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Early stages of sex chromosome differentiation in fish as analysed by simple repetitive DNA sequences *

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Abstract. Animal sex chromosome evolution has started on different occasions with a homologous pair of autosomes leading to morphologically differentiated gonosomes. In contrast to other vertebrate classes, among fishes cytologically demonstrable sex chromosomes are rare. In reptiles, certain motifs of simple tandemly repeated DNA sequences like $(gata)_n/(gaca)_m$ are associated with the constitutive heterochromatin of sex chromosomes. In this study a panel of simple repetitive sequence probes was hybridized to restriction enzyme digested genomic DNA of poeciliid fishes. Apparent male heterogamety previously established by genetic experiments in *Poecilia reticulata* (guppy) was correlated with male-specific hybridization using the $(GACA)_4$ probe. The $(GATA)_4$ oligonucleotide identifies certain male guppies by a Y chromosomal polymorphism in the outbred population. In contrast none of the genetically defined heterogametic situations in *Xiphophorus* could be verified consistently using the collection of simple repetitive sequence probes. Only individuals from particular populations produced sex-specific patterns of hybridization with $(GATA)_4$. Additional poeciliid species (*P. sphenops, P. velifera*) harbour different sex-specifically organized simple repeat motifs. The observed sex-specific hybridization patterns were substantiated by banding analyses of the karyotypes and by in situ hybridization using the $(GACA)_4$ probe.

Introduction

The class of teleost fish exhibits diverse forms of sexuality, ranging from simultaneous or successive hermaphroditism to gonochorism with early or late sexual differentiation (Price 1984). Using cytological techniques it has been demonstrated in several species that both the XY ♂/XX ♀ and the ZW ♀/ZZ ♂ mechanisms of chromosomal sex determination occur. All sex chromosomes in fish identified cytologically to date are distinctively heteromorphic in one sex and thus can be recognized by simple conventional staining methods (Ebeling and Chen 1970; Gold 1979; Beçak 1983). Yet simple karyological analyses have failed to detect heterogametic sex chromosomes in many gonochoristic fishes displaying conspiucuous sexual dimorphism. But even in the absence of size differences between gonosomes, sex determination in many teleost species can be explained by means of a chromosomal mechanism. This has been demonstrated by genetic studies on species with sex-linked colour genes (Aida 1921; Winge and Ditlevsen 1947) and breeding experiments with sex-reversed animals (Yamamoto 1961). Obviously these sex determining chromosomes are still in an initial stage of differentiation and their genetic material has not diverged considerably.

Among teleosts, the sex determination of viviparous fish of the subgenus *Poecilia* has been extensively studied. The existence of heterogamety has been suggested from studies of colour gene inheritance and other sex-linked markers in the guppy (*Poecilia reticulata*; Winge 1922) and in several species of *Xiphophorus* (*Platypoecilus*) (Gordon 1947, 1951; Kallman 1984). However, convincing evidence for cytological heterogamety is lacking (but see Foerster and Anders 1977). The present experimental approach to study the mechanism(s) underlying the differentiation of sex chromosomes utilizes sex-specifically organized DNA sequences. Singh et al. (1981) have described sex-specific satellite DNAs from snakes that hybridize preferentially to the W chromosome in the heterogametic females. The *B*anded *k*rait *m*inor satellite (Bkm) sequences are present in the heterogametic sex of many vertebrates, e.g. on the W chromosome of birds (Jones and Singh 1985) and the Y chromosome of the mouse (Epplen et al. 1982; Singh et al. 1984). A considerable part of this satellite sequence consists of particular simple repetitive sequences, $(gata)_n$ and $(gaca)_m$, which are present in a wide range of vertebrates (Epplen 1988). These simple repeats have allowed the

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detection of molecular and structural differences even between apparently homomorphic sex chromosomes in the guppy (Nanda et al. 1990). Here we present studies on *Xiphophorus* and *Poecilia* species undertaken in order (1) to analyse the association of simple repetitive sequences with the genetically apparent heterogamety and (2) to confirm the presence of simple repetitive sequences like $(gata)_n$ and $(gaca)_m$ in the early evolution of the heterogametic condition.

The platyfish *Xiphophorus maculatus* represents the most extensively studied system involving natural populations where precise genetic homology between the sex chromosomes could be derived. In natural populations there are three types of chromosomal constitution in females, WY, WX and XX, and two types in males, XY and YY (Kallman 1973, 1984). Although the sex chromosomes in this species have not been identified convincingly (but see Foerster and Anders 1977), the constant sex ratios in broods from individuals with characteristic sex-linked markers attest the existence of three types of sex chromosomes. While *X. maculatus* sex chromosomes are homologous with the sex chromosomes of several other species of *Xiphophorus*, there are also instances of atypical sex determination in *X. maculatus* where phenotypic sex does not concur with chromosomal sex (Kallman 1984). In other species of these genera sex determination has been found to conform with other chromosomal mechanisms with the extreme case of a population of *X. helleri* in which a polyfactorial mode was reported (Peters 1964). In view of the extreme form of heterogamety in *X. maculatus* we examine here the association of $(gata)_n$ and $(gaca)_m$ sequences in cytologically undefined sex chromosomes of poeciliid fishes.

Materials and methods

Mature male and female specimens of ornamental strains of *P. reticulata* (guppy), black molly (*P. shenops*, var. melanistica; the entirely black breed is known as the melanistica variant) and sailfin molly (*P. velifera*) were purchased from local animal dealers.

Fish from two populations of *X. helleri* were used. The Rio Lancetilla stock shows differing sex ratios and male size dimorphism in many broods. The differing sex ratios have been attributed to polyfactorial sex determination (Peters 1964). The population of the Laguna Catemaco has an overall sex ratio of 1:1 and no size dimorphism of males was observed in our stock. Specimens of *X. maculatus* were from populations of the Rio Papaloapan system, the Rio Coatzecoalcos system and the Rio Jamapa.

