Title
Genetic Analysis of the Sex Determination Mechanism of White Sturgeon (Acipenser transmontamus Richardson)

Author
van Eenennaam, Alison Louise

Publication Date
1997

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

Genetic Analysis of the Sex Determination Mechanism of White Sturgeon
(*Acipenser transmontanus* Richardson)

By

ALISON LOUISE VAN EENENNAAM
B.Ag.S.,Hons. (University of Melbourne) 1987
M.S. (University of California, Davis) 1990

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Genetics

in the

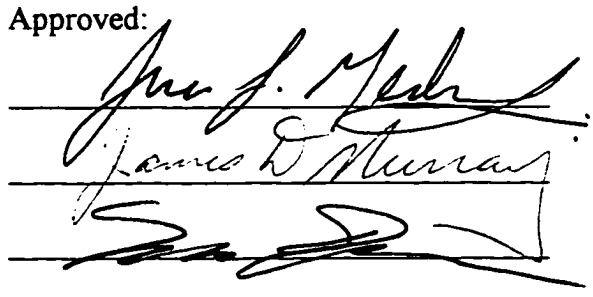
OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

Approved:



Committee in Charge

1997

ACKNOWLEDGMENTS

This research was truly a collaborative project and I wish to thank the many people and organizations who contributed to the overall success of this project. First and foremost I would like to thank Joel P. Van Eenennaam for being constantly at my side throughout the course of this research and my Ph.D. program, and for contributing invaluable technical expertise and considerable time towards the completion of the project. This dissertation would be considerably shorter without his input and extensive efforts on my behalf.

I would like to especially thank my major professor Juan F. Medrano for his friendship and guidance during the four years of my Ph.D. program and over the past decade of our acquaintance. I am grateful to him for allowing me to undertake this research topic in his previously mammalian genetics laboratory, and for giving me the freedom and latitude to develop and pursue all of the various experimental approaches that are outlined on the following pages.

I express my sincere gratitude to Drs. Jim D. Murray and Serge I. Doroshov, the remaining two members of my dissertation committee. I have utilized both their expertise and laboratories on numerous occasions during the course of this research. Special thanks go to Dr. Doroshov for his input regarding the biology and reproductive physiology of white sturgeon, and to Dr. Murray for his numerous suggestions regarding the cytogenetic analyses detailed in Chapters 4 and 5 of this manuscript.

I want to thank all three members of my dissertation committee for their timely review of each chapter presented here and for their combined editorial suggestions which greatly improved the clarity of the pages to come.

I would like to acknowledge the various funding sources which supported this research, the National Sea Grant College Program, National Oceanic and Atmospheric Administration, U.S. Department of Commerce, under grant number NA36RG0537, project R/A-99 through the California Sea Grant College System, the California State Resources Agency, the University of California Genetic Resource Conservation Program, and Jastro Shields Research Scholarships. I also wish to acknowledge the financial support I received from the Department of Animal Science and from other various sources in the form of a one year Novo Nordisk Fellowship, a one year predoctoral Biotechnology traineeship from the National Institute of General Medical Sciences (NIH T32-GM08343), and a two year California Sea Grant traineeship.

I would especially like to thank my wonderful parents, Maurice and Dawn Balson, who have always enthusiastically encouraged my academic endeavors and whose unwavering love and support have had a very positive influence on my life. It gives me particular pleasure to be obtaining my doctorate from The University of California as my father did before me in 1958. It was then, as it is now, a very long way from Australia. It must be in the genes.

Finally I would like to thank my family and close friends for their emotional support especially during the final year of my Ph.D. program. Their love and compassion helped me to confront the seemingly insurmountable challenges of the past and to gradually look forward to the possibilities and opportunities of the future. I am forever indebted to them for their friendship and support.

DEDICATION

For Zoey

We miss you special one

and all that could have been

TABLE OF CONTENTS

TABLE OF TABLES

TABLE OF FIGURES

PROJECT SUMMARY

Genetic Analysis of the Sex Determination Mechanism of White Sturgeon (*Acipenser transmontanus* Richardson)

The overall aim of this project was to understand the mode of sex determination in white sturgeon and to provide the first evidence as to what type of sex determination mechanism operates in chondrostean species. The following hypotheses were tested:

i) white sturgeon has a genetic sex determination system,

ii) one of the sexes in sturgeon is heterogametic, and

iii) sex-specific DNA polymorphisms exist between the sexes.

Several experimental approaches derived from the fields of transmission, molecular, and cytogenetics were used to examine the nature of the sex determination process.

In the first series of experiments (Chapter 2) ploidy manipulation techniques were used to produce gynogenetic and polyploid white sturgeon. A novel random amplified polymorphic DNA (RAPD)-based technique was developed to rapidly assess the overall success of treatments designed to induce gynogenesis, and measuring erythrocyte nuclei size with a Coulter Counter was found to be a rapid and accurate technique for ploidy analysis in sturgeon. Both sexes were observed in 23-24 month old gynogenetic progeny groups derived from four different females supporting the hypothesis that white sturgeon has a female heterogametic (ZW:ZZ) genetic sex determination system. The proportion of males in diploid (48%), gynogenetic (18%), and triploid (14%) progeny groups derived from the same female suggested that the sex-determining element on the W chromosome segregates independently of the centromere.

In the second series of experiments (Chapter 3) various molecular techniques were used in an attempt to isolate genetic markers for the identification of sex in white sturgeon. Subtractive hybridization, representational difference analysis and bulked segregant analysis were used, in conjunction with genetically unique groups of sturgeon, which were specifically developed to increase the probability of isolating sex-specific DNA sequences. DNA sequences associated with sex in white sturgeon were not identified. These results suggest that either 1) there are no sex-specific sequences in sturgeon, or 2) the sex-specific DNA is comprised of unusual sequences which were not complementary to the 1200 random decamer primers or the recognition sequence of the restriction endonucleases used in this set of experiments, or 3) the sex-specific sequences comprise a very small portion of the genome.

Chapter 4 details a synaptonemal complex (SC) analysis of white sturgeon spermatocytes. Synaptonemal complex analyses have not been previously reported for any sturgeon species and this study was initiated to determine if it was possible to directly identify heteromorphic sex chromosomes in meiotic prophase SC spreads. No bivalent consistently exhibited asynaptic behavior or had axes of unequal length suggesting that heteromorphic sex chromosomes are not present in white sturgeon spermatocytes which concurs with the findings of Chapter 2. Pachytene nuclei had varying numbers of univalents, self-paired foldback elements with no obvious centromeric region (1-7), and other SC peculiarities. No consistent evidence of alignment or pairing involving more than two lateral elements was found indicating that the process of diploidization is now complete in this ancient polyploid. The SC number was 139 (± 3.4) with both between and within animal variation.

The final series of experiments (Chapter 5) involved mitotic analyses of metaphase chromosome spreads from four white sturgeon individuals of each sex. Conventional mitotic analyses provided no evidence for a heteromorphic sex chromosome pair or any sex-related chromosomal polymorphism. Likewise fluorescence *in situ* hybridization of labeled male and female sturgeon genomic DNA to metaphase chromosome spreads of each sex did not reveal a chromosome that was seen to be specifically hybridizing only to the genomic DNA of one sex. The average chromosome number was 271 (range 265-276) which concurred with the meiotic count found in Chapter 4. An improved C-banding technique using propidium iodide and epifluorescence microscopy revealed between animal variation (2-7) in the number of entirely heterochromatic metacentric chromosomes. These heterochromatic chromosomes and the self-pairing elements observed in Chapter 4 were approximately equal in number and relative size and they may represent accessory B chromosomes.

In combination these results suggest that white sturgeon has a female heterogametic ZW:ZZ genetic sex determination system, and that the sex-determining element segregates independently of the centromere. No evidence of a heteromorphic sex chromosome pair was found in meiotic analyses of white sturgeon spermatocytes supporting the hypothesis of male homogamety in this species. The inability to identify sex-specific DNA sequences despite evidence of a genetic sex determination system suggests that sex-specific DNA is rare, making up a very small portion of the genome. Mitotic analyses provided no evidence for a heteromorphic sex chromosome pair suggesting that the accumulation of sex chromosomal rearrangements is insufficient to be visible at the cytogenetic level. All of these results support the contention that white sturgeon sex chromosomes are at an early stage of differentiation.

CHAPTER 1. LITERATURE REVIEW: THE ELUCIDATION OF SEX DETERMINATION MECHANISMS - HISTORICAL PERSPECTIVE AND CURRENT APPROACHES.

Introduction

The fast-growing and highly prized white sturgeon, *Acipenser transmontanus* Richardson, is an increasingly important species to California aquaculture. Confined production of white sturgeon began in the United States in 1979 with the sturgeon program of the Aquaculture and Fisheries Program at the University of California, Davis (Logan et al. 1995). There are currently 51 private aquaculture operations registered to raise white sturgeon in California[1]. In addition to its value as a commercial food fish, this species has the potential to produce high quality domestic caviar for national and international markets. With the decline of the caviar fisheries in the former-Soviet Union and China (Birstein 1993), there is now a potential high value market for domestic caviar.

In analyzing the feasibility of sturgeon flesh/caviar production in the United States, Peykani (1993) found that maximum profitability was achieved when male fish (50% of the cohort) were sold very early in the production process and only females were kept in the growout system for caviar production. The current diagnostic technique for sex identification in this sexually-monomorphic species requires a surgical biopsy of sexually-differentiated gonads (Conte et al. 1988). This procedure requires the fish to be of 7-8 kg body weight and

---

[1] State of California - Resources Agency, Department of Fish and Game, List of Registered Freshwater Aquaculturists as of December 31, 1996.

of at least three years of age. The availability of monosex female populations or an age-independent, DNA-based sex identification procedure would significantly enhance the economic feasibility of domestic caviar production systems. Sexed males could be culled and/or used for flesh production at a young age while immature females could be maintained for the production of caviar at 6-10 years of age. Economically efficient production of caviar would allow for market diversification which would enhance the overall feasibility of the domestic sturgeon industry (Peykani 1993, Logan et al. 1995).

