Bkm satellite DNA and *ZFY* in the coral reef fish *Anthias squamipinnis*

STEPHEN WACHTEL AND SUZANNE DEMAS
Division of Reproductive Genetics, Department of Obstetrics and Gynecology, University of Tennessee, Memphis, TN 38105, U.S.A.
TERRENCE TIERSCH
Ecological Research Center, Department of Biology, Memphis State University, Memphis, TN 38152, U.S.A.
PETER PECHAN
Department of Biological Sciences, Florida International University, Miami, FL 33199, U.S.A.
AND
DOUGLAS SHAPIRO
Department of Marine Sciences, University of Puerto Rico, Box 5000, Mayaguez, PR 00709, U.S.A.

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We studied DNA from the protogynous sex-changing fish *Anthias squamipinnis* to evaluate the recent observation that male-specific bands are identified after hybridization with Bkm, a probe originating in the W chromosome of the snake *Bungarus fasciatus*. Sex-specific hybridization would imply modification of DNA structure during the sex-changing process. No sex-specific Bkm fragments were identified in our study, after digestion of DNA from 15 males and 11 adult females, despite the use of 12 different restriction enzymes. However, hybridization with Bkm did produce a distinct fingerprint pattern, similar to the fingerprint patterns described for other species after hybridization with GATA (GACA) type probes. In other experiments, the pDP1007 probe, which identifies the *ZFY* gene in the male-determining region of the human Y chromosome, generated identical hybridization patterns in DNA from males and females of *A. squamipinnis* and estimation of DNA mass by flow cytometry revealed identical genome sizes.

Key words: Bkm satellite DNA, sex determination, sex change, *ZFY*, pDP1007, genome size.

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L'ADN du poisson protogyne qui change de sexe, l'*Anthias squamipinnis*, a été étudié en vue d'évaluer les observations récentes que les bandes spécifiques aux mâles ne sont identifiées qu'après une hybridation avec la sonde Bkm provenant du chromosome W du serpent *Bungarus fasciatus*. Une hybridation spécifique au sexe devrait impliquer une modification de la structure de l'ADN au cours du processus de changement de sexe. Aucun fragment de la sonde Bkm spécifique au sexe n'a pu être identifié dans cette étude, après digestion de l'ADN de 15 mâles et de 11 femelles adultes, malgré l'emploi de 12 enzymes de restriction différentes. Toutefois, l'hybridation avec la sonde Bkm a produit un profil distinct de carte moléculaire, lequel s'est avéré semblable aux profils décrits pour d'autres espèces suite à l'hybridation avec les sondes types GATA (GACA). Dans d'autres expériences, la sonde pDP1007, laquelle détermine le sexe mâle chez le chromosome Y humain, a généré des profils d'hybridation identiques dans l'ADN des mâles et des femelles de l'*A. squamipinnis* et les estimations des masses d'ADN par flux cytométriques ont révélé que les dimensions des génomes étaient identiques.

Mots clés : ADN du satellite Bkm, détermination du sexe, changement de sexe, *ZFY*, pDP1007, dimension génomique.

[Traduit par la rédaction]

Introduction

Sex reversal occurs routinely among the adults of many marine fishes. The coral reef species *Anthias squamipinnis* is a protogynous hermaphrodite species in which all fish begin life as females. Individuals live in bisexual social groups of varying size and composition (Shapiro 1988*a*). When one of the males dies or is removed, a large female changes sex (Shapiro 1980). Transformation from female to male, induced apparently by behavioral cues, is characterized by gross changes in gonadal histology, hormone production, and body color (Shapiro 1979). There are reports that sex change in fishes such as *Anthias* also involves changes in the expression of phylogenetically conservative H-Y antigen (Duchac and Bühler 1983; Pechan et al. 1986; Reinboth et al. 1987) and, in addition, the de novo appearance of male-specific DNA sequences related to the Bkm minisatellite (Shapiro 1988*b*; and see below).