Founder fish for the stocks were obtained from A. and F. Anders (Gießen, FRG), K.D. Kallman (New York, USA) or during a collection trip to Mexico in spring 1989, respectively. All experimental fish were maintained under standard conditions (Kallman 1973). The *Xiphophorus* strains were bred in closed stocks derived from at least two to three brother-sister matings to minimize polymorphism of the sex chromosomes analysed.

Chromosome preparation, banding and in situ hybridization. The techniques used for the preparation of mitotic chromosomes, demonstration of constitutive heterochromatin and in situ hybridization using the biotinylated $(GACA)_4$ oligonucleotide have been described previously (Haaf and Schmid 1984; Nanda et al. 1990).

DNA preparation and oligonucleotide hybridization. Genomic DNA was isolated from brain, liver, spleen and gill according to Blin and Stafford (1976). DNA (5 µg) from all individuals was digested with restriction endonucleases (HaeIII, HinfI, AluI, Sau3AI) according to the recommendations of the supplier (Boehringer-Mannheim, FRG) and resolved on 0.8% horizontal agarose gels in TAE buffer (40 mM Tris, 12 mM sodium acetate, 2 mM EDTA, pH 8.3) at 2 V/cm for about 40 h. Prior to hybridization the gels were dry-blotted, stained with ethidium bromide, photographed, denatured, neutralized and reequilibrated as described (Schäfer et al. 1988). The oligonucleotide probes specific for simple repeats were synthesized on an automated DNA synthesizer (Applied Biosystems 381A, Weiterstadt, FRG) and were end-labelled with [γ^{32}P]ATP (Amersham, Braunschweig, FRG) in a standard kinase reaction. Hybridizations were carried out for 3–4 h at 35° C for $(GATA)_4$, at 43° C for $(GACA)_4$, $(CA)_8$ and $(GGAT)_4$, and at 45° C for $(CAC)_5$ in 5 × SSPE (20 × SSPE is 3 M NaCl, 200 mM $NaH_2PO_4 \times H_2O$, 20 mM EDTA), 0.1% sodium dodecyl sulphate (SDS), 10 µg/ml sonicated and denatured *Escherichia coli* DNA and 1×10^6 cpm/ml of the labelled probes. After hybridization gels were washed three times for 30 min each at room temperature in 6 × SSC (0.9 M NaCl, 90 mM trisodium citrate) followed by a 1 min wash at the hybridization temperature. Gels were then exposed to Kodak XAR-5 film at room temperature. Before reprobing, the gels were washed twice for 30 min each in 5 mM EDTA at 60° C and finally reequilibrated in 6 × SSC.

Results

Sex-specific hybridization with simple repeat probes

Sex-specific hybridization using simple repeat probes was investigated in at least five guppies of both sexes (juvenile individuals were avoided whereever possible). HinfI- and/or AluI-digested genomic DNA samples from outbred male and female guppies were hybridized with the $(GATA)_4$ and $(GACA)_4$ probes. In contrast to females, males displayed a single prominent $(GACA)_4$ band in the high molecular weight range (>23 kb; see Fig. 1).

The $(GATA)_4$ probe reveals a Y chromosomal polymorphism (Fig. 1, band around 23 kb; f approx. 0.3–0.4). Using mixed oligonucleotide probes consisting of varying amounts of $(gata)_n$ and $(gaca)_m$ sequences [$(GATA)_3(GACA)_2$ and $(GATA)_2GACA(GATA)_2$], we hybridized genomic DNA from the individuals that were displaying male-specific signals with the $(GATA)_4$ probe. In all combinations of simple $(gata)_n/(gaca)_m$ repeats (mixed oligonucleotide probes) sex specificity similar to that found with $(GACA)_4$ was observed (data not shown). Thus the polymorphism in the organization of the simple sequences from the guppy Y chromosome could be demonstrated only with the pure synthetic $(GATA)_4$ probe.

The genomes of *P. sphenops* and *P. velifera* exhibit comparatively low amounts of $(gata)_n$ and $(gaca)_m$ simple sequences. In contrast to the guppy, in *P. sphenops* the longest prominent $(GACA)_4$ band appeared at around 6.6 kb. However, upon over-exposing autoradiographs it is possible to obtain faint hybridization at the high molecular weight range only in females (Figs. 1, 2). In *P. velifera* both $(gata)_n$ and $(gaca)_m$ sequences are even less abundant. After long exposure faint signals were observed with $(GACA)_4$ in the 23 kb region in both sexes (data not shown). Interestingly there was promi-

