Folia biologica (Kraków), vol. **53** (2005), No 3-4

Identification of Early and Late Replicating Heterochromatic Regions on Platyfish (*Xiphophorus maculatus*) Chromosomes*

Konrad OCALEWICZ

Accepted September 6, 2005

OCALEWICZ K. 2005. Identification of early and late replicating heterochromatic regions on platyfish (*Xiphophorus maculatus*) chromosomes. Folia biol. (Kraków) **53**: 149-153.

The platyfish (*Xiphophorus maculates*) is one of the best studied lower vertebrates, however cytogenetic data on this species is rather limited. This paper presents advances in platyfish cytogenetics, performed by classical techniques enabling the identification of heterochromatin as well as early and late replicating chromosomal regions. Analysis of chromatin resistant to restriction enzymes (*Alu I, Dde I* and *Hinf I*) showed pericentromeric, telomeric and interstitial blocks of heterochromatin. DAPI fluorochrome staining revealed that some of the pericentromeric bands are discrete AT rich clusters of chromatin. Analysis of platyfish DNA replication on the chromosomal level showed heterogeneity of fish heterochromatin regarding its replication time; moreover, the replication banding pattern distinguished pairs of homologous chromosomes.

Key words: Chromosomes, heterochromatin, platyfish, replication banding, restriction enzymes.

Konrad OCALEWICZ, Department of Ichthyology, Faculty of Enviromental Sciences and Fisheries, University of Warmia and Mazury in Olsztyn, Oczapowskiego 5, 10-718 Olsztyn, Poland.
E-mail: con@uwm.edu.pl

The platyfish (*Xiphophorus maculates*) has aroused interest for many years. At first, this aquarium species was studied because of its genetic sex determination process. The platyfish has a three chromosomal sex determination system (X, Y, W) and sex linked pigment loci (ORZACK *et al.* 1980; KALLMAN 1984). Individuals possessing XY or YY sex chromosomes develop into males and XX, XW and WY are females. Now, the platyfish and its hybrids with other *Xiphophorus* fishes are considered as fish models for investigating the etiology and genetics of melanoma formation and other diseases (SCHARTL 1995; MORIZOT *et al.* 2001; MOREDOCK *et al*. 2003; MEIERJOHANN *et al*. 2004). Several DNA sequences closely linked to genes involved in the sex determination process as well as genes involved in the process of oncogenesis have been identified (NANDA *et al*. 1996, 2000; GUTBROD & SCHART 1999). Unfortunately, success in this field does not correspond to cytogenetic studies, which seem to be the weakest point of platyfish physical gene mapping.

The karyotype of the platyfish consists of 24 pairs of small acrocentric and subtelocentric chromosomes. Chromosome number and morphology were analyzed by simple banding techniques (NANDA *et al*. 1992; OCALEWICZ 2004) which could not establish pairs of homologues. Differences in genome compartmentalization between warm-blooded and cold-blooded vertebrates (contrary to homotherms, genomes of poikilothermic vertebrates are not divided into GC-rich and GC-poor compartments), cause a lack of structural banding patterns such as G banding in fish chromosomes (MEDRANO *et al*. 1988). Despite the difficulties in establishing distinct bands, several approaches have been proposed for studying fish chromosomes. Identification of heterochromatic regions or the induction of replication bands during the course of incorporation of a thymidine analogue, BrdU, into chromosomal DNA allow for more in-depth analyses of fish chromosomes (OCALEWICZ *et al*. 2003; SALVADORI *et al*. 2003; JANKUN *et al*. 2004). In the present work, the distribution of differentially stained heterochromatin as well as early and late replicating chromosomal regions in the platyfish genome have been investigated. A combination of these approaches pointed

*Supported by grant from University of Warmia and Mazury in Olsztyn, Poland, Project No. 05071.213.

out the heterogeneity of platyfish heterochromatin. Additionally, pairs of platyfish homologous chromosomes were identified based on the replication banding pattern.

Material and Methods

Chromosome slides from 14 platyfish were obtained according to the method described by DAGA *et al*. 1996.