Sturgeon are bony fish (Class Osteichthyes) which belong to the monophyletic ancestral group of ray-finned fish, Order Acipenseriformes, Infraclass Chondrostei. None of the chondrostean species (sturgeon and paddlefish) exhibit external sexual dimorphism (Holčík 1986), and all species so far investigated appear to be ancient polyploids (Dingerkus and Howell 1976, Birstein and Vasiliev 1987, Birstein et al. 1993, Blacklidge and Bidwell 1993). No obvious morphological difference has been found between any specific chromosome pair to indicate the presence of sex chromosomes in chondrostean species (Fontana and Colombo 1974, Holčík 1986) and it is therefore not possible to cytologically identify sex in these species. The exact mechanism of sex determination in chondrostean species is unknown (Kirpichnikov 1981).

The overall aim of this project is to understand the mode of sex determination in white sturgeon. The hypotheses to be tested are:

i) white sturgeon has a genetic sex determination system,

ii) one of the sexes in sturgeon is heterogametic, and

iii) sex-specific DNA polymorphisms exist between the sexes.

Testing of these simple hypotheses will provide the first evidence as to what type of sex determination mechanism operates in chondrostean species. In addition to its basic research interest, this information could benefit California domestic sturgeon caviar producers as they contemplate the production of monosex female populations for the caviar industry.

Sex Determination Systems in Animals

Mechanisms of sex determination in animals are astonishingly diverse (Table 1; modified from Hodgkin 1992) and seemingly disparate mechanisms can be found within single taxonomic levels. The fact that extant sexual species employ a variety of primary sex determining signals suggests that this regulatory process has undergone repeated convergent evolution (Bull 1983). There is also great variation in the way that the primary sex determination signal generates sex-specific differentiation. Strikingly different developmental pathways have emerged in different species to resolve the same sex-related biological dilemmas. These dilemmas include how to use chromosomal ratios to determine sex, how to achieve heteromorphic sex chromosome dosage compensation, and the need to ensure the correct and uniform choice of sexual fate in all the tissues of an individual (McKeown and Madigan 1992, McElreavey et al. 1993, McKeown 1994). In vertebrates, in spite of notable differences in the primary sex determination signal, sex-specific differentiation depends largely on circulating sex hormones (McLaren 1991, Solari 1994).

TABLE 1. Examples of different animal sex determination mechanisms.

ANIMAL	MECHANISM	FEMALE		MALE	
VERTEBRATES					
Mouse, man	CSD[1] : dominant Y	XX	female	XY	male
Birds	CSD : ratio ?[2]	ZW	female	ZZ	male
Turtles	ESD[3] : temperature	Warm	female	Cool	male
Alligators	ESD : temperature	Cool	female	Warm	male
Fish	ESD or CSD	Variety		Variety	
INSECTS					
Drosophila melanogaster	CSD : X/A[4] ratio	XX	female	XY	male
Apis mellifera	CSD : haplo-diploidy	Diploid	female	Haploid	male
Musca domestica	CSD : dominant M locus	*m/m*	female	M/*m*	male
NEMATODES					
Caenorhabditis elegans	CSD : X/A[5] ratio	XX	hermaphrodite	XO	male
Meliodogyne incognita	ESD : population density	Sparse	female	Crowded	male

[1] CSD, chromosomal sex determination.
[2] ratio ?, it is considered likely but not proven that the ratio of Z chromosomes to autosomal chromosome sets determines sex in birds, ZZ:AA = male, Z:AA = female.
[3] ESD, environmental sex determination.
[4] X/A ratio, the ratio of X chromosomes to autosomal chromosome sets determines sex, XX:AA = female , X:AA = male.
[5] X/A ratio, the ratio of X chromosomes to autosomal chromosome sets determines sex, XX:AA = hermaphrodite , X:AA = male.

The most familiar form of genetic sex determination involves structurally distinct sex chromosomes which seem to have arisen independently in many groups. The term "sex chromosome" usually implies that sex factors are inherited within a chromosome segment that

has a low incidence of crossing over in the heterogametic sex. There appears to be a common process of sex chromosome evolution in which the X and Y chromosomes originate from autosomes and then undergo a gradual accumulation of differences until they become heteromorphic. The evolution of sex chromosomes involves a variety of DNA alterations that are probably not directly involved in the sex determination process. The first step towards sex chromosome differentiation is a reduction in the crossing-over events occurring between sex-allele carrying chromosomes following rearrangements, such as inversions (Ohno 1967). This reduction in crossing over leads to the gradual transformation of the areas that have stopped undergoing recombination into heterochromatic fractions. At least three hypotheses have been proposed as to the genetic basis of this process: Muller's rachet, the hijack model and the hitchhiking mode (Jones 1989).

Various species are known in which the sex chromosomes are indistinguishable or only slightly heteromorphic, as indicated by X-Y (Z-W) morphological similarity, X-Y (Z-W) recombination, and YY (WW) viability (Schmid and Haaf 1987, Solari 1994). Mammals and birds have very heteromorphic sex chromosomes, whereas in many fish, amphibians and reptiles sex chromosomes are only slightly heteromorphic and may represent an early stage of sex chromosome evolution (Bull 1983, Charlesworth 1991). Once extreme sex chromosome differences evolve, the evolution of new sex determining mechanisms may be prevented, because YY (WW) individuals are no longer viable and XX males may be less successful than XY males. This process tends to stabilize the sex determination mechanism such that all species descended from a common ancestor will share the same sex chromosome system (Bull 1983).

Sex Determination in Fish

Fish represent an extremely heterogeneous group with regard to their mechanism of sex determination. Social, environmental and multiple genetic factors can all act to determine or change the sex of adult fish in certain species (Kirpichnikov 1981, Yamazaki 1983). Separate sexes (gonochorism) are present in the majority of fish species, but both sequential and simultaneous hermaphroditism have been reported in a significant number of species (Solari 1994). Furthermore, in some species with separate sexes, phenotypic sex reversal is feasible by simple hormonal treatments (Yamazaki 1983). Karyological examination of most species reveals that the sex chromosomes are morphologically undifferentiated. This suggests that the majority of fish exhibit a primitive condition of sex-chromosome evolution where the accumulation of chromosomal rearrangements is insufficient to be visible at the cytogenetic level (Bull 1983, Devlin et al. 1991). Monomorphic sex chromosomes do not preclude a stable gonochoristic sex ratio of 1♂:1♀. This ratio will still result if sex is determined by male or female heterogametic sex genes on the "autosomes" (Yamazaki 1983). The few fish species that have well-differentiated sex chromosomes belong to many different orders suggesting that sex chromosomes have evolved in multiple independent instances. A variety of gonosomal systems have been reported in fish including XY-XX; ZZ-ZW; XX-XO; ZO-ZZ and multiple gonosomal systems (Table 2).

In the absence of cytologically demonstrable sex chromosomes in most fish, genetic approaches have been used to show that sex determination in many of the gonochoristic species can be explained by a chromosomal mechanism. Inheritance patterns of sex-linked traits such as pigment genes (Winge 1922, Kallman 1984) and the sex of offspring derived

from matings with experimentally sex-reversed individuals (Yamamoto 1953, Yamamoto and Kajishima 1969, Hunter et al. 1982, Hunter et al. 1983, Davis et al. 1990) can provide strong evidence as to the identity of the heterogametic sex. All-female gynogenetic offspring may also suggest female homogamety (Stanley 1976, Chourrout and Quillet 1982, Refstie et al. 1982, Howell et al. 1995, Pongthana et al. 1995), but evidence from gynogenesis alone is not conclusive. Meiotic gynogenetic *Oreochromis aureus* are nearly all females, but the interpretation of the sex determination mechanism in this species from the results of several experimental techniques is that the female is heterogametic (ZW), and that there is a high rate of recombination between the centromere and the primary sex-determining locus (Penman et al. 1986, Avtalion and Don 1990, Mair et al. 1991a).

No hermaphrodites have been recorded in domestic white sturgeon, although very rare cases of rudimentary hermaphroditism have been reported in wild chondrosteans (Atz and Smith 1976, Chapman et al. 1996). The University of California, Davis domestic broodstock appear to produce offspring with a gonochoristic sex ratio of 1♂:1♀ (S. I. Doroshov, pers. comm.), and similarly the sex ratio in adult populations of white sturgeon from the San Francisco Bay was 1♂:1♀ (N = 855, Chapman et al. 1996). These observations suggest that white sturgeon has a gonochoristic type of sexuality and that sex is inherited genetically in white sturgeon. Environmental sex determination produces variations in sex ratios when there are systematic fluctuations in the environmental factors influencing sex (Bull 1983). Observations on natural populations of other chondrosteans suggest similar patterns of gonochorism (Zubova 1971, Holčík 1986).

Sex Determination and Polyploidy

Polyploidy is the presence in the cells or tissues of an organism of three or more copies of the basic set of chromosomes (the haploid genome). Polyploidy is very common in plants and it has been estimated that as many as 50 per cent of angiosperms have gametic chromosome numbers which are simple multiples of those found in related species. Newly formed autopolyploids have decreased fertility due to multivalent pairing which leads to irregularities in chromosome segregation and unbalanced gametes. Polyploids therefore depend upon especially favorable combinations of circumstances for their survival and reproduction (Stebbins 1950). Almost all naturally occurring polyploids undergo "diploidization" over time, meaning that the chromosomes eventually pair as bivalents rather than multivalents. Following the development of regular chromosome segregation patterns, the polyploid population may outcompete the originating diploid population. Polyploid plants are more tolerant of extreme ecological conditions than their diploid relatives and polyploids are thus ideally suited to the colonization of newly opened areas (Stebbins 1976). Polyploidy increases DNA content and cell size. It has been suggested that DNA quantity *per se* may have an adaptive quantitative non-genic, or "nucleotypic", function in addition to its qualitative genic function (Cavalier-Smith 1978). This nucleotypic effect may increase the fitness of the polyploid genotype relative to the diploid genotype.