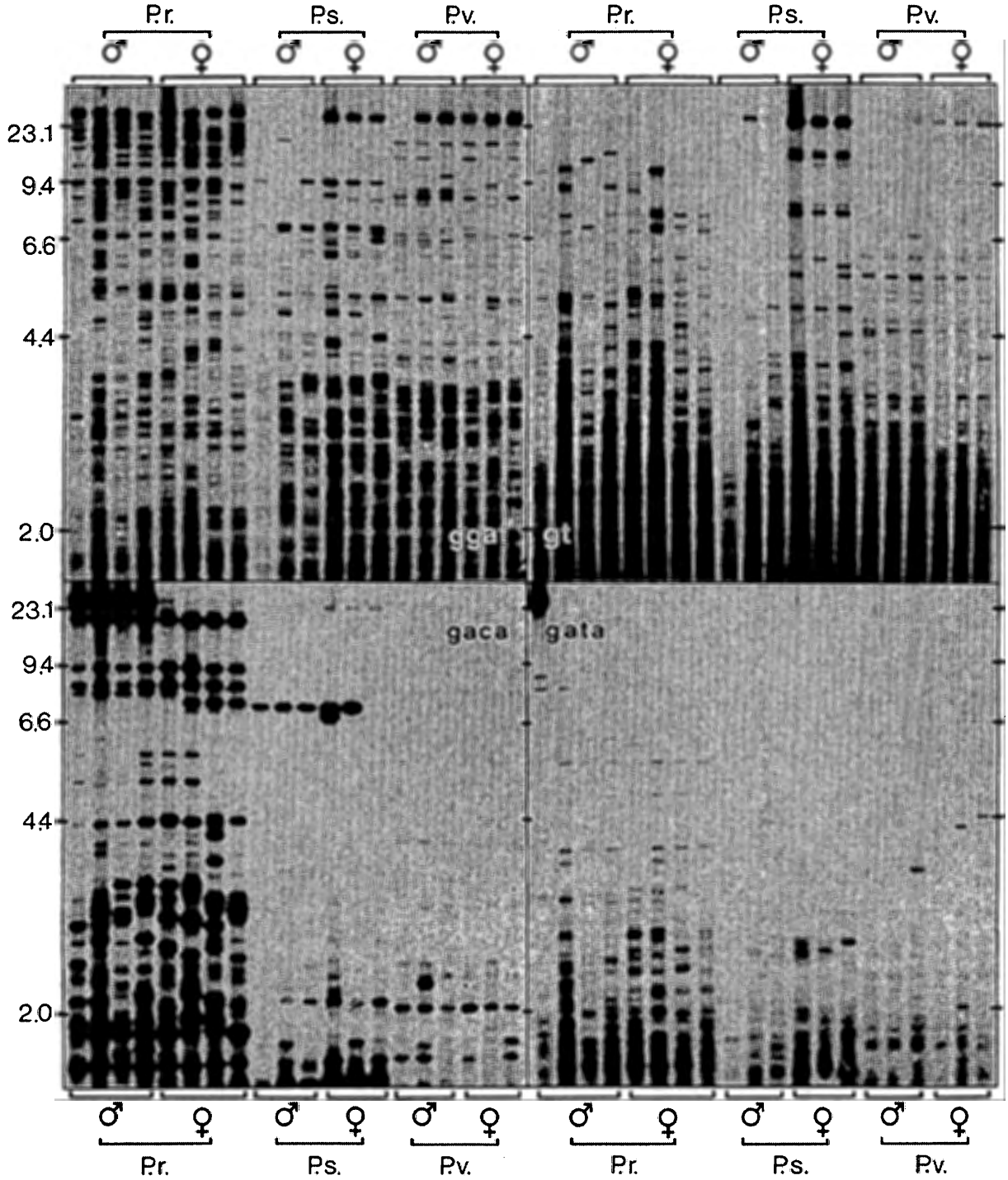

Fig. 1. In-gel hybridization of genomic DNA from *Poecilia reticulata* (*P.r.*), *P. sphenops* (*P.s.*) and *P. velifera* (*P.v.*) with the simple repetitive oligonucleotide probes $(GGAT)_4$, $(GT)_8$, $(GACA)_4$ and $(GATA)_4$. DNA was prepared from three or four male and female individuals and separated by agarose gel electrophoresis after HinfI digestion. The gel was dried and hybridized consecutively with the probes. Note the following pronounced male/female differences: (*1*) P.s. using $(GGAT)_4$ in the >23 kb range; (*2*) P.v. using $(GT)_8$ at approx. 23, 20 and 10 kb; (*3*) P.r. >23 kb using $(GACA)_4$. The prominent signal obtained with $(GATA)_4$ in one male guppy (*on the left-hand side*) is due to a Y chromosomal polymorphism. *Length markers* are indicated in kilobases

