nocturnal-diurnal changeover. Nat. Hist. Mus. Los Ang. Cty., Sci. Bull. 14:98–124.

DOMM, S. B., AND A. J. DOMM. 1973. The sequence of appearance at dawn and disappearance at dusk of some coral reef fishes. Pac. Sci. 27:128–135.

HELFMAN, G. S. 1981. Twilight activities and temporal structure in a freshwater fish community. Can. J. Fish. Aquat. Sci. 38:1405–1420.

HOBSON, E. S. 1968. Predatory behavior of some shore fishes in the Gulf of California. US Fish Wildl. Serv., Res. Rep. 73.

———. 1972. Activity of Hawaiian reef fishes during the evening and morning transitions between daylight and darkness. Fish. Bull. 70:715–740.

KÜHLMANN, D. H. H., AND H. KARST. 1967. Freiwasserbeobachtungen zum Verhalten von Tobiasfischschwärmen (Ammodytidae) in der westlichen Ostsee. Z. Tierpsychol. 24:282–297.

MACER, C. T. 1966. Sand eels (Ammodytidae) in the south-western North Sea; their biology and fishery. Fish. Invest., Minist. Agric., Fish. Food (G. B.), Lond., Ser. 2 24(6):1–55.

MCFARLAND, W. N., J. C. OGDEN AND J. N. LYTHGOE. 1979. The influence of light on the twilight migrations of grunts. Environ. Biol. Fish 4:9–22.

MAJOR, P. F. 1977. Predator-prey interactions in schooling fishes during periods of twilight: A study of the silverside *Pranesus insularum* in Hawaii. Fish. Bull., US 75:415–426.

MEYER, T. L., R. A. COOPER AND R. W. LANGTON. 1979. Relative abundance, behavior, and food habits of the American sand lance *Ammodytes americanus*, from the Gulf of Maine. Fish Bull., US 77:243–253.

MUNZ, F. W., AND W. N. MCFARLAND. 1973. The significance of spectral position in the rhodopsins of tropical marine fishes. Vision Res. 13:1829–1874.

PITCHER, T. J., AND C. J. WYCHE. 1983. Predator-avoidance behaviours of sand-eel schools: Why schools seldom split, p. 193–204. *In:* Predators and prey in fishes. D. L. G. Noakes, D. G. Lindquist, G. S. Helfman, and J. A. Ward (eds.). Dr. W. Junk Publications, Dordrecht, The Netherlands.

REAY, R. J. 1970. Synopsis of biological data on North Atlantic sand eels of the genus *Ammodytes, A. tobianus, A. dubius, A. americanus,* and *A. marinus.* FAO Fish. Synop. 82, 42 p.

SCOTT, J. S. 1973. Food and inferred feeding behavior of northern sand lance (*Ammodytes dubius*). J. Fish. Res. Board Can. 30:451–454.

SENTA, T. 1965. Nocturnal behavior of sand eels, *Ammodytes personatus* Girard. Bull. Jpn. Soc. Sci. Fish. 31:506–510.

WINSLADE, P. 1974. Behavioural studies on the lesser sandeel *Ammodytes marinus* (Raitt). I The effect of food availability on activity and the role of olfaction in food detection. J. Fish. Biol. 6:565–576.

EDMUND S. HOBSON, *Southwest Fisheries Center Tiburon Laboratory, National Marine Fisheries Service, 3150 Paradise Drive, Tiburon, California 94920.* Accepted 13 May 1985.

Copeia, 1986(1), pp. 226–231

CHROMOMYCIN A_3 STAINS NUCLEOLUS ORGANIZER REGIONS OF FISH CHROMOSOMES.—The chromomycinone antibiotics, chromomycin A_3 (CMA) and mithramycin, are thought to preferentially bind to chromosomal regions rich in guanine-cytosine (GC) base pairs and to generally yield fluorescence patterns on chromosomes which are the reverse of those produced by adenine-thymine (AT) specific fluorochromes such as quinacrine, Hoechst 33258, DIPI and DAPI (Schweizer, 1981). When stained with CMA or mithramycin, mammalian chromosomes frequently exhibit serial R-bands as well as some heterochromatin bands; the nucleolus organizer regions (NORs), however, remain undifferentially stained (Schweizer, 1976, 1981). In contrast, in organisms where serial (G-, R-, Q-) bands are either nonexistent or difficult to produce, chromosome staining with GC-specific fluorochromes, in addition to demonstrating heterochromatin bands, also differentiates the NORs and often as the brightest fluorescing bands on the chromosomes. Included in this category thus far are several species of plants (Schweizer et al., 1983), a species of isopod crustacean (Rocchi et al., 1984), four species of grasshoppers (Schweizer et al., 1983) and numerous amphibian (mostly anuran) species (Schmid, 1980a, b, 1982; Sims et al., 1984). The effects of GC-specific fluorochromes on chromosomes from birds, reptiles and fishes have not been previously documented.