Polyploidy in sexually reproducing animals is rare, with only a few well established cases on record, like South American and African frogs, and salmonid fish (Beçak et al. 1966, Allendorf and Thorgaard 1984, Kobel 1985). This striking difference between plants and animals has been attributed by some to the fact that most higher plants are hermaphroditic,

while the sexes are separate in the majority of animals. Muller (1925) offered the first explanation for this observation, suggesting that polyploidy upsets the balance of the segregation of the factors that determine sex. He argues that in diploid animals with an autosomal balance system of sex determination, there is 0.5 X chromosome: 1 autosomal set for the male (0.5X:1A), and 1 X chromosome : 1 autosomal set for the female (1X:1A). In triploid individuals, neither the X-YY:3A, or the XX-Y:3A "male" have the "correct" ratio of 0.5X:1A necessary to be fertile males. This male infertility was confirmed in *Drosophila*, where both types of triploid males (XXY, XYY) were found to be sterile (Bridges 1921). Muller further contends that in tetraploids, the ratio of gametes produced by an autotetraploid XXYY male will be 1XX:4XY:1YY. When crossed with a tetraploid female this will lead to progeny in the ratios 1XXXX:4XXXY:1XXYY. Only one-third of the offspring of these autotetraploids will return the parental sex chromosome ratios. Two-thirds will have a proportion of sex-producing gametes normal to neither male nor female. Early data from the plant kingdom tended to support this hypothesis. Hagerup (1927) found that the diploid *Empetrum niagrum* of Europe is dioecious, while the tetraploid *E. hermaphroditum* of Greenland is hermaphroditic with perfect flowers.

Warmke (1940) proved that dioecism and polyploidy are not incompatible with each other in plants by experimentally doubling the chromosome number in a species of *Melandrium*, a normally diploid dioecious plant. In the F_1 tetraploid generation, he produced fertile males of chromosome constitution 4A:XXYY and fertile females of constitution 4A:XXXX were then produced and crossed to get an F_2 generation. No intersexes or hermaphrodites were produced in this F_2 generation and it was found that the XXXY male

was 35 times more numerous than the XXYY male because the XXYY male formed an excess of XY gametes. The X chromosomes preferentially paired with each other, as did the Y chromosomes during prophase I, and this caused a greater than expected (88.8% rather than 66.6%) production of XY bearing gametes. The XXXY males were fully fertile in this experiment and formed a stable, dioecious polyploid "race". A similar pattern of preferential pairing was seen in autotetraploid silkworms, *Bombyx mori* (Kawamura et al. 1994). These results suggest that the existence of functional bisexual polyploids depends upon the presence of a strongly dominant sex-determining factor on the heteromorphic sex chromosome (Y or W). Sexually reproducing polyploid animal species do occur naturally among the amphibia and fish (Thorgaard 1977, Kobel 1985), but polyploidy in animals is often associated with parthenogenic reproduction (Gillies 1989).

Stebbins (1950) considers that another barrier to polyploidy in animals is that the sudden change in genotype causes disharmony to the complex processes of animal development. Ohno (1970) supports this concept by suggesting that the lethality of tetraploidy in mammals and higher vertebrates is due to the altered dosage of regulatory genes that results from chromosome set manipulation. He postulates that the normal diploid organism has a 1:2 dosage ratio of regulator to regulated structural gene (i.e. either the maternal or paternal copy of the regulator gene is turned off in the diploid). Evidence from the *lac*-operon system in diploid *Escherichia coli* suggests that regulated structural genes may be subjected to super-repression in a newly arisen polyploid (Sadler and Novick 1965) and Levy (1976) found that colchicine induced autotetraploidy produced a variety of qualitative alterations in the glycoflavone chemistry of a plant.

Stebbins (1950, 1976) also contends that the large amount of gene duplication that occurs with polyploidy dilutes the effect of new mutations such that polyploidy contributes little to progressive evolution. Polyploids are slow to adapt to change because a lot of their genetic variation is hidden in their polygenic loci, and therefore it is unavailable to the forces of selection. He extends this idea to suggest that the scarcity of polyploidy in animals may explain the fact that evolution in animals has tended to produce more families and orders adapted to particular modes of life, as compared to plants. He considers that this fact itself is evidence that polyploidy, although it multiplies greatly the number of plant species, retards rather than promotes progressive evolution.

Polyploidy in Acipenseriformes

Karyotypes of fish belonging to the order Acipenseriformes are characterized by a very large number of chromosomes, about half of which are microchromosomes (Table 3). The order may be divided into two groups; the first group has a chromosome number of approximately 120 and a mean genome size (1C) of 1.6-2.5 pg, and the second group has a chromosome number of 240-250 and a genome size of 4.0-4.8 pg (Birstein et al. 1993, Blacklidge and Bidwell 1993). On the basis of chromosome number, nuclear DNA content, isozyme data and the grouping of the karyotypes of some of the 120-chromosome species into quadruplets, it is considered that contemporary Acipenseriformes have a polyploid origin with the $2n \approx 120$ group being tetraploid (4N) derived, and the $2n \approx 240$-250 group being of an octoploid (8N) origin (Ohno et al. 1969, Dingerkus and Howell 1976, Birstein and Vasiliev 1987, Blacklidge and Bidwell 1993). The more ancient chondrostean polyploidization event

is thought to have occurred approximately 300 million years ago (Dingerkus and Howell 1976, Carlson et al. 1982), and the second genome doubling event leading to the *2n* ≈ 240-250 group of sturgeons is estimated to have occurred 80 million years ago (Vasiliev et al. 1980). There is some suggestion on the basis of genome size that the North American shortnose sturgeon (*A. brevirostrum*, 2C = 13.1 pg) and the Sakhalin sturgeon (*A. medirostris*, 2C = 13.9 - 14.7 pg) are dodecaploid (12N) and hexadecaploid (16N) derived species, respectively (Birstein et al. 1993, Blacklidge and Bidwell 1993). This suggestion needs to be confirmed by karyotypic analysis of these two species. On the basis of the location of nucleolar organizer regions in four sturgeon species, Fontana (1994) contends that species with 120 chromosomes should in fact be considered diploid, and species with 240-250 chromosomes should be considered tetraploid.

Isozyme systems that have variation within a polyploid-derived population provide a useful indicator as to the number of duplicated loci and whether they have either been silenced, remain undifferentiated, or have become differentiated with regard to structure, function or regulation (Markert 1975). Given the wealth of isozyme and breeding information that is available on the tetraploid-derived Salmonidae (Allendorf and Thorgaard 1984), there have been surprisingly few studies performed on the Acipenseriformes. Carlson (1982) studied 35 enzyme loci from *Polyodon spathula* (*2n* ≈ 120) and reported a 6% level of duplicate gene expression. This high level of gene silencing (94%) was considered to be consistent with the antiquity of the first chondrostean polyploidization event 300 million years ago (Dingerkus and Howell 1976, Carlson et al. 1982, Ryabova and Kutergina 1990). In contrast to this study, a high degree of duplicate gene expression was found in *A. stellatus*

(*2n* ≈ 118) in a study of 10 enzyme systems (29 loci) (Nikonorov et al. 1985, Ryabova and Kutergina 1990). The high degree of expression of duplicate genes in *A. stellatus* (31%), as compared to *P. spathula* (6%), needs explanation given the simultaneous timing of the first polyploidization event which is thought to have taken place in the ancestors of the early Acipenseriformes, not long before the divergence of the Acipenseridae and Polyodontidae families. Only two isozyme studies have been published on the *2n* ≈ 240-250 group of sturgeons (Slyn'ko 1976, Kuz'min 1991). Malate dehydrogenase was the only enzyme that was common to both studies. Unfortunately both studies did not use standardized terminology to discuss their findings and so it is difficult to compare their results. It does appear that in the *2n* ≈ 240-250 series of sturgeon that the diverse isozyme spectra can only be explained by two pairs of duplicated genes A_1, A_2, and B_1, B_2. Lactate dehydrogenase has been examined in only one species and the banding pattern was so complex that the author could not determine the extent of duplicate gene expression. Rather he concluded that "the exceptional multiplicity of the lactate dehydrogenase isozyme spectra in Russian sturgeon points to a complex structural organization of genetic systems" (Slyn'ko 1976). No study has compared the isozyme expression patterns in the postulated octoploid *(2n* ≈ 240-250) and tetraploid *(2n* ≈ 120) species of this order, and no breeding data have been reported to test for the existence of polysomic inheritance ratios in sturgeon species.

Gynogenesis and Ploidy Manipulation

Artificial ploidy manipulation techniques have been reported for many species of fish (reviewed in Purdom 1983, Thorgaard 1983, Chourrout 1986, Thorgaard 1986, Ihssen et al.

1990). Methods have been devised to suppress either normal meiosis or mitosis to retain additional sets of chromosomes, destroy the genetic information contained in the spermatozoa or egg nuclei, or both (Ihssen et al. 1990). Artificial gynogenesis, whereby inheritance is entirely maternal, is a promising technology which can enable the rapid production of highly inbred lines and can have applications in the sex control of cultured species in which one sex is more economically profitable than the other (Carter et al. 1991). To induce gynogenesis the male genetic contribution is inactivated, usually through the use of ionizing or UV-irradiation of the sperm, and diploidy is restored by treating eggs with either a temperature shock, hydrostatic pressure or chemical treatments subsequent to the entry of the inactivated sperm. The timing of this shock may be chosen to disrupt the second meiotic division (early shock) resulting in a meiotic gynogen, or it may occur during the first mitotic division (late shock) to produce a homozygous mitotic gynogen.

