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Improved methods for working with fish chromosomes with a review of metaphase chromosome banding

J. R. GOLD, Y. C. LI, N. S. SHIPLEY AND P. K. POWERS
Department of Wildlife and Fisheries Sciences, Texas A&M University, College Station, Texas 77843, U.S.A.

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Improved methods for obtaining, preparing, and staining fish chromosomes are described. Included are procedures for resolving serial or G-type bands. A brief review of various metaphase banding procedures and their use in fishes is also presented.

Key words: chromosomes; methods; metaphase banding.

I. INTRODUCTION

Studies of the chromosomes of fishes have not been as successful or widespread as in other vertebrate groups. Standard karyotypes (chromosome and chromosome arm number) are reported for less than 10% of the more than 20 000 extant species of fishes (W. H. LeGrande, Database of Fish Karyotypes, University of Wisconsin at Steven's Point), and the application of metaphase chromosome banding (*sensu* Sumner, 1977) methodologies to fish chromosomes has been minimal (Gold, 1979; Hartley & Horne, 1985). The limiting factors apparently are obtaining consistently good chromosome spreads from fish tissues and the problem that most fish complements contain a relatively large number of comparatively small chromosomes (Gold, 1979).

Work in our laboratory over the last 15 years has focused on the evolutionary cytogenetics of North American freshwater fishes, with particular emphasis on representatives of the family Cyprinidae. Most of our early work centered on establishing standard karyotypes of cyprinid and other species (Gold *et al.*, 1979*a,b*, 1981). More recently, our efforts have been directed towards metaphase chromosome banding and the identification of homologous chromosomes within and among species (Gold, 1984; Gold & Amemiya, 1986; Amemiya & Gold, 1986, 1988, 1990; Gold *et al.*, 1986; Amemiya *et al.*, 1986). In the last 2 to 3 years, we have developed a number of improved procedures for harvesting, preparing, and staining fish chromosomes. Included among these are methods for producing serial or G-type bands which thus far have proved difficult to obtain on fish chromosomes (Hartley & Horne, 1985). In the following, these procedures are outlined; a brief review of chromosome banding is also presented. The information has been collated largely at the request of colleagues to whom our procedures have been sent through the years.

II. METHODS AND PROCEDURES

OBTAINING FISH CHROMOSOMES

Methods for obtaining mitotic chromosomes directly from live specimens or embryos were reviewed by Gold (1979). Good sources of actively dividing tissue include soft-organs

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(kidney or spleen) and epithelial cells from gills, fins, scales, or cornea. Testes are also useful for obtaining mitotic chromosomes, but can be used only during active spermatogonial proliferation. Our usual preference is kidney since the renal intertubular tissue in most fishes contains the hematopoietic organs (Catton, 1951), thus providing numerous blood cells in active division. Epithelial cells from the gill, however, are preferred if the specimens are very small (< 10 mm in length). Administration of mitotic inhibitors is fairly straightforward. In the past, we used direct intraperitoneal injection of colchicine (approximately 0·1 ml of a 0·3 μg ml^{-1} solution per gramme body weight) or colcemid (0·8–1·0 μg per gramme body weight) followed by a 90–120 min interval before sacrificing the specimen. This approach, although widely used, has the disadvantage that large quantities of mitotic inhibitor must be used if specimens are large (e.g. > 500 mm in length). At present, we use a modification of the phytohaemagglutin (PHA) direct preparation method developed by Hong *et al.* (1984). The PHA presumably serves to stimulate mitotic activity (Nowell, 1960). The method (described below) was developed primarily for cyprinid fishes up to 50 mm in total length.