Cytogenetically, fishes have been the least studied of vertebrates. Because most fishes have fairly large numbers of relatively small chromosomes (Gold, 1979; Blaxhall, 1983), good metaphase spreads are difficult to obtain. In addition, chromosome banding techniques, excepting silver staining for chromosomal NORs (AgNORs), have met with comparatively few successes in fishes. Fish chromosomes are thought to be structurally similar to amphibian chromosomes, as both respond similarly to various differential staining techniques (Birstein, 1982). The finding, therefore, that CMA stain-

ing produces striking NOR differentiation on amphibian chromosomes (Schmid, 1980a, b, 1982), suggested to us that this might also be true for fish chromosomes. In this paper we document the correspondence between CMA-bright regions and chromosomal NORs (as identified by silver staining) in 21 species from four orders of fishes.

Materials and methods.—All specimens were collected by seine from populations in Texas and neighboring states, transported live to the laboratory and maintained in well-aerated aquaria prior to sacrifice. Following tissue removal for karyotyping, all specimens were individually tagged for identification and maintained in our laboratory collections. The specimens will ultimately be deposited in the Texas Cooperative Wildlife Collection at Texas A&M University. Chromosomes were obtained either by direct tissue preparations (Gold, 1984) or from cultured fibroblasts (Amemiya et al., 1984). A total of 54 fish specimens were karyotyped (Table 1) and at least ten good sequentially stained (CMA then Ag) metaphases were examined per specimen. If, however, the NORs were known to be located on certain marker chromosomes (such as the largest or smallest pair) in a given species, sequential staining was not required (i.e., Ag and CMA staining could be carried out on different slides from the same specimen).

Staining with chromomycin A_3 (Sigma) was done in one of two ways: 1) by a modification of the method of Schmid (1982), employing DAPI (Sigma, 0.03 mg/ml in ethanol) as a counterstain in place of distamycin A; or 2) by a technique (Amemiya and Gold, unpubl.) which does not require a counterstain for good differential staining. Briefly, this latter technique involves treating the slides for 2–3 min in the dark with a CMA staining solution (0.1 mg/ml in McIlvaine's buffer with 10 mM $MgCl_2$, pH 7), rinsing the slides for 10–15 sec in the above buffer, then mounting the slides in a medium of NaOH-supplemented glycerol (0.01 g/ml). Fluorochrome destaining followed Schweizer et al. (1983) and silver staining followed Howell and Black (1980) as modified by Gold and Ellison (1983).

We used a Zeiss Universal research microscope equipped with epifluorescence. For fluorescence microscopy, an XBO 75 W/AC lamp and the following filter systems were employed: 1) CMA—BP 495 exciter filter, FT 510 chromatic beam splitter and LP 530 barrier filter; and 2) DAPI—UG-1 exciter filter, FT 420 chromatic beam splitter, and LP 395 barrier filter. A general discussion of the theory and application of fluorescence microscopy can be found in Brown and Bertke (1974), Berlyn and Miksche (1976), or many cytology/histology texts. Photomicrographs were taken through 63× Planapochromat, 63× Neofluar or 100× Neofluar objectives on Kodak Technical Pan 2415 film (ASA 25–40 for bright field; ASA 125–160 for fluorescence) developed in Diafine (Acufine) or D19 (Kodak).

TABLE 1. SUMMARY OF TAXA EXAMINED.

Taxon	No. specimens	2n
Order Cypriniformes		
Family Cyprinidae		
Hybopsis aestivalis	4	50
Nocomis leptocephalus	1	50
Notemigonus crysoleucas	3	50
Notropis amabilis	2	50
Notropis buchanani	1	50
Notropis camurus	2	50
Notropis longirostris	2	50
Notropis lutrensis	8	50
Notropis oxyrhynchus	3	50
Notropis potteri	3	50
Notropis shumardi	2	50
Notropis venustus	5	50
Pimephales notatus	1	50
Family Catostomidae		
Carpiodes carpio	1	ca 100
Order Siluriformes		
Family Ictaluridae		
Ictalurus punctatus	1	58
Noturus gyrinus	2	42
Order Atheriniformes		
Family Poeciliidae		
Gambusia affinis	5	48
Order Perciformes		
Family Centrarchidae		
Lepomis humilis	1	48
Lepomis macrochirus	1	48
Family Percidae		
Percina sciera	3	48
Family Mugilidae		
Mugil cephalus	3	48