Chromosomal set manipulation can also be used to produce monosex female populations. In species having female heterogamety (ZW) gynogenesis may produce ZZ males, WW "super"females, and/or ZW females depending upon the rate of recombination between the sex determining locus and the centromere during meiotic prophase. If the WW super female is viable, then this female will produce all female offspring when crossed to a normal ZZ male. Artificial gynogenesis in species having female homogamety produces all-female progeny. The disadvantage of using gynogenesis for sex control is that the gynogenetic offspring are inbred (Thorgaard 1986). In female homogametic species this problem can be overcome by following gynogenesis with sex-reversal to produce XX males which will sire outbred monosex female populations.

Some of the earliest work on the production of meiotic gynogenetic diploid fish was reported by Romashov et al. (1960, 1963). In these early studies on loach, carp and sturgeon, X-irradiation was used to genetically inactivate sperm, and diploidy was restored to the activated egg by the application of an early temperature shock. The production of the first meiotic gynogenetic sturgeon was reported in the 1963 study, although none of the gynogenetic larvae survived beyond 192 days after hatching. In the thirty years since this early work, techniques for the production of gynogenetic fish have been reported for many species, however the literature contains little information on genetic manipulations with sturgeon species, and there are no further reports of the successful production of gynogenetic sturgeon.

Vassetzky (1966, 1967) reported the production of triploid and tetraploid sturgeon (*A. gueldenstaedti* V. Marri) through the application of a 34°C heat shock to eggs at different stages of development. Grunina and Neifakh (1991) induced diploid androgenesis in the Siberian sturgeon (*A. baeri* Brandt) by X-irradiation of the egg nuclei and diploidization of the male chromosome complex by a late 37°C heat shock. No genetic or biochemical proof was offered to support their inference that the larvae produced were fully derived from the paternal genome. These putative androgenetic larvae died before the transition to active feeding. Finally, there is a report of preliminary experiments that were performed in an attempt to induce polyploidy, gynogenesis and androgenesis in white sturgeon (Kowtal 1986). Early and late heat shocks produced viable triploid and tetraploid larvae respectively, while no larvae hatched from the gynogenetic treatment which involved UV irradiation of the sperm and an early 37-38°C heat shock. Seven viable androgens were reported following UV irradiation of unfertilized eggs, followed by fertilization and an early 37°C temperature shock.

Again, no genetic evidence was offered to support the assertion of the successful induction of androgenesis.

Identification of Sex-Specific DNA Sequences

Single-copy and repetitive sex-specific DNA sequences have been isolated and cloned using a variety of methodologies. In amniotes, the banded krait minor (Bkm) minisatellite $(GATA)_n/(GACA)_n$, the human telomeric sequence (TTAGGG), and the mammalian Y-specific genes, ZFY and SRY, are associated with a particular sex. Analyses of fish genomes with sex-specific probes from amniota have failed to identify sex-specific sequences in channel catfish *(Ictalurus punctatus)*, rainbow trout *(Oncorhynchus mykiss)*, or white sturgeon (Ferreiro et al. 1989, Lloyd et al. 1989, Tiersch et al. 1992). However, hybridization of restriction enzyme digested genomic DNA from the guppy (*Poecilia reticulata*) with the oligonucleotide probe $(GACA)_4$ revealed a Y-chromosome male-specific simple tandem repeat locus (Nanda et al. 1990, Nanda et al. 1992). A chinook salmon (*O. tshawytscha*) growth hormone pseudogene which was fortuitously discovered in an experiment that was not specifically designed for the isolation of sex-specific sequences was subsequently found to be male-specific in both chinook and coho (*O. kisutch*) salmon (Du et al. 1993).

Subtractive hybridization was the first general method devised for identifying sequences present in only one of two highly related DNA samples (Lamar and Palmer 1984). A typical subtraction experiment involves the addition of a large excess of DNA from one sample (driver) which lacks the trait of interest to a small amount of DNA from the other sample (tester) which possesses the trait of interest. The DNA mixture is then denatured and

complementary strands are allowed to reanneal. Tester DNA fragments predominately form hybrids with the vast excess of driver DNA fragments. The hybrid DNA complexes composed of sequences that are common to both the tester and the driver are then either removed by physical means; or are designed to be more difficult to clone than the complementary DNA complexes derived from sequences found only in the tester. Representational difference analysis (RDA) (Lisitsyn et al. 1993) is a modification of the subtractive hybridization process which utilizes PCR to kinetically enrich for sequences that are uniquely found in tester DNA. Lisitsyn et al. (1994) describe the use of RDA to specifically generate genetic markers linked to a trait of interest. Subtractive hybridization techniques (Lamar and Palmer 1984, Kunkel et al. 1985, Devlin et al. 1991, Fletcher et al. 1993, Nakayama et al. 1994) and representational difference analysis (Drew and Brindley 1995, Navin et al. 1996, Wigger et al. 1996) have been used to clone sex-specific DNA sequences from a variety of species.

Randomly amplified polymorphic DNA (RAPD) genetic markers were developed independently and reported simultaneously by Welsh and McClelland (1990), and Williams et al. (1990). The RAPD technique relies on the differential enzymatic amplification of DNA fragments using PCR with oligonucleotide primers of arbitrary sequence. The RAPD assay represents a particularly powerful strategy for identifying genetic markers where DNA sequence information for the genome of interest is not available to allow for the design of PCR primers. A large number of primers can be screened to generate markers in a relatively short period of time. The RAPD assay has been used to identify sex-specific markers in birds (Griffiths and Tiwari 1993, Levin et al. 1993), cattle (Antoniou et al. 1996), mice (Wardell et al. 1993) and sheep (Cushwa et al. 1996).

Michelmore et al. (1991) described an application of the RAPD technique termed bulked segregant analysis (BSA) which can be used to identify molecular markers linked to a trait of interest. The method involves analyzing two pools of DNA derived from a number of individuals that are contrasting for a particular trait (e.g. resistant and susceptible for a particular disease). The two pools differ in allelic composition within the genomic region of interest (individuals within each pool are identical for this region), while they are genetically similar outside of this region. Differences in the electrophoretic patterns of RAPD-PCR products between the pools identify polymorphisms potentially linked to the targeted region and associated with the trait used to separate the pools. In bulked segregant analyses where DNA pools differed by the trait of sex, a range of 20 to 700 different RAPD primers were required to identify at least one sex-associated DNA marker in different species (Mulcahy et al. 1992, Griffiths and Tiwari 1993, Hormaza et al. 1994, Cushwa et al. 1996).

Cytogenetics and Sex Chromosomes

The cytogenetics of fish has received little study compared to other animals; it is stated that only 850-900 of the more than 25,000 existent species have their diploid number and standard karyotype reported (Sola et al. 1981, Gold et al. 1990b). Research into the cytogenetics of fish has been limited by the difficulty of obtaining consistently good chromosome spreads from fish tissues and the fact that the chromosomal complements of most fish contain a relatively large number of comparatively small chromosomes (Gold et al. 1990a). Approximately 10% of the species that have been karyologically studied show heteromorphic sex chromosomes. The cytological detection of a heteromorphic sex

chromosome pair is the simplest way of ascertaining which is the heterogametic sex in a given species (Morescalchi 1991). However, in most fish species with separate sexes, sex alleles are localized on gonosomes which undergo regular recombination at meiosis and therefore they fail to accumulate heterochromatic fractions making it impossible to distinguish them from the autosomes. Furthermore in some cases it is not easy to distinguish chromosomal polymorphisms from presumed sex-linked heteromorphism (Mestriner et al. 1995). Fish sex chromosomes seem to contain many functional autosomal loci (Nanda et al. 1990) and in no case do they attain the degree of morphological and genetic specialization seen in the Y and W heterochromosomes of mammals, and some snakes and birds (Jones 1989, Morescalchi 1991).

The application of metaphase chromosome banding methodologies to fish chromosomes has been minimal. Chromosome banding techniques have been confined to those techniques which stain regions containing repeated sequences: C- and quinacrine (Q) banding for constitutive heterochromatin, chromomycin A_3 (CMA_3) and silver staining for nucleolar organizer regions (NORs) (Hartley 1991). Detailed linear banding patterns similar to the G and R banding patterns of higher vertebrates have not been obtained in fish chromosomes presumably due to the pattern of DNA organization in lower vertebrates (Schmid and Guttenbach 1988). The application of more elaborate chromosome analysis techniques is still quite limited in fish although some promising results have been obtained (Almeida-Toledo et al. 1988, Lloyd and Thorgaard 1988, Sánchez et al. 1990, Hartley 1991).

Most procedures for C-banding in fish follow the methods of Sumner (1972) and C-band patterns have now been documented for over 50 species of fish. Generally there is a

small amount of constitutive heterochromatin and resolved C-bands are primarily centromeric (Haaf and Schmid 1984, Hartley 1991, Vitturi et al. 1991, Mestriner et al. 1995, Fontana et al. 1996). Fluorochromes may also be used to resolve C-bands provided that 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 (Haaf and Schmid 1984, Mayr et al. 1987, Phillips and Hartley 1988); as has the GC-enhancing fluorochrome CMA_3 (Amemiya and Gold 1986). Lloyd and Thorgaard (1988) showed that treatment of rainbow trout metaphase chromosomes with specific restriction endonucleases followed by Giemsa staining produced a C-banding pattern. Recently C- and fluorescence metaphase banding techniques have been reported for sturgeon species (Sola et al. 1994, Fontana et al. 1996, Rab et al. 1996).