Banded krait minor (Bkm) DNA, recovered from the W chromosome of the snake *Bungarus fasciatus*, contains numerous repeats of the tetranucleotide GATA. The Bkm sequence is found dispersed throughout the genome in a broad spectrum of eukaryotes. In some species, concentrations of Bkm DNA are found in association with the sex-determining genes (review in Epplen, 1988). Foci of Bkm have been identified in the male-determining region of the mouse Y chromosome (Singh and Jones 1982) and our preliminary studies have revealed sex-specific Bkm fragments in the sea turtles *Chelonia mydas* and *Lepidochelys kempi* (Demas et al. 1990). Inasmuch as the sex of sea turtles is determined by the temperature of incubation, sex-specific sequences could be explained by temperature-mediated modification of DNA structure during early embryogenesis. Alternatively there is an underlying genetic mode of sex

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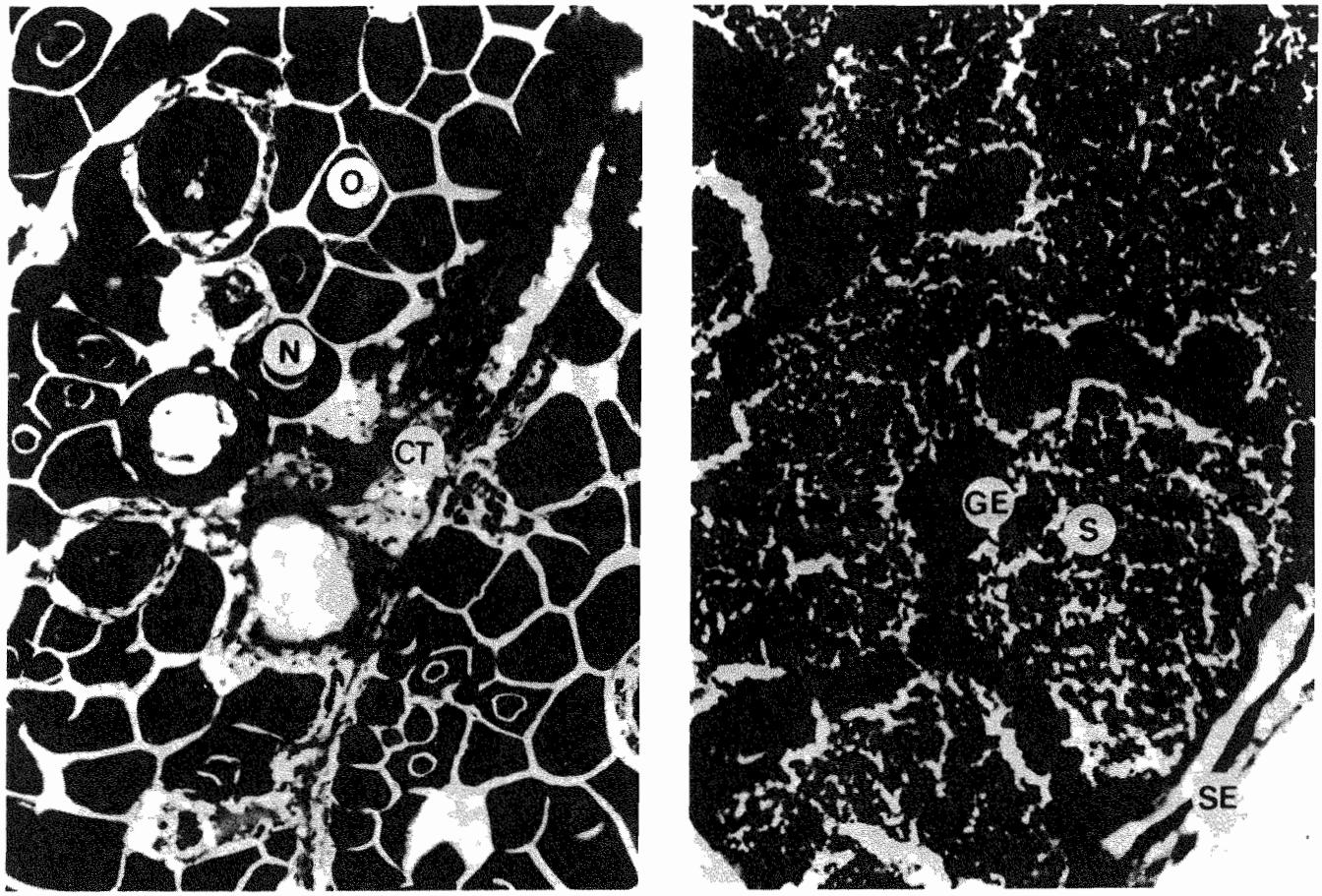