Digestion with restriction endonucleases *Alu I, Dde I* and *Hinf I* was performed according to the procedure described by OCALEWICZ (2002). Enzymes suspended in deionized water and appropriate buffers were added to metaphase slides and covered with coverslips. The enzyme concentration for *Alu I* was 0.1 U/μl and incubation lasted for 30 min, for *Dde I* it was 0.3 U/μl and lasted 45 min, and for *Hinf I* 0.5 U/μl and 60 min. After incubation in a moist chamber at 37° C the slides were washed with distilled water and stained in 20% Giemsa solution for 15 min.

For visualization of AT rich chromosome regions, the slides were mounted in antifade solution containing DAPI (4',6-diamidino-2-phenylindole) (5 μg /ml).

Replication banding was carried out following ALMEIDA-TOLEDO (1992) with some modifications: the period of BrdU incubation ranged from 4 to 6 hours. The amount of BrdU administered was 0.01 ml per fish. The FPG method (Fluorochrome plus Giemsa) was used for chromosome staining (ALMEIDA-TOLEDO 1992).

Results and Discussion

The platyfish has a diploid chromosome number of 2n= 48. Chromosomes are acro-subtelocentric and very similar to each other. Only the smallest chromosome pair could be identified (Fig. 1). Three different restriction enzymes: *Alu I*, *DdeI* and *Hinf III* recognising and cutting different DNA sequences (AG↓CT, C↓TNAG, and G↓ANTC, respectively), were used to identify chromatin resistant to enzyme digestion in the platyfish karyotype (Fig. 2). Although positive bands on chromosomes showing chromatin clusters poor in digestion sites were subtle and sometimes difficult to observe on the small platyfish chromosomes, this approach revealed the distribution of different components of the platyfish genome. *AluI* and *DdeI* did not cut pericentromeric and telomeric regions in most of the chromosomes. Additionally, interstitially located chromatin clusters resistant to enzyme digestion were observed on at least six and two chromosomes after *Alu I* and *DdeI* enzyme restriction, respectively (Fig. 2a, b). On the contrary, *Hinf I* enzyme digestion generated more interstitial darkly stained bands; large undigested blocks of chromatin were identified on at least 12 chromosomes, whereas pericentromeric and telomeric regions were stained rather pale (Fig. 2c). Different banding patterns after different restriction enzyme digestion showed heterogeneity of fish heterochromatin as revealed in chromosomes of salmonid fish species (HARTLEY *et al.* 1991; LOZANO *et al.* 1991; JANKUN *et al*. 2004). This conclusion is in agreement with results obtained with the DAPI banding procedure; only a few chromosomes showed discrete centromeric bands demonstrating that these regions belong to AT-rich DNA clusters (Fig. 2d). Weak DAPI fluorescent signals confirmed that chromosomes of Poecilidae have very small and rare AT-rich regions (SOLA *et al*. 1992). BrdU incorporation into chromosomes during late S phase (4-6 hours of treatment) identified of early (dark) and late (pale) replication bands. The distribution of these bands is characteristic for both homologous chromosomes because of the identical organization of replicon clusters (HOLMQUIST *et al*. 1982). Late replication regions of platyfish chromosomes are confined to the centromeric or pericentromeric regions of chromosomes from the 1, 2, 3, 4, 8, 9, 10, 14, 16, 18, 19, 20, 21, 22, and 24 pairs (Fig. 3). In

Fig. 1. Karyotype of the platyfish (*Xiphophorus maculates*) (2n= 48), stained conventionally by Giemsa.