The synaptonemal complex is a proteinaceous structure formed between homologous chromosomes during zygonema. The cytogenetic technique of synaptonemal complex analysis by electron microscopy offers the ability to directly observe the process of chromosome pairing from early zygotene to late pachytene in prophase I of meiosis. Studies of the synaptonemal complex in fish are limited (Wise and Nail. 1987, Yajuan and Qixing 1991, Foresti et al. 1993, Peterson et al. 1994, Mestriner et al. 1995, Oliveira et al. 1995a, Oliveira et al. 1995b, Rodionova et al. 1996) and no studies have been reported for sturgeon. Heteromorphism for the sex chromosomes is displayed at the level of synaptonemal complex analysis as heteromorphic synaptic configurations (Moses 1980). In many vertebrates, the SCs of heteromorphic sex bivalents are readily distinguished from those of the autosomes (Solari 1974, Moses 1977, Solari 1977, Hale and Greenbaum 1986, Hale et al. 1988, Dollin et al.

1989). Synaptonemal complex techniques have permitted the direct identification of heteromorphic sex chromosomes in some species of fish (Yajuan and Qixing 1991, Foresti et al. 1993, Oliveira et al. 1995a), while in others no evidence of heteromorphism was found despite prior evidence of heteromorphic sex chromosomes from mitotic chromosome studies (Mestriner et al. 1995, Rodionova et al. 1996).

In a newly arisen polyploid some meiotic multivalent formation is likely to occur and polysomic inheritance ratios are expected. Selection for decreased infertility should cause a reduction of multivalent pairing and the eventual restoration of disomic inheritance. Long-established naturally occurring autopolyploids which have almost exclusive bivalent pairing at metaphase I may be considered to have become diploidized. Incomplete diploidization of polyploid-derived species has been seen in synaptonemal complex analyses where irregular synapses during zygotene and pachytene has given rise to a variable number of multivalents (Gillies 1989). Predominate or even exclusive bivalent formation by pachytene has been reported for tetraploid-derived *Bombyx mori* females (Rasmussen and Holm 1979), nematodes (Goldstein and Triantaphyllou 1980, Goldstein and Triantaphyllou 1981), and *Xenopus laevis* (Loidl and Schweizer 1992). On the basis of this finding Loidl and Schweizer (1992) concluded that functional diploidization has reached a very advanced state in *X. laevis*. The one synaptonemal complex study done on a naturally occurring autotetraploid-derived salmonid species (*Oncorhynchus mykiss*) found irregular synapses involving three or four lateral elements in 3.4% of the cells analyzed in mid or late zygotene (Oliveira et al. 1995a) which would agree with the finding of residual tetrasomic inheritance in salmonids (Allendorf and Thorgaard 1984). There was however an absence of irregular synapsis in pachytene nuclei

suggesting that homeologous synapsis occurs before homologous synapsis in this species. Given that crossing over occurs during pachytene when only bivalents are present, it is interesting to consider and explain how residual tetrasomic inheritance is occurring in rainbow trout. Bivalents were also exclusively reported in mid to late pachytene nuclei of artificially-produced triploid *O. mykiss*, suggesting a probable extensive nonhomologous synapsis involving the third set of chromosomes in this induced autopolyploid (Oliveira et al. 1995b).

Conclusions

The sex determination system of the order Acipenseriformes has received little study. It is known that none of the species so far investigated exhibit external sexual dimorphism, all appear gonochoristic and of polyploid origin, and no morphological difference has ever been found between any specific chromosome pair to indicate the presence of heteromorphic sex chromosomes. The model of sex determination proposed for this order must be consistent with these findings. Studies on sex determination in other species of fish have drawn on techniques from the fields of transmission genetics, molecular genetics, and cytogenetics. One or all of these approaches may help to elucidate the mechanism of sex determination in white sturgeon.

TABLE 2. Known heterogametic sex determination systems in Osteichthyes.

Order, family	Species	*2n*	♀	♂	Evidence	(Reference)
SALMONIFORMES						
Salmonidae	*Oncorhynchus mykiss*	58 - 60	XX	XY	Cytogenetic evidence	(Thorgaard 1977)
	Oncorhynchus kisutch	50	XX	XY	Sex of progeny from XY females	(Hunter et al. 1982)
	Oncorhynchus tshawytscha	68	XX	XY	Sex of progeny from XX males	(Hunter et al. 1983)
	Salvelinus namaycush	84	XX	XY	Cytogenetic evidence	(Phillips and Ihssen 1985)
	Oncorhynchus nerka	58 (57)	XXXX	XXY	Cytogenetic evidence	(Thorgaard 1978)
Galaxiidae	*Galaxias platei*	30	XX	XO	Cytogenetic evidence	(Campos 1972)
Sternoptychidae	*Sternoptyx diaphana*	36 (35)	XX	XO	Cytogenetic evidence	(Chen 1969)
Bathylagidae	*Bathylagus wesethi*	34	XX	XY	Cytogenetic evidence	(Chen 1969)
	Bathylagus stilbius	64	XX	XY	Cytogenetic evidence	(Chen 1969)
	Bathylagus milleri	60	XX	XY	Cytogenetic evidence	(Chen 1969)
	Bathylagus ochotensis	54	XX	XY	Cytogenetic evidence	(Chen 1969)
Synodontidae	*Saurida undosquamis*	48	ZW	ZZ	Cytogenetic evidence	(Nishikawa and Sakamoto 1978)
	Saurida elongata	48	ZW	ZZ	Cytogenetic evidence	(Nishikawa and Sakamoto 1978)
MYCTOPHIFORMES						
Neoscopelidae	*Scopelengys tristis*	48	XX	XY	Cytogenetic evidence	(Chen 1969)
Myctophidae	*Symbolophorus californiensis*	48	XX	XY	Cytogenetic evidence	(Chen 1969)
	Lampanyctus ritteri	48 (47)	XX	XO	Cytogenetic evidence	(Chen 1969)
	Parvilux ingens	50 (49)	XX	XO	Cytogenetic evidence	(Chen 1969)
ANGUILLIFORMES						
Anguillidae	*Anguilla japonica*	38	ZW	ZZ	Cytogenetic evidence	(Park and King 1979)
Congridae	*Astroconger myriaster*	38	ZW	ZZ	Cytogenetic evidence	(Park and King 1979)

TABLE 2 (*continued*). Known heterogametic sex determination systems in Osteichthyes.

Order, family	Species	*2n*	♀	♂	Evidence	(Reference)
PLEURONECTIFORMES						
Cynoglossidae	*Symphurus plagiusa*	46 (45)	XX	XO	Cytogenetic evidence	(Le Grande 1975)
CYPRINIFORMES						
Cyprinidae	*Scardinius erythrophthalmus*	50	ZW ZW'	ZZ	Cytogenetic evidence	(Koehler et al. 1995)
	Ctenopharyngodon idella	48	XX	XY	Sex of gynogens	(Stanley 1976)
	Carassius auratas auratas	100	XX	XY	Cytogenetic evidence	(Ojima 1983)
	Carassius auratas aururus	100	XX	XY	Sex of progeny from XY females and XX males	(Yamamoto and Kajishima 1969)
	Puntius gonionotus Bleeker	50	XX	XY	Sex of gynogens	(Pongthana et al. 1995)
Cobitidae	*Cobitis taenia*	50 (49)	XXXX	XXY	Cytogenetic evidence	(Saitoh 1989)
SILURIFORMES						
Bagridae	*Mystus tengara*	54	ZW	ZZ	Cytogenetic evidence	(Rishi 1973)
Loricariidae	*Microlepidogaster leucofrenatus*	54	ZW	ZZ	Cytogenetic evidence	(Andreata et al. 1993)
	Plecostomus ancistroides	68	XX	XY	Cytogenetic evidence	(Michele et al. 1977)
Ictaluridae	*Ictalurus punctatus*	56	XX	XY	Sex of progeny from XY females	(Davis et al. 1990)
Mochokidae	*Synodontis* sp.	54 - 56	ZW	ZZ	Cytogenetic evidence	(Agnèse et al. 1990)
Siluridae	*Callichroms bimaculatus*	42 (41)	XXXX	XXX	Cytogenetic evidence	(Rishi 1976b)
BERYCIFORMES						
Melamphaeidae	*Melamphaes parvus*	50	XX	XY	Cytogenetic evidence	(Chen 1969)
Anoplogasteridae	*Scopelogadus mizolepis bispnosus*	46	XX	XY	Cytogenetic evidence	(Chen 1969)
	Scopeloberyx robustus	42	XX	XY	Cytogenetic evidence	(Chen 1969)
GASTEROSTEIFORMES						
Gasterosteidae	*Apeltes quadracus*	46	ZW	ZZ	Cytogenetic evidence	(Ebeling and Chen 1970)
	Gasterosteus wheatlandi	42	XX	XY	Cytogenetic evidence	(Ebeling and Chen 1970)
SCORPAENIFORMES						
Cottidae	*Cottus pollyx*	48	XX	XY	Cytogenetic evidence	(Nogusa 1957)

TABLE 2 (*continued*). Known heterogametic sex determination systems in Osteichthyes.