FIG. 1. Histological sections of gonads from female (left) and male (right) of *A. squamipinnis*. Note oocytes (O), nuclei (N), and connective tissue (CT) in female (original magnification 200×), and germinal epithelium (GE), lumen of seminiferous tubule filled with spermatozoa (S), and surface epithelium (SE) in male (original magnification 400×).

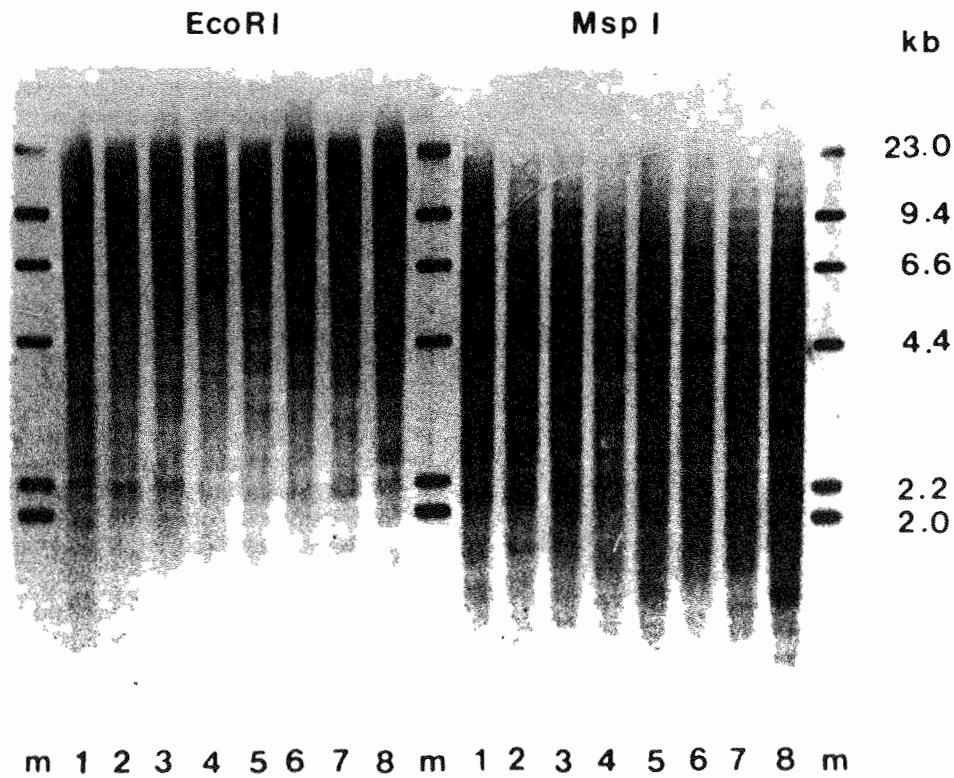

FIG. 2. Southern blot of genomic DNA from four males and four females of *A. squamipinnis* after hybridization with Bkm. The DNA was digested with the restriction enzymes *Eco*RI and *Msp*I. DNA from females was placed in lanes 1–4; DNA from males was placed in lanes 5–8; lanes marked *m* contained the marker.