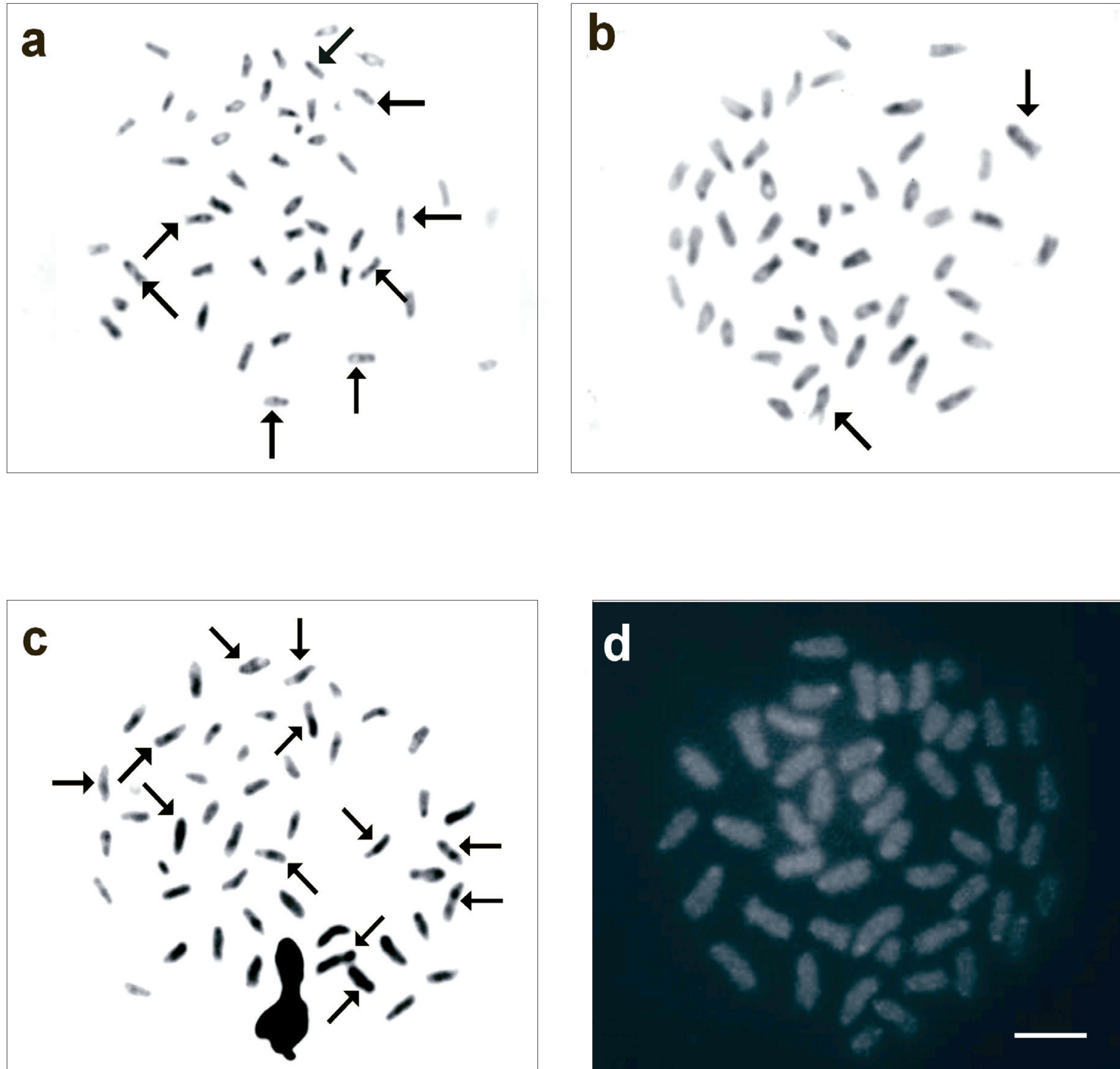

Fig. 2. Metaphase chromosomes of platyfish showing the restriction endonuclease banding pattern obtained after digestion with *Alu I (a), Dde I (b), Hinf I (c)* and DAPI banding pattern (d). Arrows indicate pairs of chromosomes with interstitial heterochromatin resistant to enzyme digestion. Bar = 10 μm.

pufferfish (*Tetraodon nigroviridis*), centromeres of all chromosomes show a late replicating pattern (GRÜTZNER *et al*. 1999), however, in spined loach (*Cobitis taenia*) centromeric regions exhibit an early replicating nature (BORON 2003). Different replication time of centromeric heterochromatin between particular chromosomes has been observed in salmonids (JANKUN *et al*. 1998) and cyprinids (DAGA *et al.* 1996).