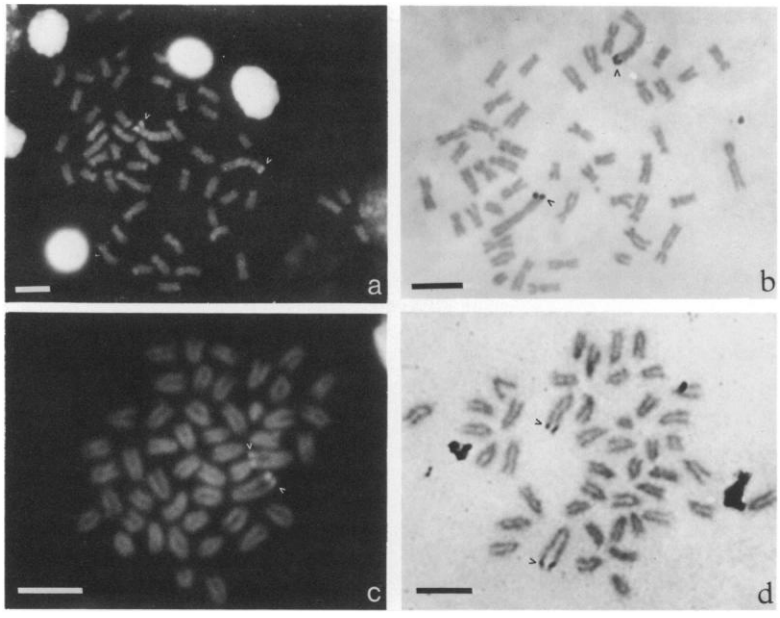

Fig. 1. Comparison of CMA and Ag staining patterns. a, b) Metaphases from *Notropis lutrensis* after CMA and Ag staining, respectively. c, d) Metaphases from *Mugil cephalus* after CMA and Ag staining, respectively. NORs are indicated by arrowheads. Bars represent 5 μm.

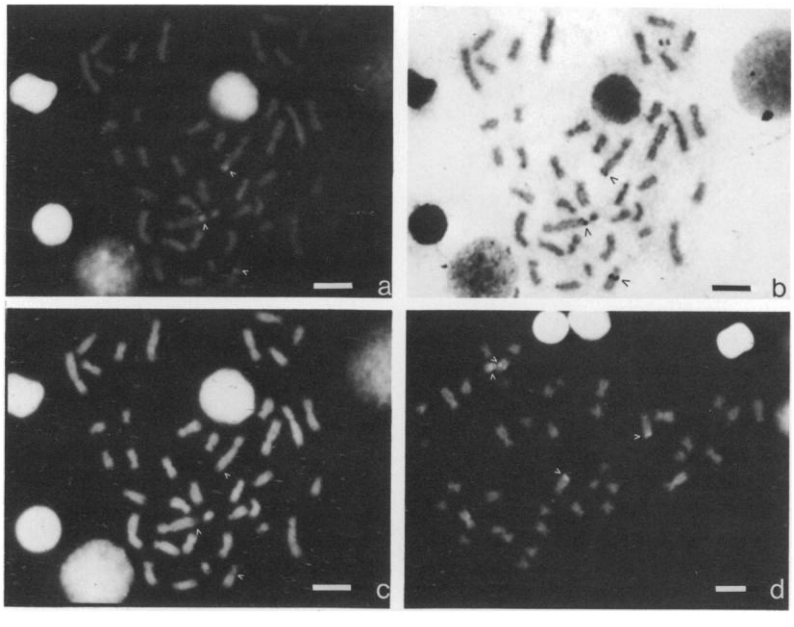

Fig. 2. Metaphase from a *Noturus gyrinus* individual stained with (a) CMA then (b) Ag. The three NORs are indicated by arrowheads. c) The same metaphase as above showing fluorescence pattern of DAPI counterstain (note the quenched regions corresponding to the NORs). d) CMA stained metaphase from a different *N. gyrinus* specimen that had four NORs (arrowheads). The difference in the number of NORs between the two specimens is due to an apparent NOR deletion. Bars represent 5 μm.