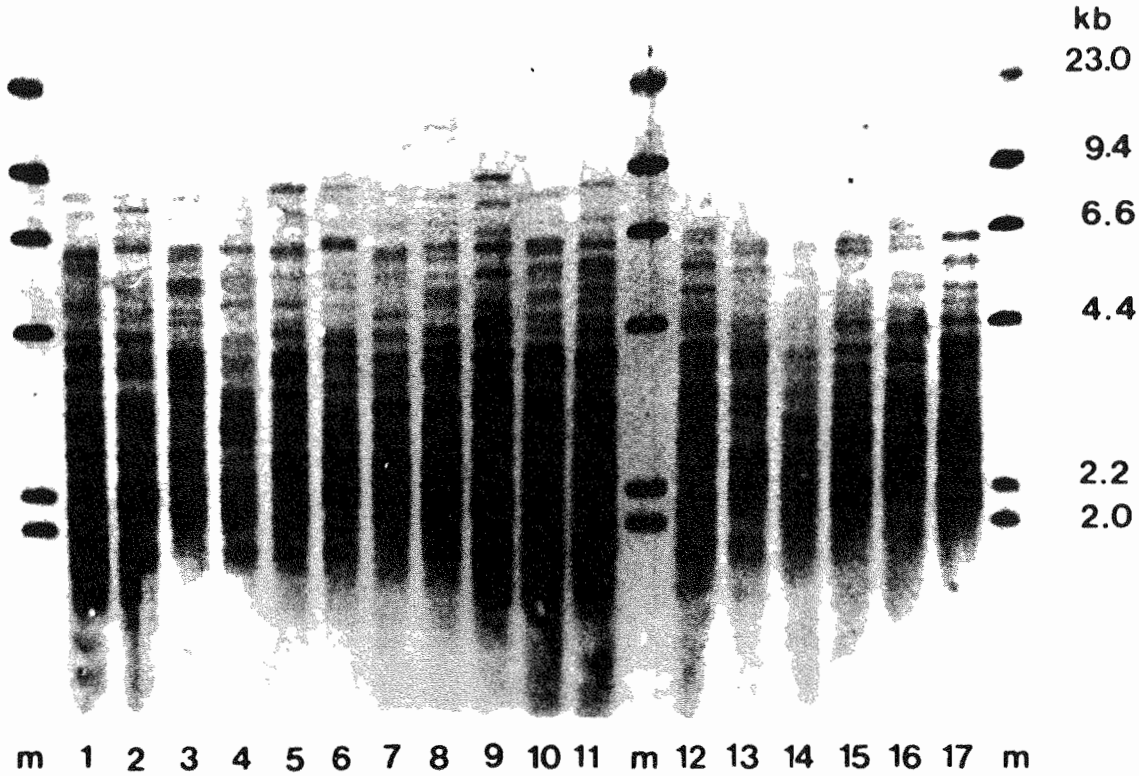

FIG. 3. Southern blot of genomic DNA from 11 males and 6 females of *A. squamipinnis* after hybridization with Bkm. The DNA was digested with the restriction enzyme *Bst*NI. DNA from males was placed in lanes 1–4, 8–11, and 15–17; DNA from females was placed in lanes 5–7 and 12–14; lanes marked *m* contained the marker.

determination in these animals and the animals tested were not sex reversed by temperature.

Another probe, pDP1007, identifies the male-specific *ZFY* gene (zinc finger Y) on the Y chromosome in mammals and the related *ZFX* sequence on the X. This probe hybridizes in a nonspecific manner with DNA in male and female birds (Page et al. 1987), reptiles (Bull et al. 1988), and fishes (Ferreiro et al. 1989).

Different banding patterns in DNA from the different sexes in *Anthias* (Shapiro 1988*b*) would signal an unexpected rearrangement of DNA in adult fish undergoing sex reversal. We therefore set out to survey a broader sample of males and females by application of the Bkm 2(8) and pDP1007 probes, and a panel of 12 and 6 restriction enzymes, respectively, including four of the six enzymes with which male-specific fragments were identified by Shapiro (1988*b*).