PHA direct preparation

Specimens are injected i.p. with 2·5 μg ml^{-1} PHA dissolved in 1 × RPMI 1640 medium (Gibco) using 0·1–0·15 ml total volume. Modified Hank's (1 ×) solution or 0·9% NaCl may be used instead of RPMI 1640 medium. Care should be taken not to puncture the intestine or air bladder. Specimens are then returned to aerated aquaria for 18–24 h. The following day, individual specimens are removed from aquaria, bled for 1–2 min by snipping the main vein in the gill slit, and opened (using scissors or scalpel) from the anal opening to the base of the head. All kidney material is removed and placed in a 10 ml beaker containing 3–5 ml of 1 × 1640 media (or modified Hank's solution) without foetal calf serum. Gill tissue may also be used, although better results in terms of acceptable metaphase spreads are generally obtained with kidney. The material is teased apart using forceps, and colchicine (0·25 g ml^{-1} in distilled water or isotonic saline) is added at the rate of 1 to 2 drops per 5 ml of media. For larger specimens (and larger quantities of tissue), additional media (and colchicine) should be used and the large tissue pieces removed and discarded. On the average, we obtain approximately 5 mm^3 of kidney from a fish of 50 mm in length. The beaker is covered with Parafilm and placed in a 30° C incubator for 1·5–2·5 h. The dissociated cells and media are then poured into 15 ml conical centrifuge tubes and centrifuged at approximately 50 **g** for 4 min. The supernatant is removed, leaving only a small amount of liquid above the pellet. The pellet is resuspended in roughly 0·5 ml of supernatant before addition of hypotonic solution (7–8 ml of 0·56% KCl). The tubes are slightly agitated to resuspend the pellet and then placed in a 30° C incubator for 30 min. The tubes are centrifuged and the supernatant removed as above. The material is then fixed and prepared as described in a following section.

In addition to direct preparation, we also employ both long- and short-term cell culture. General methods for fish cell culture are well reviewed by Wolf & Quimby (1969), Gold (1979), and Hartley & Horne (1985). The long-term (3–5 weeks) culture method used in our laboratory is described in Amemiya *et al.* (1984). Briefly, primary cultures are initiated from 70% ethanol-rinsed pieces of caudal fin or scales by placement in growth media fortified 20% with foetal calf serum or 10% foetal calf serum and 10% newborn calf serum. The tissue culture flasks are incubated at 30° C for 2–4 weeks until monolayer growth reaches confluency. The flasks are monitored daily for explant outgrowth and contamination. We now use antibiotic/antimycotic solution in the growth media in the amount of 2·5 ml of 1 × antibiotic/antimycotic (Gibco) per 500 ml of growth media. We also now use 1 × Leibovitz's L-15 medium with L-glutamine (Gibco) instead of Medium 199 as described in Amemiya *et al.* (1984). Full details regarding subculturing and the harvest and preparation of chromosomes are in Amemiya *et al.* (1984). In general, our best yields are obtained with this procedure, although the time and expense involved have limited its use in large-scale studies. One recent improvement has been the development of a simple protocol for obtaining material in the field and bypassing the necessity of returning specimens alive to the laboratory. In brief, specimens obtained in the field are rinsed thoroughly with 70% ethanol to remove surface contaminants. Pieces of caudal fin or a few scales are then

removed, rinsed with 70% ethanol in a sterile 10 ml beaker, and placed in 15 ml autoclavable snap cap tubes, each of which contains approximately 2–5 ml of sterile ' field media '. The latter is L-15 medium supplemented with 2·25 ml of 1 × antibiotic/antimycotic per 50 ml of media. The growth media (prior and subsequent to the addition of tissue) is kept on ice (4–8° C). Upon return to the laboratory, the tubes are kept at room temperature for 2 to 3 days and monitored daily for microbial contamination. Those that show evidence of contaminants are discarded. On the average, cells from well over half of the non-contaminated tubes obtained in this way can be induced to divide once placed in new growth media fortified with foetal calf serum and incubated at 30° C. From this point, the flasks are treated as in our long-term cell culture procedure.

The short-term (3–7 days) culture method used in our laboratory essentially follows Yamamoto & Ojima (1973). Specimens are thoroughly rinsed with 70% ethanol and then opened (using sterile scissors or a sterile scalpel), taking care not to accidently slice the intestine or stomach. All internal organs (except the kidney) are removed and discarded. The kidney is then removed using sterile forceps and placed in a sterile 10 ml beaker containing 3–5 ml of sterile 1640 growth medium supplemented with 20% foetal calf serum. The kidney tissue is dissociated into small pieces using sterile forceps and a sterile scalpel. The resulting cell suspension is decanted into a 25 cm^2 tissue culture flask and additional growth media is added to make a final volume of roughly 10 ml. The flasks are incubated at 30° C for 3–7 days. The pH of the medium should be maintained at 7 or slightly lower. Each flask is gently agitated once a day and checked for pH and contamination. Chromosomes are harvested in the same way as from long-term cultures (Amemiya *et al.*, 1984).

One last point to note is that the incubation temperature used in our laboratory for the PHA direct preparation and cell culture procedures was resolved empirically only for cyprinid fishes. Temperature optima for other fish groups may need to be resolved by individual investigators. The same may also be true for salt concentration, particularly if the species or group is primarily marine.