Order, family	Species	*2n*	♀	♂	Evidence	(Reference)
TETRAODONTIFORMES						
Aluteridae	*Stephanolepis cirrhifer*	34 (33)	XXXX	XXY	Cytogenetic evidence	(Murofushi 1980)
CYPRINODONTIFORMES						
Cyprinodontidae	*Oryzias latipes*	48	XX	XY	Sex of progeny from XY females	(Yamamoto 1953)
	Fundulus diaphanus	48	XX	XY	Cytogenetic evidence	(Ebeling and Chen 1970)
	Fundulus parvipinnis	48	XX	XY	Cytogenetic evidence	(Ebeling and Chen 1970)
	Mexican cyprinodontid	48 (47)	XXXX	XXY	Cytogenetic evidence	(Uyeno and Miller 1971)
Poecilidae	*Gambusia affinis*	48	ZW	ZZ	Cytogenetic evidence	(Chen and Ebeling 1968)
	Gambusia holbrook	46	ZW	ZZ	Cytogenetic evidence	(Yosida and Hayashi 1970)
	Poecilia sphenops	46	ZW	ZZ	Cytogenetic evidence	(Haaf and Schmid 1984)
	Poecilia reticulata	46	XX	XY	Sex-linked color inheritance	(Winge 1922) (Nanda et al. 1990)
	Xiphophorus maculatus	48	WY WX XX	XY YY	Breeding experiments	(Kallman 1965)
	Mollienisia sphenops	46	ZW	ZZ	Cytogenetic evidence	(Rishi and Gaur 1976)
Goodeidae	*Mexican goodeid*	48 (47)	XXXX	XXY	Cytogenetic evidence	(Uyeno and Miller 1972)
Anostomidae	*Leporinus reinhardti*	54	ZW	ZZ	Cytogenetic evidence	(Galetti and Foresti 1986)
	Leporinus obtusidens	54	ZW	ZZ	Cytogenetic evidence	(Galetti et al. 1981)
	Leporinus elongatus	54	ZW	ZZ	Subtractive hybridization	(Nakayama et al. 1994)
	Leporinus macrocephalus	54	ZW	ZZ	Cytogenetic evidence	(Galetti and Foresti 1986)
	Leporinus trifasciatus	54	ZW	ZZ	Cytogenetic evidence	(Moreira-Filho et al. 1993)
	Leporinus conirostris	54	ZW	ZZ	Cytogenetic evidence	(Moreira-Filho et al. 1993)
	Leporinus lacustris	54	XX	XY	Cytogenetic evidence	(Galetti et al. 1981)
GYMNOTIFORMES						
Sternopygidae	*Eigenmannia* sp.	32 (31)	XXXX	XXY	Cytogenetic evidence	(Almeida-Toledo et al. 1984)

TABLE 2 (*continued*). Known heterogametic sex determination systems in Osteichthyes.

Order, family	Species	*2n*	♀	♂	Evidence	(Reference)
ZEIFORMES						
Zeidae	*Zeus faber*	44 (42)	XXXX	XY	Cytogenetic evidence	(Vitturi et al. 1991)
PERCIFORMES						
Percidae	*Perca fluviatilis*	48	XX	XY	Cytogenetic evidence	(Lieder 1963)
	Acerina cernua	48	XX	XY	Cytogenetic evidence	(Lieder 1963)
Gobiidae	*Gobiodon citrinus*	44 (43)	XX	XO	Cytogenetic evidence	(Arai and Swander 1974)
	Gobionellius shufeldti	48 (47)	XXXX	XXY	Cytogenetic evidence	(Pezold 1984)
Belontiidae	*Colisa lalius*	45 (46)	ZO	ZZ	Cytogenetic evidence	(Rishi 1976a)
	Colisa fasciatus	48	ZW	ZZ	Cytogenetic evidence	(Rishi 1979)
Scatophagidae	*Scatophagus argus*	48	XX	XY	Cytogenetic evidence	(Khuda-Bukhsh and Manna 1974)
Osphronemidae	*Trichogaster fasciatus*	48	ZW	ZZ	Cytogenetic evidence	(Rishi 1975)
Cichlidae	*Geophagus brasilliensis*	48	XX	XY	Cytogenetic evidence	(Michele and Takahashi 1977)
	Oreochromis mossambicus	44	WY WX XX	XY YY	Various breeding experiments	(Hammerman and Avtalion 1979)
	Oreochromis aureus	44	ZW	ZZ	Breeding experiments	(Avtalion and Don 1990)
Channichthyidae	*Chionodraco* sp. *Chaenocephalus* sp. *Pagetopsis* sp.	48 (47)	XXXX	XXY	Cytogenetic evidence	(Morescalchi et al. 1992)
	Chionodraco hamatus	48 (47)	XXXX	XXY	Hybridization of Bgl II satellite DNA	(Capriglione et al. 1994)
CHARACIFORMES						
Parodontidae	*Parodon hilarii*	54	ZW	ZZ	Cytogenetic evidence	(Moreira-Filho et al. 1993)
	Aparetodon affinis	55 (54)	ZWW	ZZ	Cytogenetic evidence	(Moreira-Filho et al. 1993)
Characidae	*Triphortheus albus*	52	ZW	ZZ	Cytogenetic evidence	(Moreira-Filho et al. 1993)
	Triphortheus signatus	52	ZW	ZZ	Cytogenetic evidence	(Moreira-Filho et al. 1993)
	Triphortheus elongatus	52	ZW	ZZ	Cytogenetic evidence	(Moreira-Filho et al. 1993)
	Triportheus guentheri	52	ZW	ZZ	Cytogenetic evidence	(Bertollo and Cavallaro 1992)

TABLE 3. Karyotypic summary of the chromosome complement of Acipenseriformes[1].

Species	*2n*	m + sm[2]	a (+ mc)[3]	mc[4]	Literature
Acipenser					
baeri	249 ± 5			120	(Vasiliev et al. 1980)
	246 ± 8	98	150		(Fontana 1994)
gueldenstaedti	250 ± 8	92 ± 4			(Birstein and Vasiliev 1987)
	249.9 ± 2.2	97.6 ± 0.4	152.2 ± 2.6		(Arefjev and Nikolaev 1991)
	256 ± 8				(Fontana et al. 1995)
	258 ± 4	106	152		(Fontana et al. 1996)
naccarii	239 ± 7	150	88		(Fontana and Colombo 1974)
	246 ± 8				(Fontana 1994)
	241 ± 3	88 ± 2	153		(Arlati et al. 1995)
	246 ± 8				(Fontana et al. 1995)
nudiventris	118 ± 3	54 ± 4	4	60 ± 3	(Arefjev 1983)
	118 ± 2				(Holčík 1986)
oxyrhynchus	99 - 112				(Li et al. 1985)
ruthenus	116 ± 4	66	40		(Fontana et al. 1975)
	118 ± 2	82 ± 4			(Birstein and Vasiliev 1987)
	117.3 ± 0.6	57.2 ± 0.3	60.1 ± 0.7		(Arefjev 1989)
	118 ± 4	58	4	56 ± 4	(Rab 1986)
	118 ± 4				(Fontana 1994)
	118 ± 9				(Fontana et al. 1995)
schrencki	240				(Vasiliev et al. 1980)
sinensis	264	98	166		(Yu et al. 1987)
stellatus	118 ± 2	70 ± 4			(Birstein and Vasiliev 1987)
sturio	116 ± 4	70	42		(Fontana and Colombo 1974)
transmontanus	237-243				(Hedrick et al. 1991)
	248 ± 8	104	144		(Fontana 1994)

TABLE 3 (*continued*). Karyotypic summary of the chromosome complement of Acipenseriformes[1].

Species	*2n*	m + sm[2]	a (+ mc)[3]	mc[4]	Literature
Huso					
dauricus	120				(Burtzev et al. 1976)
huso	116 ± 4	68	46		(Fontana and Colombo 1974)
	118 ± 2	60 ± 2			(Birstein and Vasiliev 1987)
	118.6 ± 0.5	61.5 ± 0.3	57.9 ± 0.5		(Arefjev 1989)
	117.6 ± 0.4	61.2 ± 0.2	57.6 ± 0.6		(Arefjev and Nikolaev 1991)
	118 ± 3	62 ± 4	6	50 ± 3	(Serebryakova et al. 1983)
Scaphirhynchus					
platorhynchus	112	50	14	48	(Ohno et al. 1969)
Polyodon					
spathula	120	40	8	72	(Dingerkus and Howell 1976)

[1] Based on information compiled by F. Fontana and available from the following URL: http://dns.unife.it:/geneweb/sturgeon.html.

[2] m + sm = number of metacentric and submetacentric chromosomes.

[3] a (+mc) = number of acrocentric (+ microchromosomes). These two chromosome types have been combined in many species where there is no separate count listed for mc.

[4] mc = microchromosomes.

CHAPTER 2. INDUCTION AND GONADAL SEX OF MEIOTIC GYNOGENETIC AND POLYPLOID WHITE STURGEON (*Acipenser transmontanus* RICHARDSON).

Alison L. Van Eenennaam, Joel P. Van Eenennaam, Juan F. Medrano, and Serge I. Doroshov

Published in part in Aquaculture (1996) **147**: 177-189.

Introduction

Ploidy manipulation has been reported for many species of fish (reviewed by Purdom 1983, Thorgaard 1983, Chourrout 1986, 1986, Ihssen et al. 1990). Artificial induction of gynogenesis and polyploidy in sturgeon species is of interest to both commercial aquaculturists and researchers investigating the developmental biology and genetics of chondrostean fish. The earliest work using artificial techniques for the production of meiotic gynogenetic sturgeon was reported by Romashov (1963). In their study none of the gynogenetic larvae survived beyond 192 days after hatching. A preliminary attempt to induce gynogenesis in white sturgeon was unsuccessful (Kowtal 1986). Vassetzky (1966, 1967) reported the successful production of triploid and tetraploid sturgeon (*Acipenser gueldenstaedti* Brandt), while putative androgenetic larvae of the Siberian sturgeon (*A. baeri* Brandt) died before the transition to active feeding (Grunina and Neifakh 1991). No genetic or biochemical proof was offered to support the hypothesis that these larvae were fully derived from the paternal genome. White sturgeon and many other fish have no obvious single locus morphological traits (e.g. color) that can be used to provide evidence of gynogenesis,

and heterologous sperm from other chondrostean species is not readily available to allow for the visual recognition of paternal inheritance.

The specific mechanism of sex determination in Chondrosteans is not known and the effects of ploidy manipulation on sex segregation ratios in sturgeon have not been described. Chromosomal set manipulation can be used to produce monosex female populations, with the potential production of all female populations being of obvious interest to sturgeon caviar producers. In species with ZW:ZZ female heterogametic sex determination systems, gynogenesis may produce ZZ males, WW "super"females, and/or ZW females depending upon the rate of recombination between the sex-determining element and the centromere during meiotic prophase. If the WW "super"female is viable, then this female will produce all female offspring when crossed to a normal ZZ male. Artificial gynogenesis in species having female homogamety will produce all-female progeny. The disadvantage of using gynogenesis for the production of monosex populations is that the gynogenetic offspring are inbred (Thorgaard 1986). In female homogametic species this problem can be overcome by following gynogenesis with sex-reversal to produce XX males which will sire outbred monosex female populations.