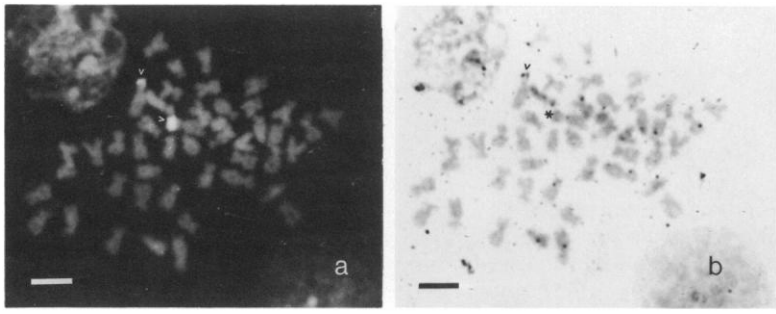

Fig. 3. Demonstration of a NOR activity heteromorphism. Chromosomes from a *Notropis shumardi* specimen stained with (a) CMA then (b) Ag. NORs are indicated by arrowheads. The "*" in (b) indicates an inactive NOR. All metaphases from this individual had only one AgNOR but two CMA-NORs. Bars represent 5 μm.

Results.—Table 1 gives the diploid number of all species examined. Both *Notropis buchanani* (diploid number previously unreported) and *Pimephales notatus*, a species whose diploid number was reported to be 52 (Legendre and Steven, 1969) had 2n = 50 chromosomes. The *Lepomis humilis* specimen karyotyped had 2n = 48 chromosomes rather than the previously reported diploid number of 46 (Roberts, 1964). All other taxa had diploid numbers in agreement with those previously reported (Gold et al., 1980).

In all cases, the most conspicuous CMA-bands corresponded unequivocally to AgNORs. Figs. 1 (a–d) and 2 (a–b) show karyotypes from representative fishes stained with CMA and/or Ag. Staining with CMA (GC-specific) or DAPI (AT-specific) show fluorescent patterns that were the reverse of one another as expected (Fig. 2a, c).

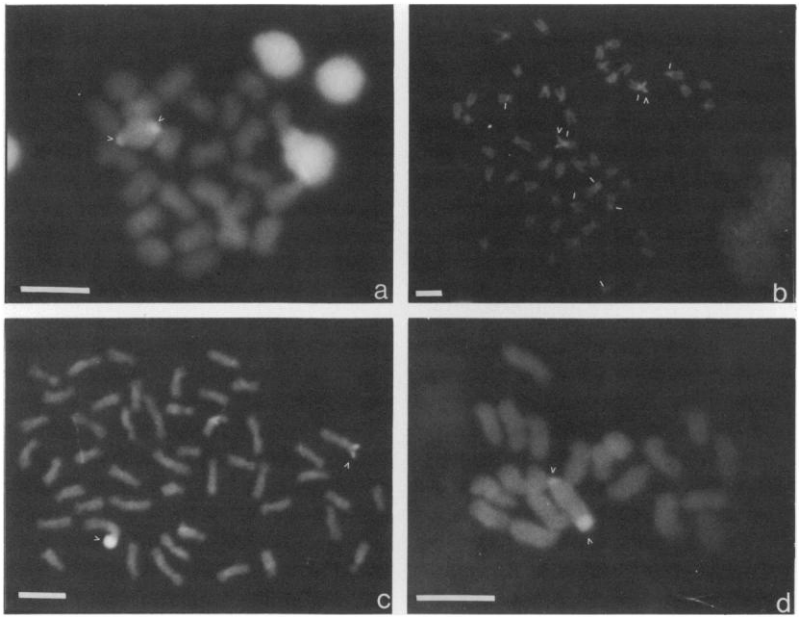

Fig. 4. Chromomycin staining of: a) *Notropis potteri* metaphase I (meiotic) chromosomes; b) *Percina sciera* mitotic chromosomes, showing some heterochromatin differentiation (small bars); c, d) *N. shumardi* mitotic and metaphase I (meiotic) chromosomes, respectively. This individual had a NOR size heteromorphism which was observed in all mitotic and meiotic spreads. NORs are indicated by arrowheads. Bars represent 5 μm.