PREPARING FISH CHROMOSOMES

Following hypotonic treatment, the majority of animal cytogeneticists employ a fixative solution of freshly prepared methanol–glacial acetic acid in a 3 : 1 ratio. In theory, the alcohol component hardens the tissue and also causes shrinkage; the acetic acid component, alternatively, counteracts some of the shrinkage caused by the alcohol and is desirable because of its rapid penetrating ability (Humason, 1979). We have recently adopted a new fixation schedule developed by Islam & Levan (1987) for use with mammalian chromosomes. This fixation procedure (described below) has several desirable attributes including increased removal (clearing) of cytoplasmic constituents. It may also enhance G-banding with trypsin (Islam & Levan, 1987).

Fixation procedure

Following hypotonic treatment in 15 ml conical centrifuge tubes (as employed in all our procedures), 3–5 ml of freshly prepared 9 : 1 (methanol–glacial acetic acid) fix is added drop by drop while gently shaking the tube on a vortex mixer. The final volume of 9 : 1 fix is brought to 7–8 ml. The tubes are incubated at room temperature for 2–3 min and then centrifuged at approximately 50 **g** for 4 min. The supernatant is removed, leaving only a small amount of liquid above the pellet, and 7–8 ml of glacial acetic acid is slowly added. The tube is gently agitated by hand and not with the vortex mixer. The cell suspension is incubated for 10 min at room temperature and centrifuged as before. Following removal of the supernatant, 7–8 ml of 3 : 1 (methanol–glacial acetic acid) fix is added and the tube vigorously shaken by hand. The cell suspension is incubated for 10 min at room temperature and then centrifuged as above. The supernatant is removed and fresh 3 : 1 fix is added. At this point, slides can be made or material stored at −20° C for later use.

Microscope slides are prepared using modifications of the methods described in Kligerman & Bloom (1977). The modifications are fully described in Rayburn & Gold (1982) and include the following.

Slide preparation

Small pieces of fixed tissue (from PHA direct preparations) or 1 to 2 drops of fixed cells (from cell culture) are placed in a depression well of an agglutination microslide. Four to five drops of 50% glacial acetic acid are added and the mixture gently agitated with the tip of a Pasteur pipette. Forty to fifty microlitres of the mixture are then withdrawn into a 50 µl disposable accupette pipette with a rubber suction tube and expelled gently onto a pre-cleaned microscope slide heated to approximately 46–48° C on a slide warmer. After 25–30 s, the suspension is withdrawn back into the accupette leaving a ring of cells roughly 1–1·5 cm in diameter. The process is repeated once or twice generating two or three such rings per slide. The slides are removed from the slide warmer after 2–3 min and either stained or stored. Details on storage conditions for the various metaphase banding procedures used in our laboratory are given in subsequent sections.

STAINING FISH CHROMOSOMES

Non-differentially stained (i.e. standard) preparations follow procedures used in most cytogenetics laboratories. Air-dried preparations are stained in 4–5% Giemsa (in 0·01 M phosphate buffer, pH 6·8) for 5 min, rinsed briefly in distilled water, and air dried. All preparations are made permanent by clearing slides in xylene for 10 min followed by mounting in Permount.

In 1968, Caspersson *et al.* (1968) opened a new era of cytogenetics research by showing that individual metaphase chromosomes could be differentiated following exposure to specific DNA-binding fluorescent stains or fluorochromes. Since then, a multitude of 'banding' techniques have been developed that enormously increase the power and sensitivity of chromosome analysis (Srivastava & Lucas, 1976; Macgregor & Varley, 1983). The three major categories of metaphase chromosome banding generally employed are nucleolus organizer region banding (NOR-bands), constitutive heterochromatin banding (C-bands), and serial or fluctuant banding (G- or R-bands). In the following, the methods used in our laboratory to produce metaphase bands on fish chromosomes are described or outlined. In addition, a brief review of chromosome banding and of the current successes in banding fish chromosomes is also included.