Materials and methods

Histology

Initially gender was assigned on the basis of external phenotype: males were red purple and larger than females; the females were orange. Gender was later confirmed histologically. The gonads were removed with the aid of a dissecting microscope and placed into Bouin's fixative. They were embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Molecular genetics

Genomic DNA was extracted by application of guanidine hydrochloride in whole blood from 15 males and 11 (adult) females of *A. squamipinnis*. The extraction technique was modified after Bowtell (1987). Three males and two females were taken from a population maintained at the University of Puerto Rico and 12 males and nine females were purchased from Quality Marine, Los Angeles; the latter fish originated in the Fiji Islands. The DNA was digested with endonucleases according to the recommendations of the manufacturers and separated by electrophoresis on 0.8% agarose gels. Southern blotting onto nitrocellulose was performed overnight in the presence of 10× SSC (1× SSC: 0.15 M NaCl plus 0.015 M sodium citrate). The samples were prehybridized for 1 h at 65°C in 4× SSCP, 0.1% SDS, 10× Denhardt's solution. This was followed by hybridization in 4× SSCP (0.6 M NaCl, 0.06 M sodium citrate, 0.1 M phosphate, pH 7.0), 0.2% SDS, 10× Denhardt's solution overnight at 65°C.

The Bkm 2(8) probe that we used (CS314) was recovered after screening of a *Drosophila* genomic library (Singh et al. 1984). Probe pDP1007 is a 1.3-kb *Hin*dIII human fragment originally recovered from phage λOX82 and subcloned into the pUC13 plasmid (Page et al. 1987). Both probes were biotinylated by nick translation before addition to the hybridization buffer. After hybridization, washes were performed at 65°C in 3× SSCP, 0.1% SDS, 1% Denhardt's solution (higher stringency washes resulted in loss of signal strength, although there was no change in the hybridization patterns). Filters were developed by use of Streptavidin – alkaline phosphatase conjugate and the colorimetric agents nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Our past experience with the Bkm 2(8) probe has shown that signal strength and clarity of the fingerprint pattern are improved when competitive DNA is omitted.

Flow cytometry

Diploid (2C) nuclear DNA content was estimated by flow cytometry for the fish from the Fiji Islands. Erythrocytes from individual fish were mixed with erythrocytes from a chicken in 0.5 mL of a 50 mg/L solution of propidium iodide containing 25 μL RNase (1.0 mg/mL), 0.1% sodium citrate, and 0.1% Triton X-100 and analyzed in a Coulter EPICS V flow cytometer, according to the method detailed in Tiersch et al. (1989). In brief, the argon-ion laser was set at 488 nm and 300 mW. Individual nuclei of lysed erythrocytes were analyzed at a rate of 100–200 per second. The fluorescence of each nucleus was converted to an analog signal,

TABLE 1. Restriction enzymes and patterns produced by hybridization of DNA from *A. squamipinnis* with Bkm

Enzyme	Recognition sequence	Pattern produced	*N*	Male-specific band (kb)	
				This study	Shapiro (1989)
*Hin*dIII	Å·AG$\overset{+}{C}$TT	Fingerprint	15	None	~3
*Hae*III	GG·$\overset{+}{C}\overset{\circ}{C}$	Fingerprint	26	None	~0.5
*Pvu*II	CAG·CTG	Fingerprint	14	None	~6
*Eco*RI	G·A$\overset{+}{A}$TT$\overset{+}{C}$	Fingerprint	8	None	~4
*Sst*I	GAGCT·C	NT (this study)	0	NT	~3
*Hin*cII	GTPy·PuAC	NT (this study)	0	NT	~3
*Msp*I	$\overset{+}{C}\cdot\overset{\circ}{C}$GG	Fingerprint	8	None	NT
*Mbo*I	·G$\overset{+}{A}$T$\overset{\circ}{C}$	Fingerprint	26	None	NT
*Sau*3AI	·G$\overset{\circ}{A}$T$\overset{+}{C}$	Fingerprint	9	None	NT
*Bst*NI	$\overset{\circ}{C}\overset{\circ}{C}\cdot\overset{(A)}{(T)}$GG	Fingerprint	26	None	NT
*Eco*RII	·$\overset{\circ}{C}\overset{+}{C}\overset{(A)}{(T)}$GG	Fingerprint	9	None	NT
*Eag*I	C·GGCCG	Isolated bands	9	None	NT
*Not*I	GC·GGCCGC	Isolated bands	9	None	NT
*Alu*I	AG·$\overset{+}{C}$T	Fingerprint	26	None	NT

NOTE: ·, cleavage site; +, methylation sensitive; °, unaffected by methylation; NT, not tested; Py, any pyrimidine; Pu, any purine.

which was transmitted to a computer, where it was digitized and used to generate pulse-height histograms. At least 25 000 cells were scored for each mixture of cells from chicken and fish.