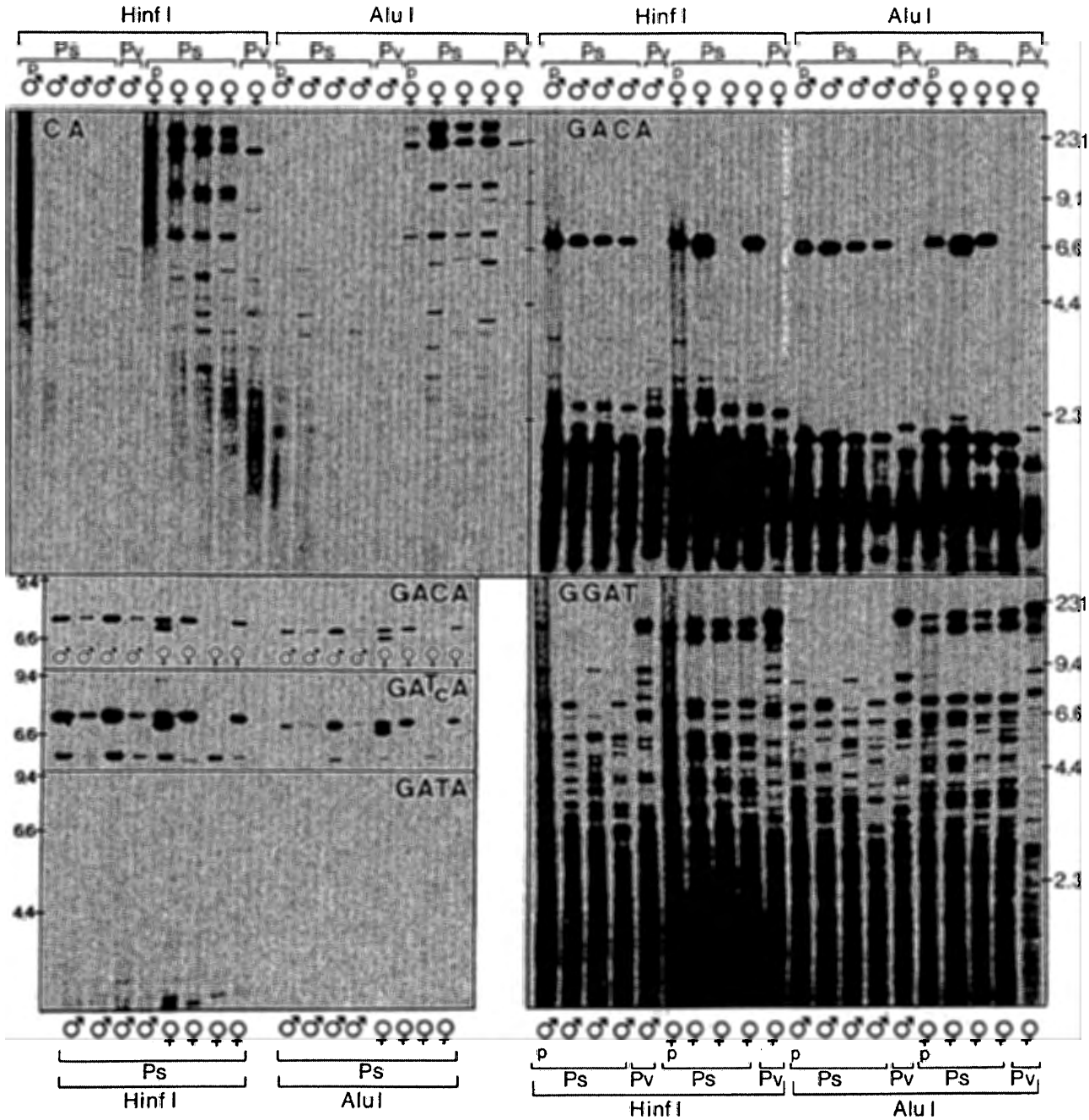

Fig. 2. In-gel hybridization of genomic DNA from *Poecilia sphenops* (*Ps*) and *P. velifera* (*Pv*) using the oligonucleotides $(CA)_8$, $(GACA)_4$ and $(GGAT)_4$ as probes. At the *bottom* left is shown a section of a second gel containing *P. sphenops* DNA samples after hybridization with $(GACA)_4$, $(GATA)_3$ $(GACA)_2$ and $(GATA)_4$. The restriction enzymes HinfI and AluI both produce very similar banding patterns with all three oligonucleotide probes. In separate pools (*p*) of DNA samples from *P. sphenops* males and females uncharacteristic smear signals appeared throughout the respective lanes. Note the different patterns between males and females. The hybridizations shown at the *bottom left* were conducted to separate physically the two 7.5 kb fragments in DNA of one *P. sphenops* female and to investigate the effects of different compositions of $(gata)_n/(gaca)_m$ probes. The polymorphic $(GACA)_4$ bands at about 7.5 kb do not harbour significant amounts of pure $(GATA)_4$ simple repeat units. *Length markers* are indicated in kilobases

nent sex-specific hybridization in the high molecular weight range in females of *P. sphenops* when the genomic DNA was hybridized with the $(GGAT)_4$ and $(GT)_8/(CA)_8$ probes. This clearly illustrates female heterogamety in *P. sphenops* and it predicts the presence of a W chromosome (Figs. 1, 2). The specific $(ggat)_n$ signal in females is confined to the 23 kb region while the dimeric repeat $(GT)_8/(CA)_8$ displays multiple bands ranging from 7 kb to extremely high molecular weights (Figs. 1, 2). On the other hand in *P. velifera* two less prominent but consistent sex-specific signals were noted with $(CA)_8$ in females only. Thus the W chromosome of *P. velifera* may not have attained the same degree of sequence diversification from the Z as observed in the case of *P. sphenops* or as the Y chromosome in *P. reticulata* from the X.

In *X. maculatus* examples of male and female heterogamety with three types of sex chromosomes are known to exist in one and the same population. In the primitive form of chromosomal sex determination sex chromosomes differ from their homologues probably only in a limited number of gene loci. The application of simple repeat probes to the *X. maculatus* model system was based on the idea of allocating certain specific bands to Y, W or X chromosomes. A series of hybridizations with various simple repeats $[(GT)_8/(CA)_8$, $(CAC)_5$, $(GGAT)_4$, $(GACA)_4]$ failed to assign any sex-specific band to any of these chromosomes (Fig. 3).

Upon digesting genomic DNA with a panel of restriction enzymes, fish from the Rio Papaloapan population of *X. maculatus* carrying a definite Y chromosome (marked by the Sp colour gene) exhibited an intense smear signal in males with $(GATA)_4$ (see Fig. 4 with three enzymes). The smear could be due to the occurrence of many restriction sites spaced at various distances on different Y chromosomal DNA fragments. More likely, however, very long, native DNA fragments harbour no restriction enzyme recognition sites but are just randomly broken due to the DNA preparation method. Their separation results in the smear signal. Interestingly the Y chromosomal polymorphism in *X. maculatus* which is phenotypically represented by different colour genes and P alleles (onset of sexual maturation; Kallman 1984) is also evident at the molecular level owing to the simple repeats. While the smear signal was readily detected in the presence of the Rio Papaloapan Y^{Sp} chromosome, no such hybridization could be assigned to the Rio Jamapa Y^{Sp} chromosome (data not shown).