NOR banding

NOR-bands (with silver) are thought to represent the chromosomal sites of the 18S and 28S ribosomal RNA genes (=rDNA) which presumably were actively transcribed at a preceding interphase (Howell, 1977, 1982). The silver-staining reaction itself is apparently specific for a NOR-associated, non-histone protein that selectively binds (and reduces) ionic silver. NOR-banding with the GC-base pair binding fluorochromes chromomycin A_3 (CMA) or mithramycin has been observed in nearly all vertebrate groups except mammals (Schmid, 1982; Phillips & Ihssen, 1985; Amemiya & Gold, 1986; Mayr *et al.*, 1986; Schmid & Guttenbach, 1988). Unlike silver, CMA and mithramycin apparently stain DNA and will differentiate NORs regardless of previous genetic activity or chromosomal stage (Amemiya & Gold, 1986; Schmid & Guttenbach, 1988). Both fluorochromes, however, can also selectively stain heterochromatin (Amemiya & Gold, 1986; Schmid & Guttenbach, 1988), suggesting that some caution is advisable before considering a CMA or mithramycin-bright region on a chromosome as a NOR.

NOR-banding patterns are now known for well over 200 species of fishes. In most cases, emphasis has been placed on documenting NOR-bands on fish chromosomes rather than using the chromosome banded phenotypes to address systematic, populational, or cytogenetic problems. Notable exceptions include work in our laboratory on cyprinid fishes (reviewed in Buth *et al.*, 1990) and several studies by Phillips and colleagues on salmonid fishes (Phillips *et al.*, 1986, 1988, 1989).

The procedures for NOR-banding are extremely straightforward and simple. For silver-staining, most researchers employ Howell & Black's (1980) one-step method using a colloidal developer. Gold & Ellison (1983) outlined a few modifications of this procedure, the most important of which was a post-staining fixation step using 5% sodium thiosulphate in

order to ensure the removal of residual ionic silver. Our procedure for NOR-banding with CMA or mithramycin is fully documented in Amemiya & Gold (1987).

C-banding

C-bands are regions of constitutive heterochromatin (Sumner, 1977) and predominantly contain transcriptionally inactive, highly repeated DNA sequences (Peacock *et al.*, 1977; John & Miklos, 1979). Most C-banding techniques involve chromatin depurination (with acid), denaturation (with base) and preferential extraction of non-heterochromatic DNA in hot salt solutions (Comings, 1978; Kongsuwan & Smyth, 1978; Holmquist, 1979). The cellular functions of constitutive heterochromatin are not known, although a number of hypotheses have been forwarded (Yoon & Richardson, 1978; John & Miklos, 1979; Brutlag, 1980).

C-band patterns are documented for over 50 species of fishes, although like NOR-banding, the primary emphasis has been on documenting the existence and location of C-bands on fish chromosomes. With notable exceptions (e.g. salmonids), constitutive heterochromatin amounts appear small and resolved C-bands are primarily centromeric (Haaf & Schmid, 1984; Sola *et al.*, 1984; Li *et al.*, 1985; Mayr *et al.*, 1987; Lopez *et al.*, 1989). The most extensive work on C-banding in fishes has been carried out on salmonids by Phillips and colleagues (Phillips & Ihssen, 1986; Phillips & Hartley, 1988; Pleyte *et al.*, 1989). C-bands in salmonids are found both interstitially and at the telomeres, and in some species, e.g. the Arctic charr, *Salvelinus alpinus*, both the number and location of chromosomal C-bands may be stock specific (Pleyte *et al.*, 1989). Considerable quantities of constitutive heterochromatin also appear to occur in blenniid fishes (Garcia *et al.*, 1987).

Most procedures for C-banding in fishes essentially follow the methods of Sumner (1972). The procedure used in our laboratory is outlined in Gold *et al.* (1986). In brief, slides (chromosomes) are hydrolysed for 15–20 min in 0·2 N HCl at 37° C, denatured in 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 & Hsu, 1974) using 2 × SSC. Empirically, we have found it important to use slides which have been stored dessicated at room temperature for three or more days prior to C-banding, and to rinse the slides thoroughly with distilled water and allow them to partially air dry following each step in the procedure. In addition, staining with Giemsa (4–5% in 0·01 M phosphate buffer) usually takes 10–15 min or longer before C-bands are fully resolved.

Fluorochromes may also be used to resolve C-bands provided the heterochromatic regions are differentially rich in AT- or GC- base pairs relative to the remainder of the chromatin. AT-enhancing fluorochromes such as quinacrine or DAPI have been used to resolve C-bands in salmonids, a poeciliid, and several European percids (Haaf & Schmid, 1984; Mayr *et al.*, 1987; Phillips & Hartley, 1988); whereas the GC-enhancing fluorochrome CMA has been used to resolve C-bands in a North American percid (Amemiya & Gold, 1986). The appropriate staining (and counterstaining) methods may be found in the cited references.