Most of the telomeric or subtelomeric regions in platyfish chromosomes are late replicating what is in agreement with previous observations (MCCARROLL & FANGMAN 1988). However, in the case of some chromosomes, reduced p arms are darkly stained after FPG staining showing their early replicating character. As GC-rich chromatin is thought to replicate early during S phase and AT-rich chromatin clusters are rather late replicating, some of the platyfish subtelomeric regions can be considered as constituting GC-rich clusters other than NORs, which in fish are heterochromatic and seem to replicate at the end of the S phase (AMORES *et al*. 1995; JANKUN *et al*. 1998; BORON 2003). The lack of either the early or late replicating pattern in pericentromeric and telomeric heterochromatin is further proof of the heterogeneity of this component in the platyfish genome.

Early replicating clusters apart from the pericentromeric and telomeric regions of several chromosomes appeared in interstitial positions of most of the chromosomes. This pattern in addition to the length of chromosomes enabled the identification of homologous chromosomes and their arrangement into a karyotype (Fig. 3). A similar approach has been proposed for karyotyping other fish in-

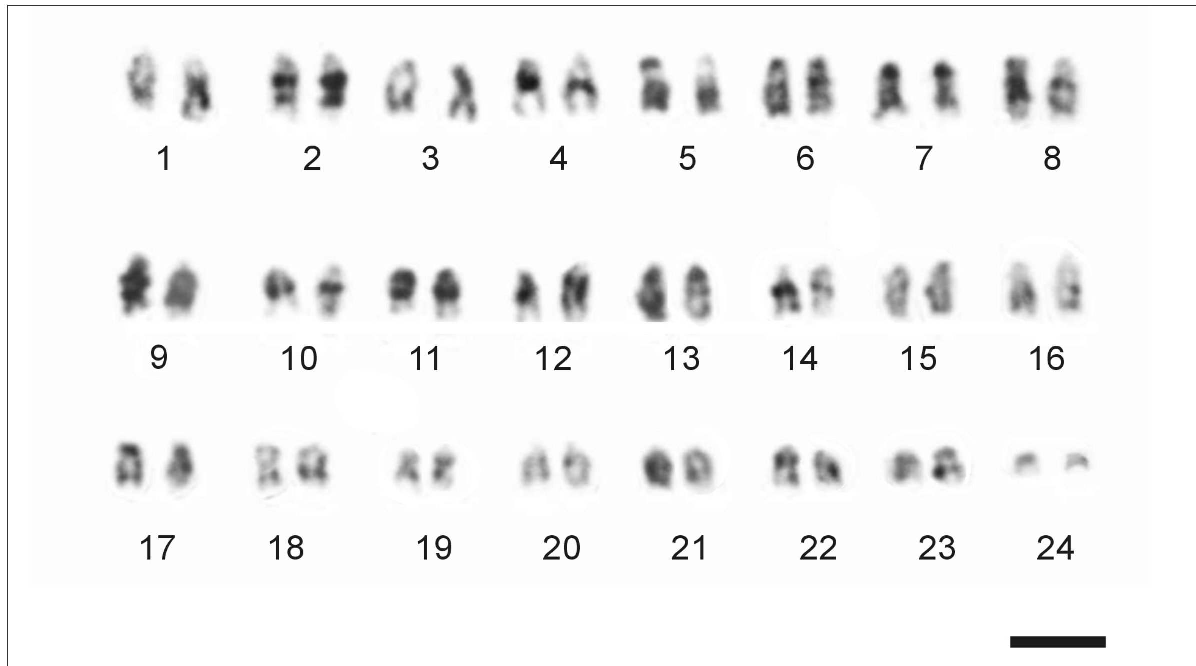

Fig. 3. Early (dark) and late (pale) replication bands on platyfish homologous chromosomes. Bar = 10 μm.