The usefulness of CMA in detecting the intraspecific NOR heteromorphisms frequently seen in fishes is shown in Fig. 2a and 2d (NOR deletion), Fig. 3 (activity heteromorphism) and Fig. 4c (size heteromorphism). CMA also differentiates NORs on metaphase I (meiotic) chromosomes (Fig. 4a, d). GC-rich heterochromatin bands with fluorescence intensities slightly less than those of the NORs also were produced by CMA staining of chromosomes from certain fish species (Fig. 4b). Lastly, it should be noted that staining with mithramycin (Sigma) produced results identical to those obtained with chromomycin A_3. Mithramycin was used in place of CMA in approximately one-fourth of all specimens. Hereafter, discussion of CMA staining refers to both CMA and mithramycin.

Discussion.—The use of chromomycin A_3 as a fluorescent NOR stain offers much promise in fish cytogenetics and in some respects is preferred over silver staining. Silver apparently does not stain NORs which were genetically inactive at the preceding interphase, nor does it generally stain NORs on chromosomes in late prophase I to metaphase II or meiosis (Howell, 1982; Schmid et al., 1982; Satya-Prakash and Pathak, 1984). Unlike silver, which presumably stains a NOR-associated protein (Howell, 1982), CMA apparently stains DNA and will differentiate NORs regardless of previous genetic activity or chromosomal stage. This is particularly useful in fishes where the frequency of intraspecific NOR heteromorphisms is apparently high (Foresti et al., 1981; Gold, 1984; Moreira-Filho et al., 1984; Amemiya and Gold, unpubl.). Exactly what component of the NOR (i.e., coding ribosomal RNA sequences, spacer sequences, or NOR-associated heterochromatin) is stained by CMA remains unknown. Schmid (1982) has suggested that several factors may contribute to the exceptionally bright CMA fluorescence of the NORs of amphibian chromosomes.

The frequency of intraspecific NOR heteromorphisms is reportedly high in fishes (Foresti et al., 1981; Gold, 1984; Moreira-Filho et al., 1984) and we have thus far identified three different types of intraspecific NOR heteromorphisms: 1) size heteromorphisms—where NORs of homologous chromosomes are of very different sizes; 2) NOR deletions—where an entire NOR has been deleted from one of the two homologues; and 3) activity heteromorphisms—where only one NOR of a homologous chromosome pair is transcriptionally active (i.e., synthesizing rRNA). Silver staining alone will not distinguish NOR activity heteromorphisms from NOR deletions since both result in the absence of binding to the NOR chromosomal site. Discerning these two types of heteromorphisms in the past has required the relatively tedious technique of in situ hybridization using radioactively-labeled rRNA or rDNA probes (Howell, 1982). Chromomycin, with its apparent high affinity for NORs on fish chromosomes, can be used (together with silver staining) to discern activity heteromorphisms from NOR deletions since the former will bind CMA but not silver, whereas the latter will bind neither CMA nor silver (see Figs. 2a–b, d and 3). In organisms with CMA-bright NORs, this should alleviate the need for in situ hybridization to differentiate these two types of NOR heteromorphism.

The question as to whether intraspecific NOR heteromorphisms in fish are heritable and constitute true chromosomal polymorphisms has been raised (Gold, 1984). A requisite for the inheritance of these heteromorphisms is their presence on meiotic chromosomes. For example, Fig. 3c shows a NOR size heteromorphism observed in all somatic metaphases of a *N. shumardi* specimen as well as on metaphase I configurations from testes of the same individual (Fig. 3d). We should note, however, that these results merely suggest that such heteromorphisms are heritable; unequivocal proof is possible only through crossing or breeding experiments.

Finally, in certain fish species, chromomycin A_3 also appears to differentiate GC-rich heterochromatin (Fig. 3b). These bands were identified as heterochromatin since they exhibited enhanced fluorescence relative to the majority of the chromosomal DNA. Although most of the species examined did not appear to exhibit such heterochromatic regions, this might be an artifact of the difficulty in discerning fluorescent bands on the relatively small chromosomes of these fishes. Indeed, the majority of the heterochromatin bands seen were on the larger chromosomes of the complements. Alternatively, in cyprinids (which comprised the greatest number of species examined), data from genomic DNA melting profiles of over 25 species indicate fairly low (ca 36%) GC base pair compositions (Karel and Gold, unpubl.). Since none of the cyprinids examined here showed differential GC-rich heterochromatin staining, this would suggest, with the exception of NORs, that the distribution of GC base pairs is fairly

uniform along cyprinid chromosomes and that cyprinid chromosomal heterochromatin is not particularly enriched in GC base pairs.

Acknowledgments.—We thank the following people for assistance in the collection and identification of specimens: James Barlow, Kristine Chenicek, William Karel, Tsutomu Miyake, Jaime Modiano, Chara Ragland, Mike Retzer and Joseph Warner. Support was provided by the National Science Foundation (DEB-8022173) and by The Texas Agricultural Experiment Station (projects H-6187 and H-6703).