Finally, Lloyd & Thorgaard (1988) and Cau *et al.* (1988) have recently shown that treatment of metaphase chromosomes on microscope slides with specific restriction endonuclease enzymes (followed by Giemsa staining) produced C-bands on the chromosomes of the rainbow trout and the muraenid, *Muraena helena*, respectively. This type of approach has yet to be fully evaluated in other fishes, although in mammals such ' banding ' appears to often yield a mixture of both C- and serial-bands (Miller *et al.*, 1983; Kaelbling *et al.*, 1984).

Serial or fluctuant banding

Serial or fluctuant bands (commonly called G- or R-bands) are lateral striations or transverse bands along the arms of chromosomes and in mammals are thought to represent regions of either differential DNA base sequence composition or differential chemical and/or thermal sensitivity (Comings, 1978; Jorgenson *et al.*, 1978; Holmquist *et al.*, 1982). G-band regions in mammals are known to be rich in AT-base pairs, resistant to protein-denaturing or detergent treatment, sensitive to thermal denaturation, and late replicating;

R-(reverse) bands are known to be rich in GC-base pairs, sensitive to protein denaturing or detergent treatments, resistant to thermal denaturation, and early replicating (Kato & Moriwaki, 1972; Comings, 1978; Jorgenson *et al.*, 1978; Neumann *et al.*, 1980; Latt *et al.*, 1980; Holmquist *et al.*, 1982; Bickmore & Sumner, 1989). Because of their AT-richness, G-bands in mammals can often be resolved with AT-enhancing fluorochromes such as quinacrine or DAPI. Similarly, GC-enhancing fluorochromes such as CMA or mithramycin will often resolve R-bands (*op. cit.*). The functions of serial bands are not fully known. Suggested functions include both a role in chromosome contraction (Sumner, 1977) and as basic organizational units in cellular DNA replication (Holmquist *et al.*, 1982). One last type of serial banding to note is replication banding where the compound 5-bromodeoxyuridine (BrdU) is substituted for thymidine during a portion of the S phase of the cell cycle, followed by staining of metaphases using a fluorochrome such as Hoechst-33258 (Latt, 1973; Schempp & Schmid, 1981). The bands produced by this procedure, at least in higher vertebrates, are essentially analogous to the G- or R-bands depending on when, during DNA replication, the BrdU was incorporated.

Serial bands produced by protein or heat denaturation treatments have been reported in only a few fishes (Wiberg, 1983; Sola *et al.*, 1984; Hartley & Horne, 1985 and references therein; Amemiya, 1987; Almeida Toledo *et al.*, 1988). However, in most of these cases, the resolution of individual bands was sufficiently poor as to preclude definitive karyotyping. *Anguilla* species appear to be exceptions (Wiberg, 1983; Sola *et al.*, 1984), a finding which has been attributed to the DNA base pair heterogeneity and possible presence of GC-rich isochores in *Anguilla* genomes (Medrano *et al.*, 1988). The fact that most fish genomes are remarkably homogeneous in DNA base pair composition (Gold & Karel, 1988) may explain why trypsin/heat denaturation produced serial bands are so difficult to obtain on fish chromosomes. Alternatively, good quality serial bands using replication banding procedures have been produced on the chromosomes of the rainbow trout (Delany & Bloom, 1984), two scorpion fishes (Giles *et al.*, 1988), a loach and the swamp eel (Liu, 1986), two species from the genus *Eigenmannia* (Almeida Toledo *et al.*, 1988), and the silver carp (Zhou *et al.*, 1989). Replication banding, however, requires good cell culture synchronization techniques in order to insure that the bands resolved are homologous since both G- or R- (or both) bands could be produced.

At present, three methods of serial banding are used in our laboratory. The first, Z-banding, was accidentally discovered while attempting to carry out restriction endonuclease banding using the methods of Mezzanotte *et al.* (1983) and Lloyd & Thorgaard (1988). In brief, we were unable to obtain reproducible restriction bands on cyprinid chromosomes. However, on control slides, containing buffer but no enzymes, it was noticed that serial-type bands were often produced. Further experimentation revealed that the ' banding ' was produced as a function of the magnesium ion concentration and the pH of the working solution (see below). A comparison of Z-bands on human chromosomes with those produced using trypsin indicated that the Z-bands are the same as G-bands. The second method is a trypsin G-banding procedure modified from Davisson & Akeson (1987). The third method is a BrdII replication banding procedure adapted from Ronne (1983), Tucker (1986) and Zhou *et al.* (1989). All three are described below. In our hands, the trypsin G-banding method is the most reproducible.

