can unambiguously differentiate a minimum of nine homologous pairs in *N. lutrensis* and eight in *N. venustus*.

Homologization of chromosomes between (or across) the two species is more difficult. The largest chromosome in both species definitely appears the same by relative size and C-band criteria, and is also known to carry the single nucleolar organizing region (NOR) on the tip of the short arm in both species (Gold 1984). Other putative homologies between the species may include pairs four, twelve, twenty-two, and twenty-five, in *N. lutrensis* with pairs six, fifteen, nineteen, and

twenty-five, respectively, in *N. venustus*. However, without additional verification using any of the other chromosome banding methods (e.g., G-bands), these homologies are best considered tentative. Alternatively, it is clear from inspection of the karyograms that many chromosomes in both species are very similar in relative size, centomere position, and C-banding. This is especially true for most of the chromosomes with short-arm heterochromatin (see below). On a chromosome by chromosome basis, we estimate minimally that perhaps 60–70% of the heterochromatin is homologous between the two species by the measurement criteria employed here.

The above notwithstanding, what is striking is the apparent amount and chromosomal distribution of the heterochromatin in both species. Nearly every chromosome, even those with no detectable heterochromatin elsewhere, possessed C-bands at or around the centromere; on some chromosomes (e.g., pairs one—three in both species), the procentric bands extended well beyond the centromeres on one or both arms. Telomeric heterochromatin was relatively sparse and was detected only on the long arm of the largest chromosome pair in both species and on the long arm of one of the smaller submetacentric pairs in *N. venustus*. Eight pairs of chromosomes in *N. lutrensis* and five pairs in *N. venustus* posessed interstitial C-bands; whereas seventeen pairs of either submetacentric or acrocentric chromosomes in *N. lutrensis* and nineteen pairs in *N. venustus* possessed entirely heterochromatic short

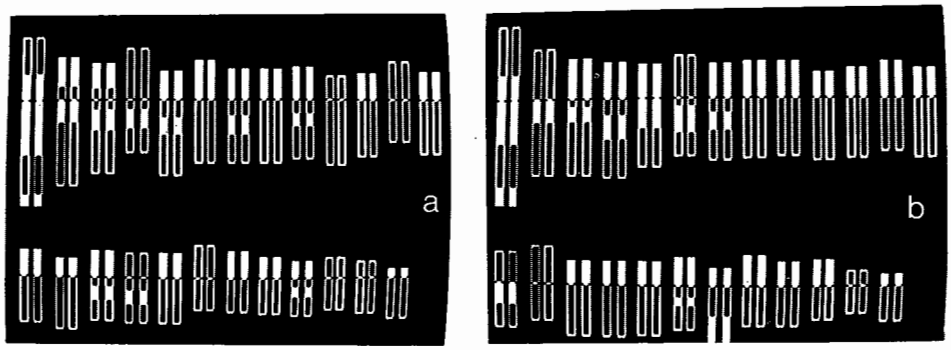

Fig. 2. Computer-assisted C-band karyograms from (a) *N. lutrensis* and (b) *N. venustus*.

arms. Such extensive short-arm heterochromatin is of interest in view of our previous findings (Gold *et al.* 1979, 1981) that almost all of the gross chromosomal variation in North American Cyprinidae involves chromosome arm number differences rather than chromosome number differences. Recent studies in the rodents *Peromyscus* and *Onychomys* have shown that most of the remarkable chromosome arm number variation which differentiates species in both genera is due primarily to additions or deletions of short-arm heterochromatin (Robbins and Baker 1981, Baker and Barnett 1981). Very possibly, a similar situation exists in North American Cyprinidae and the karyotype differences thus far observed are due to short-arm heterochromatin variation. Further studies will directly address this issue.

The total amount of heterochromatin in the two species measured as the total length of all heterochromatic regions divided by the total length of all chromosomes was 35% in *N. lutrensis* and 39% in *N. venustus*. Comparable quantitative data

from other fish are not available, but it would appear from visual scrutiny of the published C-band karyotypes (references in Table 1) that the amount of heterochromatin in these two cyprinid species considerably exceeds that found in almost all other fish examined to date. Moreover, since the density-distribution of DNA is probably greater in heterochromatin than in euchromatin (Narayan and Durrant 1983), it is likely that half or more of the total DNA in the two species is localized in chromosomal heterochromatin.