A similar situation became evident when two different populations of *X. helleri* were compared (Fig. 5). The population from Laguna Catemaco differs from that of Rio Lancetilla with respect to the smeared signal in males only. Even after prolonged exposure the same pattern could not be obtained in females. Furthermore the typical smear due to the particular organization of (ga-

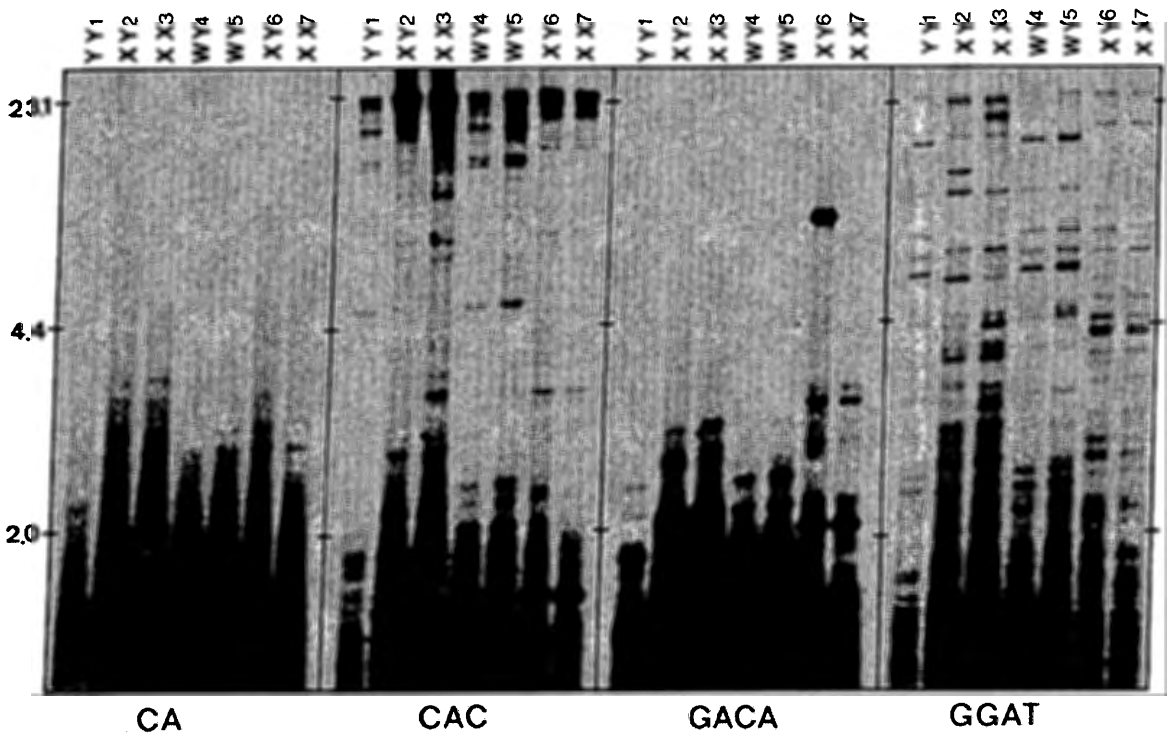

Fig. 3. In-gel hybridization of different *Xiphophorus maculatus* DNA samples to the oligonucleotide probes $(CA)_8$, $(CAC)_5$, $(GACA)_4$ and $(GGAT)_5$. The specimens included *YY* and *XY* males, *XX* females and *WY* females (Rio Coatzecoalcos) as well as *XY* males and *XX* females (Rio Papaloapan): (*1*) male, Y^{Sp9}/Y^{Sp9}; (*2*) male, X^+/Y^{Sr}; (*3*) female, X^{Dr}/X^{Dr}; (*4*) female, W/Y^+; (*5*) female W/Y^+; (*6*) male, X^+/Y^{N2}; (*7*) female X^+/X^+. DNA (6 µg) of each individual was digested with the restriction enzyme HinfI. No sex chromosome specific hybridization patterns were revealed with the probes representing simple repeat motifs. Differences in the banding patterns are mostly due to autosomal polymorphisms. *Size markers* are indicated on the left in kilobases