cluding zebrafish (*Danio rerio*) (DAGA *et al.* 1996), pufferfish (GRÜTZNER *et al*. 1999), eels (SALVADORI *et al*. 2003) and salmonid fish species (ABUIN *et al*. 1994; JANKUN *et al*. 2004), among others. Constructing standard karyotypes is indispensable if physical gene mapping programmes are proposed. Replication banding can be used simultaneously with fluorescence in situ hybridization (FISH). Incorporation of BrdU and hybridization with molecular probes indicates the location of a given DNA sequence on a particular chromosome or chromosomes (FUJIWARA *et al.* 2001). A similar approach can be proposed in platyfish where simple repetitive DNA sequences will be mapped. Merging the replication banding technique and FISH with 45S or 5S rDNA probes should show the distinct chromosomal location of major and minor ribosomal genes.

In summary, combining restriction enzyme digestion and replication banding techniques showed differences between the replication time of pericentromeric as well as telomeric heterochromatin among platyfish chromosomes. Moreover, analysis of platyfish DNA replication time on a chromosomal level was very efficient in finding pairs of homologous chromosomes. This has crucial importance in the process of physical gene mapping in platyfish and analysis of chromosome rearrangements in platyfish melanoma cells.

Acknowledgements

I thank Dr. M. JANKUN and Dr. P. WOZNICKI (UWM in Olsztyn) for helpful suggestions and technical assistance.

References

ABUIN M., AMARO R., SANCHEZ L. 1994. Improving *Salmo salar* karyotype: restriction enzyme and replication banding; Cytobios **78**: 143-152.

ALMEIDA-TOLEDO L. 1992. BrdU replication bands. First Intern. Workshop on Fish Cytogenetic Techniques, Concarneau 992, pp. 67-76.

BORON A. 2003. Replication banding patterns in the spined loach, *Cobitis teania* L. (Pisces, Cobitidae). Genetica **119**: 51-55.

AMORES A., BEJAR J., ALVAREZ M. C. 1995. Replication, Cand Ag-NOR chromosome banding in two anquilliformes fish species. Marine Biology **123**: 845-849.

DAGA R. R., THODE G., AMORES A. 1996. Chromosome complement, C-banding, Ag-NOR and replication banding in the zebrafish (*Danio rerio*). Chromosome Research **4**: 29-32.

FUJIWARA A., NISHIDA-UMEHARA C., SAKAMOTO T., OKAMOTO N., NAKAYAMA I., ABE S. 2001. Improved fish lymphocyte culture for chromosome preparation. Genetica **111**: 77-89.

GRÜTZNER F., LÜTJENS G., ROVIRA C., BARNES D. W., ROPERS H-H., HAAF T. 1999. Classical and molecular cytogenetics of the pufferfish (*Tetraodon nigroviridis*). Chromosome Research **7**: 655-662.

GUTBROD H., SCHARTL M. 1999. Intragenic sex-chromosomal crossovers of *Xmrk* oncogene alleles affect pigment pattern formation and the severity of melanoma in *Xiphophorus*. Genome Research **6**: 102-113.

HARTLEY E. S. 1991. C, Q and restriction enzyme banding of the chromosomes in brook trout, *Salvelinus fontinalis* and Arctic charr *Salvelinus alpinus*. Hereditas **114**: 252-261.

HOLMQUIST G., GRAY M., PORTER T., JORDAN J. 1982. Characterization of Giemsa dark- and light-band DNA. Cell **31**: 121-129.

JANKUN M., OCALEWICZ K., WOZNICKI P. 1998. Replication, C and fluorescent chromosome banding patterns in European whitefish (*Coregonus lavaretus* l.), from Pomeranian Bay, Poland. Hereditas **128**: 195-199.

JANKUN M., OCALEWICZ K., MOCHOL M. 2004.Chromosome banding studies by replication and restriction enzymes treatment in vendace (*Coregonus albula*) (Salmonidae, Salmoniformes). Folia biol. (Kraków) **52**: 47-52.

KALLMAN K. D. 1984. A new look at sex determination in poeciliid fishes. (In: Evolutionary Genetics of Fishes, TURNER B. J. ed. Plenum, New York): 95-171.