Literature Cited

Amemiya, C. T., J. W. Bickham and J. R. Gold. 1984. A cell culture technique for chromosome preparation in cyprinid fishes. Copeia 1984:232–235.

Berlyn, G. P., and J. P. Miksche. 1976. Botanical microtechnique and cytochemistry. The Iowa State University Press, Ames, Iowa.

Birstein, V. J. 1982. Structural characteristics of genome organization in amphibians: differential staining of chromosomes and DNA structure. J. Mol. Evol. 18:73–91.

Blaxhall, P. C. 1983. Chromosome karyotyping of fish using conventional and G-banding methods. J. Fish Biol. 22:419–424.

Brown, W. V., and E. M. Bertke. 1974. Textbook of cytology. The C. V. Mosby Company, St. Louis, Missouri.

Foresti, F., L. F. Almeida Toledo and S. A. Toledo. 1981. Polymorphic nature of nucleolus organizer regions in fishes. Cytogenet. Cell Genet. 31:137–144.

Gold, J. R. 1979. Cytogenetics, p. 353–405. *In:* Fish physiology. Vol. 8. W. S. Hoar, D. J. Randall and J. R. Brett (eds.). Academic Press, New York, New York.

———. 1984. Silver-staining and heteromorphism of chromosomal nucleolus organizer regions in North American cyprinid fishes. Copeia 1984:133–139.

———, and J. R. Ellison. 1983. Silver-staining for nucleolar organizer regions of vertebrate chromosomes. Stn. Technol. 58:51–55.

———, W. J. Karel and M. R. Strand. 1980. Chromosome formulae of North American fishes. Prog. Fish-Cult. 42:10–23.

Howell, W. M. 1982. Selective staining of nucleolus organizer regions (NORs). The Cell Nucleus 11: 89–142.

———, and D. A. Black. 1980. Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia 31:260–262.

Legendre, P., and D. M. Steven. 1969. Denombrement des chromosomes chez quelques cyprins. Naturaliste Canada 96:913–918.

Moreira-Filho, O., L. A. C. Bertollo and P. M. Galetti. 1984. Structure and variability of nucleolar organizer regions in Paradontidae fish. Can. J. Genet. Cytol. 26:564–568.

Roberts, F. A. 1964. A chromosome study of twenty species of Centrarchidae. J. Morph. 115:402–418.

Rocchi, A., G. Prantera, V. Lanza and M. Di Castro. 1984. Incipient sex chromosome differentiation in an isopod crustacean species, *Asellus aquaticus*. Chromosoma 89:193–196.

Satya-Prakash, K. L., and S. Pathak. 1984. Silver staining pattern of male meiosis in the house cricket. J. Hered. 75:319–320.

Schmid, M. 1980a. Chromosome evolution in Amphibia, p. 4–27. *In:* Cytogenetics of vertebrates. Vol. 1. H. Müller (ed.). Birkhauser, Basel, Switzerland.

———. 1980b. Chromosome banding in Amphibia. IV. Differentiation of GC- and AT-rich chromosome regions in Anura. Chromosoma 77:83–103.

———. 1982. Chromosome banding in Amphibia. VII. Analysis of the structure and variability of NORs in Anura. *Ibid.* 87:327–344.

———, C. Löser, J. Schmidtke and W. Engel. 1982. Evolutionary conservation of a common pattern of activity of nucleolus organizers during spermatogenesis in vertebrates. *Ibid.* 86:149–179.

Schweizer, D. 1976. Reverse fluorescent chromosome banding with chromomycin and DAPI. *Ibid.* 58:307–324.

———. 1981. Counterstain-enhanced chromosome banding. Human Genet. 57:1–14.

———, M. Mendelak, M. J. D. White and N. Conteras. 1983. Cytogenetics of the parthenogenetic grasshopper *Warramaba virgo* and its bisexual relatives. X. Patterns of fluorescent banding. Chromosoma 89:227–236.

Sims, S. H., H. C. MacGregor, P. W. Pellatt and H. A. Horner. 1984. Chromosome 1 in crested and marbled newts (*Triturus*): an extraordinary case of heterochromatin and independent chromosome evolution. *Ibid.* 89:169–185.

Chris T. Amemiya and John R. Gold, *Department of Wildlife and Fisheries Sciences, Texas A&M University, College Station, Texas 77843.*
Accepted 13 April 1985.