Z-banding

Prepared slides are dried overnight on a 65–85° C slide warmer or in an incubator. The following day, the slides are incubated for 15 min in a coplin jar containing absolute ethanol which has been stored at least 24 h in a −70° C ultracold freezer. The coplin jar should be packed in ice for the duration of the incubation and care should be taken to keep the interior of the coplin jar dry. Slides are removed from the alcohol, blown dry and placed into humidity chambers (after Arrighi & Hsu, 1974). The filter paper in each humidity chamber is saturated with 0·01 M phosphate buffer at pH 6·8. Approximately 100–150 µl of working solution is pipetted onto each slide, a coverslip is added, and the chamber is incubated for 2 h at 37° C. The working solution is a 1 : 10 dilution (using sterile, distilled water) of a stock solution made up of equal volumes of 0·1 M $MgCl_2$ (pH 8·6) and 0·5 M Tris-HCl (pH 8·4). The stock solution should be made fresh each week. Following incubation, the coverslips

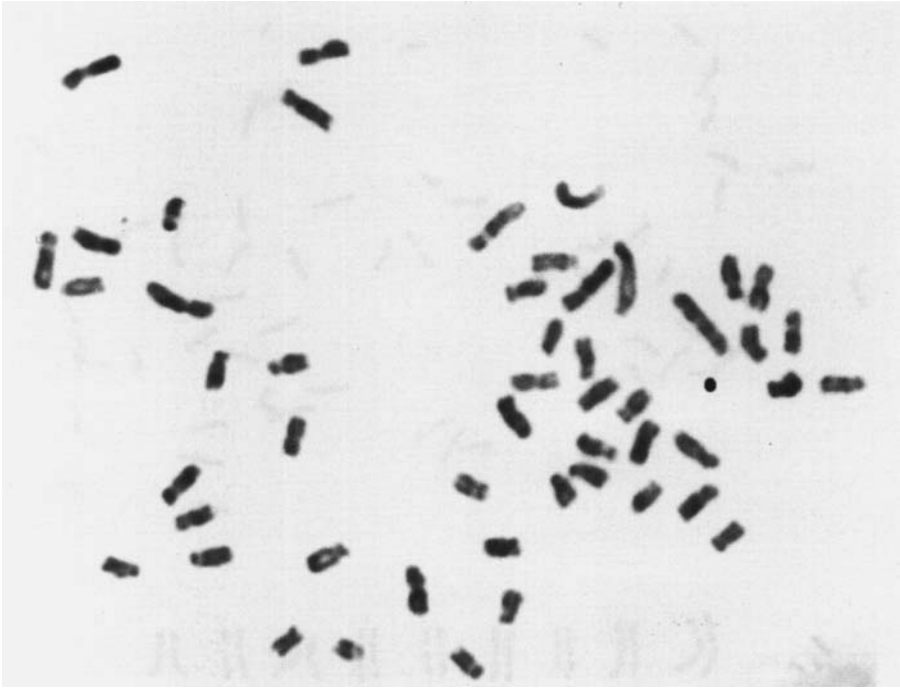

FIG. 1. Z-banded metaphase from the cyprinid fish *Notemigonus crysoleucas*.

are gently washed off with distilled water, and the slides are stained 2–3 min in 3–5 ml of 5% Giemsa stain in 0·01 M phosphate buffer at pH 6·8. An example of Z-banding is shown in Fig. 1.

G-banding

Prepared slides are stored in a 60° C oven for at least 3 days or at room temperature for at least a week. The slides are incubated in 2 × SSC for 2 h at 60° C. A coplin jar is used for the incubation and no more than six slides should be placed in a single jar. The slides are rinsed individually with 0·9% NaCl, partially air dried, and treated/stained in a trypsin/Giemsa solution for 15 min at room temperature. The trypsin/Giemsa solution contains 600 µl of a trypsin stock solution and 1·5 ml Giemsa stain in 45 ml 0·01 M phosphate buffer at pH 7·2. The trypsin stock solution contains 0·1 ml of 2·5% trypsin (Gibco) in 2·4 ml distilled water and should be made just prior to use. Following treatment/staining, slides are individually rinsed twice in distilled water, air-dried, cleared in xylene and mounted in Permount. An example of our trypsin G-banding is shown in Fig. 2(a); a completely banded karyotype is shown in Fig. 2(b).