LOZANO R., RUIZ REJON C., RUIZ REJON M. 1991. An analysis of coho salmon chromatin by means of C-banding, Ag and fluorochrome staining, and in situ digestion with restriction endonucleases. Heredity **66**: 403-409.

MCCAROLL R. M., FANGMAN W. L. 1988. Time of replication of yeast centromeres and telomeres. Cell **54**: 505-513.

MEDRANO L., BERNARDI G., COUTURIER J., DUTRILLAUX B., BERNANRDI G. 1988. Chromosome banding and genome compartmentalization in fishes. Chromosoma **96**: 178-196.

MEIERJOHANN S., SCHARTL M., VOLFF J-N. 2004. Genetic, biochemical and evolutionary facets of Xmrk-inducedmelanoma formation in the fish *Xiphophorus*. Comp. Biochem. Physiol., Part C **138**: 281-289.

MORIZOT D. C., NAIRN R. S., SIMHAMBHATLA P., COLETTA L. D., TRONO D., CHOVANEC L., WALTER R. B., KAZIANIS S. 2001. *Xiphophorus* genetic linkage map: beginnings of comparative gene mapping in fishes. Marine Biotechnology **3**: 153-161.

MOREDOCK S., NAIRN R. S., JOHNSTON D. A., BYROM M., HEATON G., LOWERY M., MITCHELL G. L. 2003. Mechanisms underlying DNA damage resistance in a *Xiphophorus* melanoma cell line. Carcinogenesis **24**: 1967-1975.

NANDA I., SCHARTL M., FEICHTINGER W., EPPLEN J. T., SCHMID M. 1992. Early stage of sex chromosomes differentiation in fish as analyzed by simple repetitive DNA sequences. Chromosoma **101**: 301-310.

NANDA I., WEIS S., FORNZLER D., ALTSCHMIED J., SCHARTL M., SCHMID M. 1996. Clustered organization and conservation of the *Xiphophorus maculatus* D-locus which includes two distinct amplified sequences. Chromosoma **105**: 242-249.

NANDA I., VOLF J. N., WEIS S., KORTING C., FROSCHANER A., SCHMID M., SCHARTL M. 2000. Amplification of a long terminal repeat-like element on the Y chromosome of the platyfish (*Xiphophorus maculates*). Chromosoma **109**: 173-180.

OCALEWICZ K. 2002. Cytogenetic markers of X chromosome of rainbow trout (*Oncorhynchus mykiss*) from Rutki strain, Poland. Folia biol (Kraków) **50**: 13-16.

OCALEWICZ K. 2004. Cytogenetic analysis of the platyfish (*Xiphophorus maculatus*) shows location of major and minor rDNA on chromosomes. Hereditas **141**: 333-337.

OCALEWICZ K., JANKUN M., LUCZYNSKI M. 2003. Homologous chromosome characteristics by sequential banding procedures in rainbow trout *Oncorhynchus mykiss*. Folia biol. (Kraków) **51**: 41-46.

ORZACK S., SOHN J., J., KALLMAN K. D., LEVI A., JOHNSTONE R. 1980. Maintenance of the three sex chromosomes polymorphism in the platyfish (*Xiphophorus maculatus)*. Evolution **34**: 663-672.

SALVADORI S., COLLUCIA E., CANNAS R., CAU A., DEIANA A. M. 2003. Replication banding in two Mediterrenean eels: chromosomal characterization and comparison. Genetica **119**: 253-258.

SCHARTL M. 1995. Platyfish and swordtails: a genetic system for the analysis of molecular mechanism in tumor formation. TIG **5**: 185-189.

SOLA L., ROSSI A. R., IASELLI V., RASCH E. M., MONACO P. J. 1992. Cytogenetics of bisexual / unisexual speciec of *Poecilia*. II. Analysis of heterochromatin and nucleolar organizer regions in (Poecilia mexicana mexicana) by C- banding and DAPI, quinacrine, chromomycin A_3, and silver staining. Cytogenet. Cell Genet. **60**: 229-235.