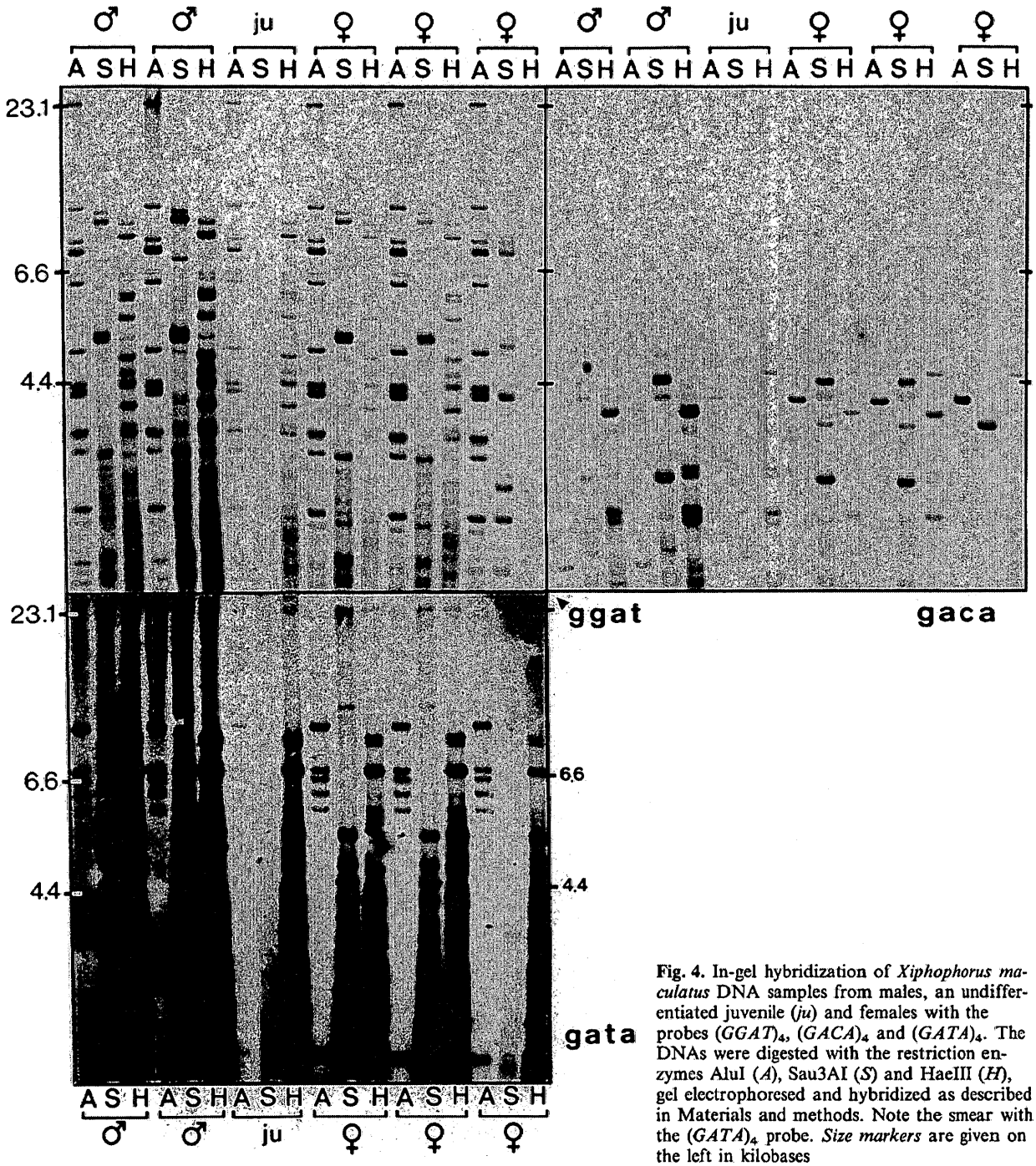

Fig. 4. In-gel hybridization of *Xiphophorus maculatus* DNA samples from males, an undifferentiated juvenile (*ju*) and females with the probes $(GGAT)_4$, $(GACA)_4$ and $(GATA)_4$. The DNAs were digested with the restriction enzymes AluI (*A*), Sau3AI (*S*) and HaeIII (*H*), gel electrophoresed and hybridized as described in Materials and methods. Note the smear with the $(GATA)_4$ probe. *Size markers* are given on the left in kilobases

ta)$_n$ sequences was confirmed by hybridizing the same gel with related simple repeat probes where this characteristic smear was not seen. In contrast to the situation in *X. maculatus* where (gaca)$_n$ sequences are poorly represented, in the *X. helleri* population a smear was noted in males above the 23 kb range. In addition a high molecular weight band characteristic of this population (Laguna Catemaco) appeared in both sexes (Fig. 5). On the other hand, in the population from Rio Lancetilla where sex is determined polygenically (Peters 1964), no sex-specific hybridization to any of the simple repeats was evident. Thus in comparison with the guppy and other *Poecilia* fish, an entirely different form of Y chromosome organization with respect to simple repetitive sequences could be revealed in populations of *Xiphophorus*.

Cytogenetic evidence for sex chromosomes in poeciliid fishes

C-banding conducted on chromosomes from numerous specimens of *P. sphenops* revealed a distinct heteromor-