BrdU replication banding

Our BrdU replication banding procedure is carried out on long-term cell cultures that have reached 60–75% confluency. The media from such confluent cultures is decanted from the tissue culture flasks and the cells are washed with 5 ml of a sterile modified Hank's (1 ×) working solution. The latter is made by mixing 25 ml of sterile Hank's salt solution and 25 ml of sterile Hank's sugar solution with 450 ml of sterile distilled water. Hank's salt solution contains 80 g NaCl, 4 g KCl, 0·32 g Na_2HPO_4, and 0·06 g KH_2PO_4 in 500 ml distilled water; Hank's sugar solution contains 10 g dextrose in 500 ml distilled water. Following the wash, 5 ml of fresh L-15 media (supplemented 20% with foetal calf serum) containing BrdU is added and the cultures are incubated in the dark at 30° C for 12 h. The

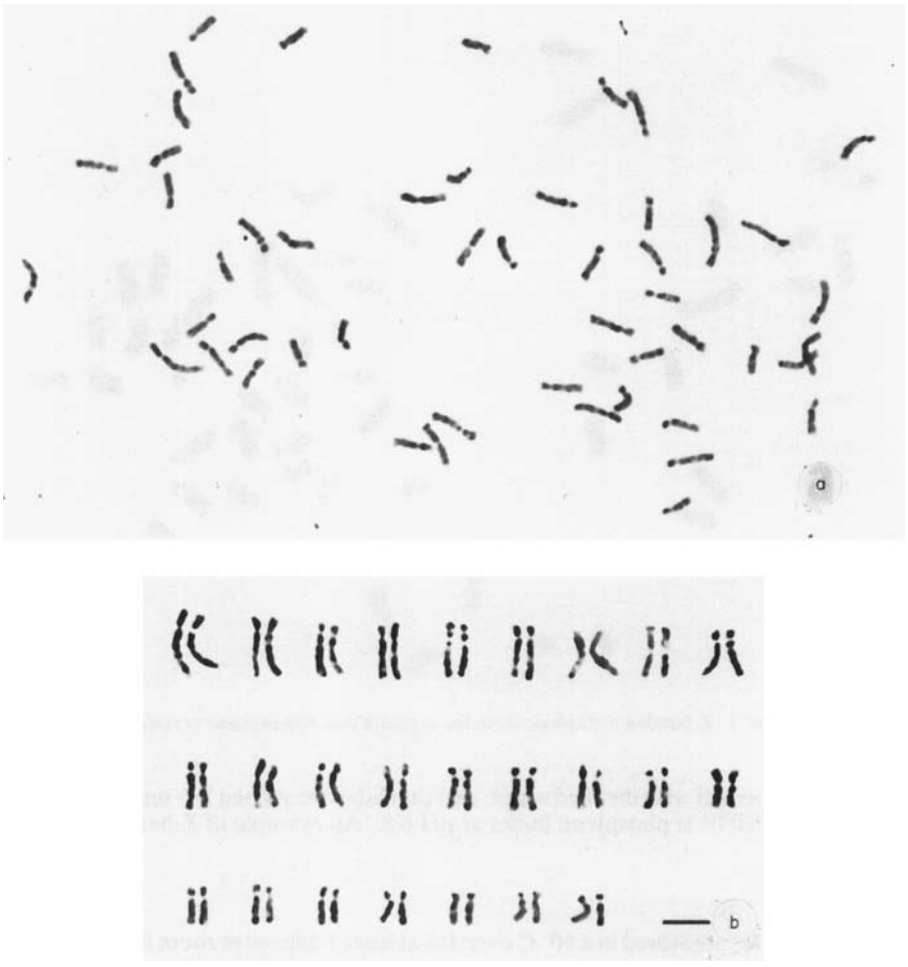

FIG. 2. (a) Trypsin G-banded metaphase from the cyprinid fish *Opsopoeodus emiliae*. (b) Karyogram of metaphase from Fig. 2(a). Scale bar equals 5 μm.