In this experiment gynogenetic, triploid and normal diploid white sturgeon were produced to allow for the examination of the sex ratios present in these genetically distinct groups of fish thereby providing information regarding the sex determination mechanism of this species. Fish were sexed at 23-24 months of age on the basis of gonadal morphology and histological analysis. A novel technique to rapidly screen putative gynogenetic progeny of known paternity for the inheritance of sire-specific RAPD markers is also described.

Materials and Methods

Experimental Design

Table 1 summarizes the experimental treatments used for the induction of gynogenesis and triploidy. All experiments included a diploid control group (untreated eggs and sperm), a haploid control group to determine the efficacy of UV inactivation of the sperm (untreated eggs and UV-irradiated sperm), a triploid control group to determine the efficacy of second polar body retention (temperature shocked eggs and untreated sperm), and a treatment designed to induce gynogenesis (temperature shocked eggs and UV-irradiated sperm). Treatments not detailed in Table 1 included a control in experiment 1 to determine if the temperature shock alone resulted in spontaneous diploid gynogenesis (temperature shocked eggs and no sperm added), and a control to examine for the presence of viable sperm in the semi-recirculating hatchery system (untreated eggs and no sperm added) in experiments 1-3. Temperature shocks were applied at 15 min post-fertilization in experiments 1 and 4, and at 12 min post-fertilization in experiments 2 and 3.

Gamete Collection

Experiments were conducted at the University of California, Davis Aquatic Center (Aquaculture and Fisheries Program) in the Spring of 1995, using as a broodstock the first hatchery generation of white sturgeon raised in captivity. Spawning induction and gamete collection was as described by Conte et al. (1988). Each of four females (experiments 1 through 4, respectively) were induced to spawn by injections of 40 μg/kg body weight of the gonadotrophin-releasing hormone analogue (D-Ala^6, Pro^9-NEt)-GnRH (Sigma Chemical

Company, St. Louis, MO). Males were induced to spermiate by a single injection of 1.5 mg/kg common carp pituitary extracts.

Sperm Treatment

For UV-irradiation, 2 ml of sperm stored in an atmosphere of pure oxygen at 4°C was diluted with 18 ml of seminal fluid (supernatant from surplus semen centrifuged at 6000 rpm for 20 min) and put into a rectangular pyrex dish (17 x 12 cm) to a depth of approximately 1 mm. This dish was placed on a gently rotating platform (90 rpm) 26-28 cm below two 15 W UVC bulbs (NIS G15T8) which provided an incident light intensity of 1200 $\mu W/cm^2$ as measured by the UVC probe of a UVX radiometer (UVP, San Gabriel, CA). The duration of UV-irradiation was adjusted for each batch of sperm such that the motility of the activated sperm was reduced to approximately 50 %. Sperm was treated with UV irradiation for 180 sec in experiment 2, 195 sec in experiments 1 and 4, and 270 sec in experiment 3. At the completion of irradiation, 380 ml of 16°C water from the incubation system was added to the irradiated sperm suspension, and this mixture was immediately added to ova. The addition of the sperm suspension to the ova was considered to be the time of fertilization. Procedures following the UV-irradiation of the sperm were performed in the absence of visible light to prevent the possible photoreactivation of the sperm's DNA (Ijiri and Egami 1980). A portion of each batch of untreated sperm was used to fertilize ova in the control diploid and polyploid treatment groups. The fertilization technique for these groups was as described except that 2 ml of undiluted sperm was added to 398 ml of incubation water immediately prior to fertilization.

Ova Treatment

Ova in coelomic fluid were maintained in a 4 l glass beaker at 16°C. For each treatment group 10-25 ml of ova were poured into a 190 mm diameter crystallizing dish and 400 ml of the appropriate sperm suspension was immediately added and the mixture was slowly stirred for three minutes. At this time the eggs were rinsed with 800 ml of fresh water four times to ensure that no viable sperm entered the incubation system. The eggs became sticky upon activation and they were quickly dispersed with a plastic transfer pipette to form a single layer across the bottom of the crystallizing dish. All dishes were kept at 16°C until the temperature shock. Heat shock treatments involved pouring the 16°C water off the eggs adhered to the bottom of the bowl, and immediately immersing the bowl in an oxygenated water bath set at the appropriate temperature. At the termination of the temperature shock the bowl was immediately immersed in a 15 l round fiberglass tank located within a semi-recirculating hatching system (16°C ± 0.5°C). Eggs that received a cold temperature shock were treated as described, using prechilled oxygenated water and a 3°C incubator for the duration of the cold shock treatment.

Neurulation and Hatching Success

Percent neurulation was determined by observation of the neural tube closure at 50-60 hours post fertilization (Dettlaff et al. 1993). Unfertilized and dead embryos were removed at this time to prevent fungal infection. At 9-10 days after fertilization the number of normal, free-swimming larvae was counted for each treatment, and the remainder of the abnormal larvae and unhatched eggs were removed from the tank and counted.

Rearing of the Larvae and Juveniles

Hatched larvae from each of the individual treatments were transferred from the hatching system to a rearing system with flow-through aerated well water (18°C) where they remained until the blood sampling for ploidy analysis. Ad libitum feeding (Silvercup semi-moist diet, Sterling H. Nelson and Sons, Inc., Murray, Utah) was initiated at 5 days post-hatch, using 24-hour automatic feeders. Experiment 4 diploid (n=1157), triploid (n=1191) and gynogenetic (n=2141) larvae were stocked into separate tanks at three days post-hatch, and daily mortalities were recorded for 40 days. At this time 300 randomly chosen juveniles from each of the experiment 4 control diploid and triploid treatment groups and all of the gynogens produced in the four experiments were set aside to be reared until gonadal sexual differentiation at 23-24 months of age. They were raised in flow-through tanks under conditions of seasonal photoperiod and water temperatures ranging from 18 to 21 °C.

Verification of Gynogenesis

Gynogenesis was verified by the analysis of sire and dam-specific random amplified polymorphic DNA (RAPD) markers generated by the polymerase chain reaction (PCR) using arbitrary primers (Welsh and McClelland. 1990, Williams et al. 1990, Scott et al. 1992). DNA was obtained from a single barbel, fresh or previously frozen, which was rinsed in 200 µl sterile H_2O, and placed in a 500 µl eppendorf tube with 200 µl of 5% chelex (Bio-Rad Laboratories, Hercules, CA), and 20 µg Proteinase K (Fisher Scientific, Pittsburgh, PA). The tube was then vortexed, incubated at 55°C for 2-3 hours, heated to 95°C for 12 minutes, and spun at 14,000 rpm in a microcentrifuge for 10 minutes. One hundred microliters of the clear

supernatant was then removed and diluted 1:50 in 0.1X TE prior to use in the RAPD PCR reaction.

PCR analysis was performed as described by Horvat and Medrano (1996). The PCR components consisted of 1.2 units of AmpliTaq DNA polymerase Stoffel fragment (Perkin Elmer Cetus, Norwalk, CT), 0.4 μM 10mer RAPD primer (OP, Operon Tech., Alameda, CA, or UBC, University of British Columbia), 1X Stoffel Buffer, 100 μM each of dNTPs, 4 mM $MgCl_2$, and 4 μl of DNA (1:50 dilution of supernatant as described previously) in a final volume of 12 μl. The amplification profile was one cycle at 94°C (2 min, 3 cycles at 94°C (1 min), 35°C (1 min), 72°C (2 min), followed by 32 cycles at 94°C (10 sec), 35°C (30 sec), 72°C (1 min, 5 min in the last cycle). PCR reactions were run in 96-well V-bottom plates (MJ Research, Watertown, MA) on a PTC-100-96V Thermal cycler (MJ research). PCR products were separated and analyzed in gels of 2% agarose (IBI, New Haven, CT) stained with ethidium bromide.

Sire and dam-specific markers were identified for the parents in each of the four crosses that were involved in the production of gynogens by comparing the products obtained by the PCR amplification of parental DNA using randomly selected RAPD primers. Primers that produced a clear sire or dam-specific polymorphic band for the parents of a given cross were recorded, and screening was continued until at least two primers that resulted in sire-specific polymorphisms had been identified. A total of 48 different RAPD primers were appraised in this preliminary screening. Ten diploid control progeny from each experiment were then screened to determine whether each identified sire or dam-specific RAPD marker was in the heterozygous or homozygous condition in the parent. To test the methodology,

four of the six predominantly-haploid mosaic fish, the three tetraploid fish, all surviving gynogens from the first three experiments, and 40 gynogens from the fourth experiment were screened for the presence of the appropriate sire and dam-specific markers. Every gel analyzing progeny included two lanes containing the appropriate parental RAPD PCR amplification products.

Verification of Polyploidy

Blood was collected from the caudal vein when the experimental animals were approximately 4 months old. The ploidy of blood cells was determined by nuclear volume analysis using a Coulter Counter Model ZM and 256 Channelyzer (Coulter Electronics, Hialeah, Florida, USA), according to the method of Wattendorf (1986). For each blood sample analyzed, the channelyzer was used to record the first channel to accumulate 1000 observations, and the mean of these channel values was used to determine the nuclear volume for the different ploidy levels. In a subsample of 162 fish, ploidy was determined by both cell nuclear volume and cell DNA content to verify the accuracy of the Coulter Counter rapid but indirect ploidy determination for this species. Blood samples were sent to the Department of Pathology, University of Washington School of Medicine where they were analyzed on an ICP-22 flow cytometer following the procedures of Thorgaard et al. (1982), and Utter et al. (1983). The mean flow cytometer channel data values for haploid, diploid, triploid and tetraploid erythrocyte nuclei were calculated, and the nuclear DNA content was estimated for each ploidy level using rainbow trout (*Oncorhynchus mykiss*) blood cells as the standard (2C = 5.5 pg, Tiersch et al. 1989).