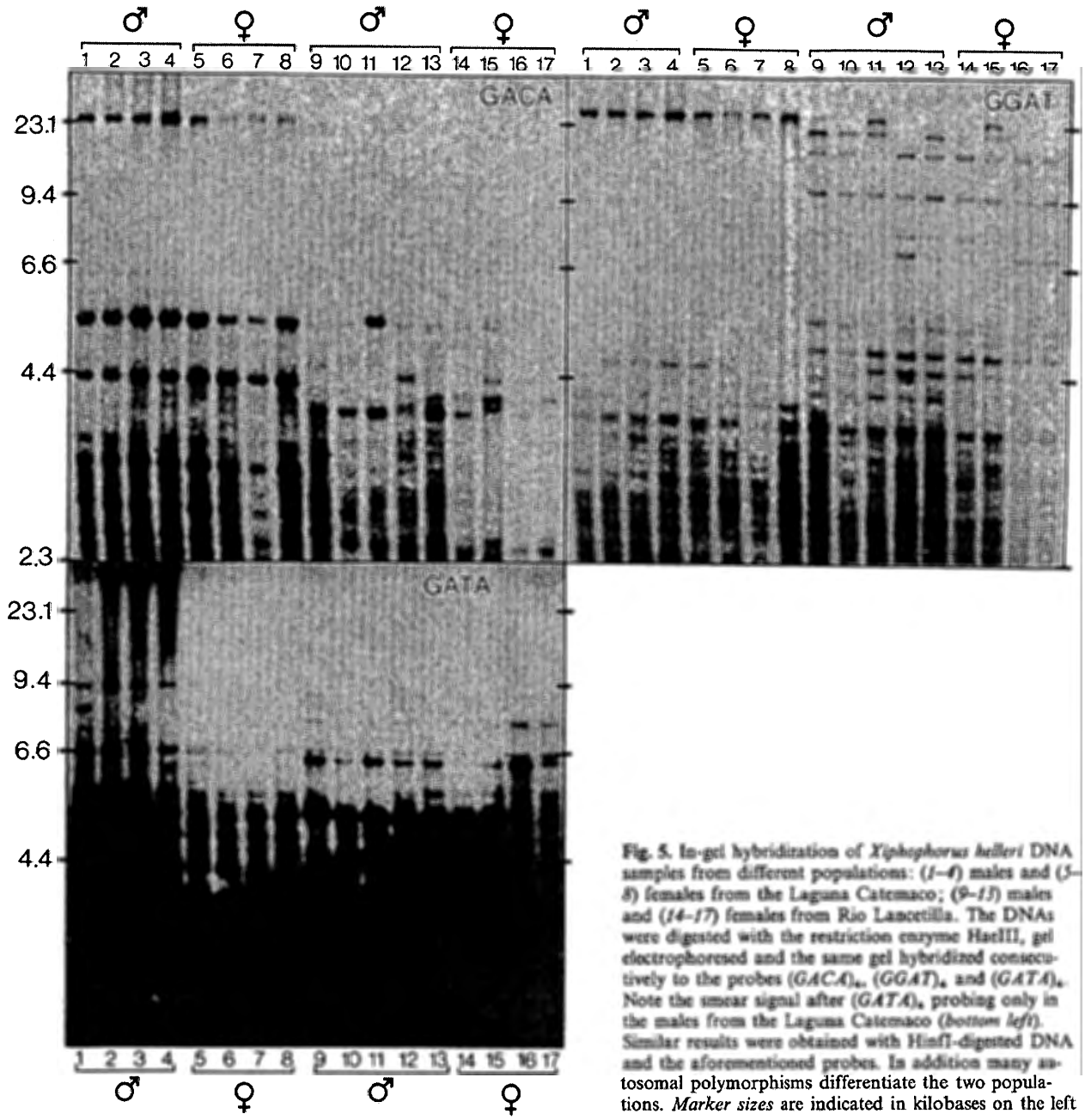

Fig. 5. In-gel hybridization of *Xiphophorus helleri* DNA samples from different populations: (*1–4*) males and (*5–8*) females from the Laguna Catemaco; (*9–13*) males and (*14–17*) females from Rio Lancetilla. The DNAs were digested with the restriction enzyme HaeIII, gel electrophoresed and the same gel hybridized consecutively to the probes $(GACA)_4$, $(GGAT)_4$ and $(GATA)_4$. Note the smear signal after $(GATA)_4$ probing only in the males from the Laguna Catemaco (*bottom left*). Similar results were obtained with HinfI-digested DNA and the aforementioned probes. In addition many autosomal polymorphisms differentiate the two populations. *Marker sizes* are indicated in kilobases on the left

phism between the homologous No. 1 chromosomes in females (Haaf and Schmid 1984). Only one homologue has intensely stained telomeric heterochromatin. Such a C-band difference leads to the conclusion that these are sex-specific chromosomes of the ZW ♀/ZZ ♂ type. In the C-banded preparations the only visible difference between the Z and W chromosomes is the telomeric W heterochromatin. A similar slight difference in the C-banding patterns has been observed between the telomeres of the homologous No. 1 chromosomes in the male guppy (Nanda et al. 1990). In one of the homologues (Y) there is always distinctly more telomeric heterochromatin than in the other (X). The size of the Y-specific heterochromatin shows interindividual variability. Hybridization of $(GACA)_4$ to the chromosomes displays the characteristic peroxidase reaction at the telomeric end of only one of the No. 1 homologues in males (Nanda et al. 1990). Thus the male-specific hybridization pattern with the $(GACA)_4$ probe is due to the accumulation of simple repeats on the telomeric heterochromatin of the Y chromosome. In contrast to the aforementioned situations, in *P. velifera* a similar heteromorphism has not been observed indicating that the Z and W chromosomes are still structurally equal. Furthermore C-banding reveals no consistent differences between any of the homologous chromosome pairs in *X. maculatus* (Rio Papaloapan population). Only in fish from the Laguna Catemaco population of *X. helleri*, where male specific hy-

bridization was observed with $(GATA)_4$, was a very small difference observed between the telomeres of the No. 6 chromosomes in the males analysed. However, this evidence for the beginning of heterochromatization of the Y chromosome in *X. helleri* must be confirmed in more individuals.

Discussion

Several vertebrate classes including fish, amphibians and reptiles harbour species with sex chromosomes in an early stage of differentiation (Bull 1983). Morphologically differentiated sex chromosomes, a characteristic of higher vertebrates, are believed to have evolved from identical homologues (Ohno 1967; Jablonka and Lamb 1990; Charlesworth 1991). Comparative studies from different reptiles have been interpreted as indicating that $(gata)_n$/$(gaca)_m$ sequences have been important in sex chromosome evolution. In many animals these sequences hybridize to the sex chromosomes carrying the largest amount of heterochromatin (Singh et al. 1976; Jones 1983). Here experimental evidence is presented that various simple repeats are implicated in the early stages of sex chromosome differentiation. We detected a Y chromosome in *P. reticulata* with the $(GATA)_4$ and $(GACA)_4$ probes. The same simple sequence failed to reveal sex-specific distinct bands in all populations and species of *Xiphophorus* investigated. Even in the population where $(gata)_n$/$(gaca)_m$ abundance is evident, a completely different pattern of hybridization was obtained. This phenomenon together with that from the guppy perhaps entail different stages in the incipient process of Y chromosome specialization. The lack of sex-associated differences in $(gata)_n$/$(gaca)_m$ sequence organization in *P. sphenops* and *P. velifera* may have the following explanation: (1) accumulation of these sequences is not an important step in sex chromosome differentiation. (2) Since there are only small amounts of heterochromatin on the sex chromosomes (*P. velifera*, as evidenced by C-banding), there may be an insufficient amount of the simple sequences present on the sex chromosome to be detected. However, this explanation does not seem to be correct as sex-specific hybridization signals are found with other simple repeats. Moreover our data suggest that $(gata)_n$/$(gaca)_m$ sequences are not the exclusive sequences involved in sex chromosome evolution. Supporting evidence for this has been found in the rainbow trout (*Salmo gairdneri*; Lloyd et al. 1989) and *Leporinus obtusidens* of the family Anostomidae (our own unpublished data), where sex chromosomes could be identified by cytological staining but sex-specific differences could not be observed using cloned Bkm sequences or oligonucleotides as hybridization probes. Furthermore other recent observations from mammalian systems question the previous speculation of the general relevance of $(gata)_n$/$(gaca)_m$ in sex chromosome evolution (Miklos et al. 1989). In addition distinct sex-specific hybridization with $(CA)_8$ and $(GGAT)_4$ reveals that several different simple repeat elements may have accumulated independently on the sex chromosomes. This phenomenon is not exclusive to poeciliid fishes but has also been observed in rodents of the genus *Mus*. In these, typical Y-specific hybridization with $(gata)_n$/$(gaca)_m$ simple repeats is highly variable (Epplen et al. 1988). Therefore association of other types of simple repeats has been suggested to explain male specific "satellite bands" observed on ethidium bromide stained gels (Platt and Dewey 1987).