Results

Histology

Sections of male and female gonads are depicted in Fig. 1. Note the prominent oocytes in the female and the seminiferous tubules in the male. In each fish examined, the histological sex was the same as the gross external sex. There was no evidence of bisexuality in any case.

Molecular genetics

When restricted with endonucleases and hybridized with Bkm, DNA from *A. squamipinnis* produced a distinct fingerprint pattern, which is similar to the patterns described for other species with oligonucleotide GATA (GACA) type probes (Jeffreys et al. 1986). Figure 2 shows the results obtained with DNA from four males and four females after digestion with the enzymes *Eco*RI and *Msp*I. Although sex-specific bands were observed with *Eco*RI in the study by Shapiro (1988*b*), there was no indication of sex-specific hybridization with either enzyme in the present study. Figure 3 shows the results obtained with DNA from 11 males and 6 females after digestion with *Bst*NI (hybridization with Bkm has produced sex-specific bands in reptile DNA after digestion with *Bst*NI; see Discussion). Sex-specific hybridization was not evident (Table 1).

Methylation of DNA sequences has been implicated in the inactivation of certain genes (Lock et al. 1986). To evaluate the possibility that differences in methylation might exist in males and females of *A. squamipinnis* the isoschizomers *Sau*3AI–*Mbo*I and *Bst*NI–*Eco*RII were selected. Although there was evidence of methylation within the sequences recognized by these enzymes, especially at the *Sau*3AI–*Mbo*I site, no consistent sex-associated fragments were revealed (Table 1).

Figure 4 shows the results obtained with *Pst*I digests of DNA from five females and five males after hybridization with pDP1007. A single fragment of 6.1 kb was evident in all samples, regardless of sex. Digestion with *Kpn*I generated a similar picture, but multiple fragments were obtained with *Eco*RI and *Hin*dIII. The results are summarized in Table 2.

Flow cytometry

The results of our tests for nuclear DNA content in blood cells of *A. squamipinnis* are summarized in Table 3. Absence of sex-specific hybridization was consistent with absence of sex-specific DNA. The mean value for DNA content was 1.775 ± 0.013 pg for 10 females and 1.770 ± 0.014 pg for 12 males. These values are indistinguishable ($P = 0.506$, *t*-test) and provide no basis for assuming male and female differences in DNA content in the order of 10^{-3} pg (10^{-15} g), or about 10^6 base pairs.

The mean genome size for the combined values of male and female was 1.772 ± 0.014 pg DNA per diploid cell (range, 1.750–1.802 pg). The intraspecific variation in genome size (2.97%) for this species is lower than the variation reported for other species of fish, such as the mean of 4.86% reported for 49 species of cyprinids (Gold et al. 1990); the mean of 4.7% reported for 12 species of salmonids (Johnson et al. 1987); and the mean of 6.24% reported for nine species of centrarchids (Ragland and Gold 1989).

Discussion

Demas et al. (1990) identified male-specific Bkm fragments in the sea turtles *Lepidochelys kempi* and *Chelonia mydas* and female-specific fragments in *C. mydas*. Sex-specific hybridization would be unexpected in sea turtles because their sex is ostensibly temperature determined, but the results could be explained if there were an underlying genetic mode of sex determination in these species or, alternatively, if temperature-influenced sex determination involved structural modification in DNA sequences adjacent to, or directly concerned with, the sex-determining sequences.