BrdU is added at the rate of 0·2 ml of a BrdU stock solution per 10 millilitres of growth media. The BrdU stock solution is 0·5 mg ml^{-1} (in distilled water) and is stored in the dark at −20° C. Colchicine from a 0·25 mg ml^{-1} stock solution (in sterile distilled water) is added at a rate of 1 drop (50 μl) per 5 millilitres media for the last 2 h. Cells are harvested by normal procedures (Amemiya *et al.*, 1984), treated with 0·56% KCl at room temperature for 25–30 min, and fixed by the method outlined above. Prepared slides are incubated in the dark for 30 min in a coplin jar containing 500 μl of a Hoechst-33258 stock solution in 45 ml distilled water. From four to six slides may be stained per coplin jar. The Hoechst-33258 stock solution contains 1 mg Hoechst-33258 dissolved in 30 ml absolute ethanol and is stored in the dark at −20° C. Following incubation, slides are rinsed with 0·01 M phosphate buffer at pH 8·2. Approximately 150 μl of fresh Hoechst-33258 is then pipetted onto each slide, coverslips are added, and the slides are illuminated for 30 min with a UV hood lamp at a distance of 2·5 cm. Following UV exposure, the slides are rinsed with distilled water to remove the coverslips and incubated in 2 × SSC at 60° C for 60–90 min. Slides are then washed in 0·01 M phosphate buffer at pH 8·2 and air-dried. The slides are stained in a trypsin/Giemsa solution as described above for the G-banding procedure. An example of serial bands produced by this method is shown in Fig. 3.

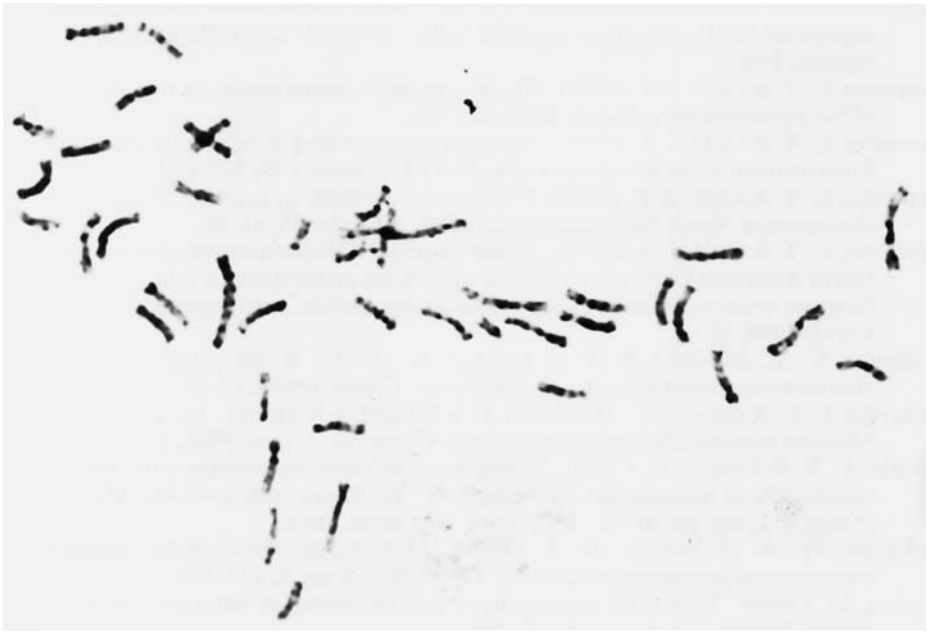

FIG. 3. Replication bands from the cyprinid fish *Cyprinella lutrensis*.

III. SUMMARY

As pointed out by Gold (1979) and Hartley & Horne (1985), the study of fish chromosomes has lagged far behind those of other vertebrates. Based on our work, the reasons for this appear primarily to be methodological. Fish obviously possess NORs, constitutive heterochromatin, and serial bands on their chromosomes, all of which can be produced by methods commonly used in other vertebrates. The only 'complete failures' at this point appear to be the resolution of serial bands using AT- or GC-enhancing fluorochromes. This may suggest that serial bands in fishes (although present) are neither AT- or GC-rich relative to the surrounding chromatin. If true, this would support the hypothesis of Holmquist *et al.* (1982) that the organization of eukaryotic genomes into temporal clusters of DNA replication units preceded the evolution of AT- and GC-rich regions which correspond to the G- and R-bands, respectively. Regardless, it is clear that most types of metaphase chromosome banding can be carried out on fish chromosomes, some (or most) of which should prove useful for identifying homologies both within and between fish species.

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