There is as yet no adequate explanation for the mode of simple repeat accumulation in the eukaryote genome (Epplen 1988). It is envisaged that simple repeats are randomly generated on all chromosomes at a high frequency due to anomalous replication from a pool of low molecular weight forms (Tautz and Renz 1984). It is likely that owing to point mutations, slipped strand mispairing and crossover events during meiosis these sequences may be reduced in the genome while sequences on the Y chromosome will be preserved for longer time intervals as a result of its perpetually haploid state: therefore the three above mentioned mechanisms may still be in operation on poeciliid sex chromosomes with their high overall homology except for nascent differentiation.

Our observations illustrate various stages of sex chromosome differentiation specific for each poeciliid species or population: (1) in the guppy, where the genetic basis of heterogamety has already been established, the nature of the Y chromosome can be confirmed by molecular and cytogenetic means. In some inbred lines of the guppy male specific hybridization in the high molecular weight region is absent, which may be related to the Y chromosome polymorphism in natural populations (Nanda et al. 1990). (2) In those systems where genetic experiments on the sex chromosome mechanisms have not been reported, the sex chromosome status could be inferred by specific hybridization patterns (*P. velifera* and *P. sphenops*). (3) In *Xiphophorus* the sex determination polymorphism has been attributed to the varying combinations of different sex chromosomes. The present study demonstrates evidence for the occurrence of a Y chromosome on the basis of hybridization patterns. Therefore, the lack of sex-specific hybridization in many populations may point to different stages of sex chromosome evolution in *Xiphophorus*.

X. helleri is well known for its polygenic mode of sex determination where many male or female determining genes are distributed on several chromosomes (Orzack et al. 1980). The determination of sex depends on the cumulative expression of any one set of these genes. In the phylogeny of sex chromosomes such a mechanism is considered to be most primitive. Eventually it is replaced by a genic system among fishes (Kirpichnikov 1981). Recent linkage analyses and mathematical models suggest that such a polygenic system is unstable and it has been shown to acquire "sexually antagonistic" genes (Rice 1986). The sex-specific signal with $(GATA)_4$ in the Laguna Catemaco population of *X. helleri* could be explained by a similar mechanism. We present here for the first time evidence that in the swordtail a heterogametic system is present besides the commonly known polygenic mode. This is in perfect confirmation of genet-

ic experiments and sex ratios on different *X. helleri* populations (Kallman 1984).

Based on studies in snakes Ohno (1967) stressed pericentric inversion as the probable mechanism of generation of heteromorphic sex chromosomes from a homomorphic sex element. This suggestion may be valid for those systems where size differences between the sex chromosomes are established. *Poecilia* fishes and many amphibians on the contrary do not display apparent morphological differences between the sex chromosomes. Therefore other mechanisms must have been operating in these systems. One possible mechanism is based on cytological heteromorphism where one homologue has dramatically different C-band characteristics compared with its partner in one sex but never in the other (Schmid 1980; King 1980). While the cytological evidence for the incipient W chromosome in *P. sphenops* (see Haaf and Schmid 1984) fits directly into this model our observations on the guppy as well as on *P. velifera* may imply that the initial heterochromatization in primordial sex chromosomes is not the result of a single structural event. Instead several different processes may be involved.

The mechanism of sexual differentiation and development of heterogamety was initially investigated via genetic experiments in several teleosts. This cumbersome method was later replaced by application of modern cytogenetic techniques which to some extent revealed heteromorphic sex chromosomes in certain species (Thorgaard 1977; Galetti and Foresti 1986). Other non-cytogenetic parameters such as screening for H–Y antigen were applied to different poeciliid systems which coincided with the known heterogametic situations (Pechan et al. 1979; Nakamura and Wachtel 1984). Owing to methodological difficulties this could not be applied extensively to other poeciliid fishes where genetic heterogamety had not been demonstrated. Recently the so-called ZFY sequence, a "zinc finger" protein encoded by the human Y (Page et al. 1987), has been postulated to be associated with the sex chromosomes of many diversified vertebrates. This view, however, remains controversial because of reports on marsupials (Sinclair et al. 1988) and reptiles (Bull et al. 1988). In addition ZFY sequences are present but also not informative with respect to the sex chromosomes in *Xiphophorus* (S.M. Robertson and M. Schartl, unpublished data) and in the guppy and *P. sphenops* (I. Nanda et al., unpublished data). Hence the present approach using simple repeats proves to be superior for the unravelling of the heterogametic status in lower vertebrates. It is also useful for revealing extensive polymorphism for individualization purposes. For example the $(GGAT)_4$ sequence reveals numerous polymorphic bands in each poeciliid fish studied and thus provides highly informative DNA fingerprints.

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