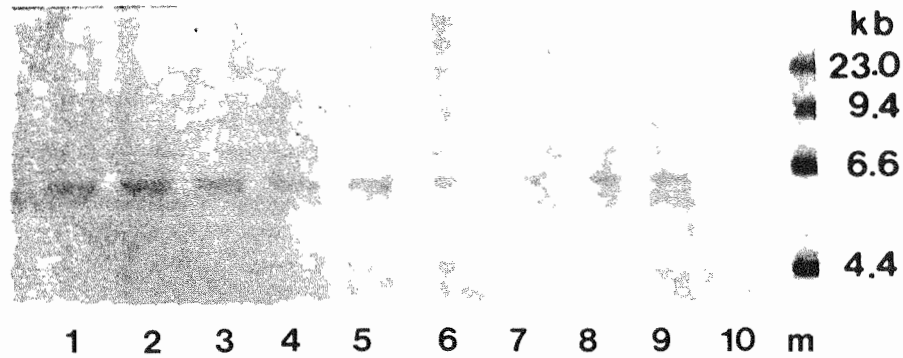

FIG. 4. Southern blot of genomic DNA from five males and five females of *A. squamipinnis* after hybridization with pDP1007. The DNA was digested with *Pst*I. DNA from females was placed in lanes 1–5; DNA from males was placed in lanes 6–10; lane *m* contained the marker.

TABLE 2. Restriction enzymes and patterns produced by hybridization of DNA from *A. squamipinnis* with pDP1007

Enzyme	Recognition sequence	Hybridization pattern	Fragment size (kb)	Polymorphism in pattern
*Pst*I	CTGCA▼G G▲ACGTC	Single fragment	6.1	No
*Kpn*I	GGTAC▼C C▲CATGG	Single fragment	5.3	No
*Eco*RI	G▼AATTC CTTAA▲G	Diffuse, multiple fragments	23–6.6	Yes
*Hin*dIII	A▼AGCTT TTCGA▲A	Diffuse, multiple fragments	23–6.6	Yes
*Hae*III	GG▼CC CC▲GG	None	—	—
*Taq*I	T▼CGA AGC▲T	None	—	—

NOTE: ▼ and ▲, cleavage sites.

TABLE 3. Descriptive statistics for diploid (2C) nuclear DNA content in *A. squamipinnis*

Sex	*n*	DNA content, pg		
		Mean ± SD	SE	Range
Females	10	1.7746 ± 0.0134	0.0043	1.7496–1.8025
Males	12	1.7691 ± 0.0144	0.0043	1.7496–1.7888
Combined	22	1.7717 ± 0.0139	0.0030	1.7496–1.8025

Although concentrations of Bkm have been identified in the male-determining region of the mouse Y chromosome (Singh and Jones 1982), and although sex-associated sequences have been found in sea turtles, we found no evidence of male-specific Bkm hybridization in our survey of DNA from males and females of *A. squamipinnis*. Distinct fingerprint patterns were generated with most of the restriction enzymes that we used, but it was not possible to draw conclusions on the heritability of the patterns because the pedigrees of the fish that we surveyed were unavailable.

Recently Lloyd et al. (1989) studied Bkm in DNA from the rainbow trout, *Oncorhynchus mykiss*. They hybridized Bkm probe CS316 with genomic DNA from males and females after digestion of the DNA with *Eco*RI, *Hae*III, or *Hin*fI. As in the present study, there was no example of sex-specific hybridization, but DNA fingerprints were generated. These appeared to be inherited "in a stable manner."

Given the results of the present report and those of Lloyd et al. (1989), the question arises why male-specific bands were evident in DNA from *A. squamipinnis* in the earlier study by Shapiro (1988*b*). The apparent disparity could be due to differences in sample size or, alternatively, to differences of method, which might generate technical artifacts. DNA was isolated from blood in our study, for example, and from whole fish in the study by Shapiro (1988*b*).

The *ZFY* gene was postulated to be identical with *TDF*, the testis-determining gene of the human Y chromosome, and although a significant function of *ZFY* is indicated by widespread phylogenetic conservatism, recent observations would seem to rule out a primary sex-determining role for the *ZFY* sequence (Palmer et al. 1989; Koopman et al. 1989; Sinclair et al. 1990). Occurrence of sequences in *A. squa-*

mipinnis hybridizing with pDP1007 is consistent with the notion that *ZFY* has an important function. What that function might be remains to be determined.

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