The fact that the genomes of these two cyprinid species contain such an appreciable amount of heterochromatin is of interest to our long term goals of understanding the chromosomal evolution and cytotaxonomy of the group. If nothing else, the extensive heterochromatin content indicates a significant fraction of the cyprinid genome with considerable potential to vary, and which is clearly different in both amount and chromosomal position between the two very closely related species studied here. Thus far, we have examined several different levels of genomic organization in North American cyprinids, including standard karyotypes (Gold *et al.* 1981), genome sizes (Gold and Price 1985), and AT/GC contents (Karel and Gold unpbl.), and in each case have found that the differences between species were generally insufficient to account for the rapid evolutionary change which characterizes the group. Avise (1977a, b) came to the same conclusion regarding the protein coding gene component. As a specific example, the two species studied here have nearly identical standard karyotypes, genome sizes, AT/GC contents, DNA melting rate profiles, and isozyme/allozyme profiles. The NOR-bearing chromosome is also the same in both species (Gold 1984), yet the two differ appreciably in C-band amount and position. Along these lines, two additional points should be noted. First, two other cyprinids from Asia (*Carassius auratus* and *Cyprinus carpio*) have been examined for C-band patterns and in both the amount of heterochromatin detected was considerably less than that exhibited by the two North American forms. Obviously, it will be of future interest to further sample Asian and North American cyprinids. The second point is that the only other fish species thus far discovered to possess appreciable amounts of heterochromatin are from the very chromosomally active families Salmonidae and Gobiidae (Gold 1979b). A close relationship between heterochromatin and classical chromosomal rearrangement (i.e., inversions, translocations, etc.) has been suggested from studies on other animal groups including vertebrates (Hatch *et al.* 1976, Yoon and Richardson 1978), and indirectly supports the thesis that chromosomal mechanisms may have contributed significantly to the extensive North American cyprinid speciation.

Regarding cytotaxonomy, the C-band data are as yet too few in terms of number of species to allow an accurate assessment of whether the approach will suitably demonstrate phyletic affinities between species. There also is the problem that several chromosomes both within and between the two species are very similar in C-band pattern and hence cannot be unequivocally homologized. However, certain marker chromosomes in North American cyprinids are becoming evident, and in the future it will be informative to compare such chromosomes across species. As one example, the single NOR-bearing chromosomes in both *N. lutrensis* and *N. venustus* appear to be homologous by virtue of similarity in overall size, centromere position,

NOR-location, and C-banding patterns (Gold 1984, Fig. 3). These two species are thought to be very close relatives in the diverse *Notropis* subgenus *Cyprinella* (Gibbs 1957). A third *Cyprinella* species (*Notropis camurus*), however, appears to possess a different NOR-bearing chromosome (Amemiya and Gold unpbl.) which is in accord with the present classification of the species since *N. camurus* is thought to be a distant relative of the other two (Gibbs 1957, 1961). Given the general difficulty in working with fish chromosomes, however, it is probable that most fish cytotaxonomy will initially have to be practiced on a chromosome by chromosome basis.

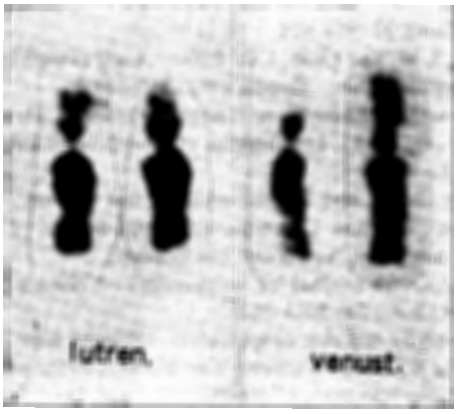

Fig. 3. C-bands with Giemsa on the NOR chromosome pair of *N. lutrensis* and *N. venustus*.

Summary

Chromosomal heterochromatin was differentiated in two closely related species of the North American cyprinid genus *Notropis*. The amounts of heterochromatin in the genomes of the two species were appreciable: 35% in *N. lutrensis* and 39% in *N. venustus*. The chromosomal distribution of heterochromatin was similar in both species: most or all chromosomes contained procentric bands, a few possessed terminal and/or interstitial bands, and there was a preponderance of submetacentric and acrocentric chromosomes with entirely heterochromatic short arms. Several chromosomes could be putatively homologized either within or between the two species, although in general the amount and type of heterochromatin made precise homologization difficult. The total amount of heterochromatin resolved in these two species is considerably more than that found among most of the relatively few fish species studied to date. Heterochromatin appears to be one of the few potentially variable fractions of the cyprinid genome which may have contributed to the extensive speciation exhibited by these fish in North America. A few marker chromosomes, specifically the one bearing the nucleolus organizer region or NOR, may prove valuable in cyprinid taxonomy.

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