Analysis of Gonadal Sex

Gynogens from all four experiments and experiment 4 diploid and triploid progeny groups were euthanized at 23-24 months of age. Gonads were removed and sex was identified by the observation of either yellowish grainy ovigerous folds on the lateral sides of the ovaries in the females, or white and smooth germinal tissue on the dorsolateral side of the testes in the males. Both gonads from each fish were examined for evidence of hermaphroditism. The sex was verified by histological analyses of gondal sections which had been fixed in 10% phosphate buffered formalin. Gonadal tissues were passed through a series of alcohols and xylenes, embedded in paraffin, sectioned at a thickness of 5-6 μm, and stained by hematoxylin and eosin. Slides of the stained tissue were examined using a light microscope (Olympus BHS). All 39 male gynogens were screened for the presence of sire-specific RAPD markers. Chi-square analysis was used to determine if the ratios obtained were significantly different from those expected for different sex determination systems.

Results

Table 1 summarizes survival to neurulation and hatching, and the ploidy of 4 month offspring from the four experiments. The neurulation and hatching rates of all diploid control groups were high, indicating that the gametes were of high quality and the fertilization and embryo incubation methods were optimal. Untreated and temperature shocked eggs in the absence of sperm failed to develop, and no viable larvae were produced in the haploid control treatment group (untreated eggs, UV-irradiated sperm) of the first three experiments indicating effective sperm inactivation by the UV-irradiation. The percentage of triploids in

the triploid treatment groups varied from 1 to 100% (Table 1). There was high variability in the success of second polar body retention when similar treatments were applied to different batches of eggs.

Triploid juveniles were phenotypically identical to the diploid fish, and no difficulties were experienced in raising them. The juvenile weight at 4 months of age was 39.6 ± 19.6 g (mean ± s.d, n = 1948), and no significant difference was found between diploid, triploid, and gynogenetic fish at this age (data not shown). Gynogenetic larvae displayed a behavioral peculiarity in that they appeared to swim randomly throughout the water column and at the water surface while the diploid and triploid control larvae exhibited the "normal" behavior aggregating on the bottom of the tank. This aberrant behavior persisted in the gynogenetic juveniles throughout the first 40 days but was not as pronounced thereafter. At 40 days post-hatch average survival was 87%, 84%, and 62%, in the experiment 4 diploid, triploid and gynogen treatments, respectively. Our observations do suggest a lower survivability for gynogenetic individuals, although the large decrease in the viability of gynogenetic sturgeon reported by Romashov et al. (1963) was not observed.

RAPD markers showed simple Mendelian segregation patterns in diploid control progeny (data not shown). Figure 1 illustrates a typical RAPD gel pattern obtained with four different RAPD primers and DNA derived from the sire and dam of experiment 3, and four putative gynogenetic progeny. The first three primers, UBC661, UBC497, and OPB8 amplify a clear sire-specific band at 850-bp, 650-bp and 350-bp, respectively. Putative gynogen "4" is not a true gynogen as it clearly received some, if not all, of the paternal genome. Primer OPC7 amplifies two dam-specific bands one of which was homozygous and the other of

which was heterozygous in the dam. The 900-bp homozygous band can be seen in all of the progeny, while the 1250-bp heterozygous band can be seen only in putative gynogen "1". A total of 108 juvenile (4 months of age) putative gynogens were screened from the four experiments using at least two RAPD primers which were known to generate sire-specific bands. One hundred and five of these fish showed no evidence of paternal inheritance, two individuals from experiments 2 and 3 showed amplification of at least one of the paternal-specific markers, and the barbel-derived DNA from one fish did not amplify in the PCR reaction.

Figure 2 illustrates the RAPD analysis of DNA derived from four of the predominantly-haploid mosaic progeny. There was no evidence of paternal-transmission in these fish, but maternally-derived RAPD bands are evident in all cases, suggesting that these individuals inherited only the maternal genome. The equivalent analysis for the three tetraploids produced in experiment 3 revealed that these individuals had received genetic markers from both the sire and the dam (data not shown).

A total of 2469 samples were analyzed with the Coulter Counter and of these 2468 gave an unambiguous ploidy determination. There was no overlap between the median channel data values observed for haploid, diploid, triploid, and tetraploid blood cell nuclei (Figure 3). Flow cytometry analysis of a random subsample of 162 blood samples agreed with the Coulter Counter ploidy determination. Mosaic individuals with both predominantly-haploid (less than 35% diploid) and diploid-tetraploid blood cells were included in this concordantly classified subset. The mean flow cytometer channel data values for haploid,

diploid, triploid and tetraploid erythrocyte nuclei were 25.5, 49.5, 72.2, and 93.7 which corresponds to estimated nuclear DNA contents of 5.5, 10.7, 15.6, and 20.2 pg, respectively.

Histological analyses of male gonadal tissue from 23-24 month fish showed a distinctively smooth epithelial layer, beginning of testicular cyst formation and a few cysts with primary spermatocytes (Figure 4a,b). Female gonadal tissue had well-defined ovigerous folds on the lateral side of the ovary, clusters of oogonia, and small oocytes adjacent to the epithelial layer (Figure 4c,d). Table 2 summarizes the sex ratios and average weights of the 23-24 month offspring from the four experiments. The body weights of male and female fish did not differ significantly. A total of 12, 15, 23 and 123 gynogens were sexed from experiments 1-4, respectively. In addition 85 diploids and 123 confirmed triploids from experiment 4 were sexed. The sex ratio of the experiment 4 control diploids did not differ significantly from 1♂:1♀. Both sexes were observed in the gynogenetic progeny groups derived from the four different females (experiments 1-4), with the percentage of males ranging from 18%-50%. The percentage of males found in the experiment 4 triploid group (14%) was not significantly different from that of the gynogenetic group (18%). Preliminary analyses revealed no evidence of hermaphroditism in the triploid group. Sire-specific RAPD markers were not found to be present in any of the male or experiment 4 gynogens.

Discussion

We observed both sexes in gynogenetic white sturgeon derived from four different females. Similar results have been observed in gynogenetic plaice (*Pleuronectes platessa*) (Purdom and Lincoln 1973), and tilapia (*Oreochromis aureus*, *O. niloticus*) (Penman et al.

1986, Avtalion and Don 1990, Mair et al. 1991a, Mair et al. 1991b). The frequency of ZW heterozygotes in gynogenetic offspring from species with female heterogamety is expected to vary according to the frequency of second division segregation (Table 3). No heterozygotes are expected if a crossover never takes place between the sex-determining element and the centromere, 100% heterozygotes are expected if an obligate single crossover takes place, and about 2/3 heterozygotes are expected if the sex-determining element is assorting independently of the centromere (Nace et al. 1970). In the absence of interference the expected proportion of females in species where the sex-determining element segregates independently of the centromere is (2/3 ZW + 1/6 WW) = .83 females, assuming that the WW "super"female is viable. One fifth of these gynogenetic females (i.e. the "WW" females) would be expected to produce all female progeny when crossed to normal ZZ males. In the absence of Z-chromosome specific markers, progeny testing is the only way to differentiate between the more numerous heterozygous ZW females and the valuable WW "super"females. If the "super"female is inviable then the ratio of males to females will range from 1:0 to 1:4 as the sex-determining element becomes more distally located from the centromere.

Our data support a female heterogametic (ZW:ZZ) genetic sex determination system for white sturgeon. The sex ratios found in the three large experiment 4 progeny groups most closely match the ratios expected for species with genetic sex determination where the sex-determining element segregates independently of the centromere (1/6 ♂:5/6 ♀; Table 3). The sex ratios obtained for the gynogenetic progeny groups from experiments 2-3 do not significantly differ from 1/6 ♂:5/6 ♀, however the 1♂:1♀ sex ratio found in experiment 1 does

differ significantly from 1/6 ♂:5/6 ♀. This ratio may be the result of the very small sample size (n = 12) in the experiment 1 gynogenetic progeny group.

The fact that the sex ratio of the experiment 4 triploid group did not differ significantly from that of the gynogenetic group provides further evidence in support of a ZW:ZZ sex-determination system where the sex-determining element segregates independently of the centromere (1/6 ZZZ males: 4/6 ZZW females: 1/6 ZWW females). The data do not allow us to distinguish between a dominant "W" sex determination switch mechanism and an autosomal balance system where the ratio Z/A < 0.67 generates females, or to determine whether the WW "super"female is viable. This latter question is of commercial importance as WW "super"females would be the ideal broodstock for monosex culture caviar production facilities. The data do not support ZO:ZZ female heterogamety as this system would generate gynogenetic sex ratios of 100% ZO females if disjunction of the single replicated Z chromosome occurred during the first meiotic division, and 50% ZZ males: 50% OO females (possibly inviable) if disjunction occurred during the second meiotic division.

The fact that the sex-determining element on the W chromosome generally appears to be segregating independently of the centromere suggests that the sex chromosomes in white sturgeon are undergoing frequent recombination. This supports the idea that the sex chromosomes are at an early stage of differentiation such that there appears to be little structural divergence to impede the process of crossing over. This agrees with cytogenetic studies which have not revealed heteromorphic sex chromosomes in either sex of any sturgeon species (Fontana and Colombo 1974, Holčík 1986, Chapters 4 and 5) suggesting that the accumulation of sex chromosomal rearrangements is insufficient to be visible at the

cytogenetic level. If the Z and W chromosomes are at an early stage of differentiation such that they have undergone little divergence then there is an increased probability that the WW "super"female remains viable in this species.

In this study a novel technique to identify paternal inheritance in gynogenetic diploids was described. Screening for sire-specific RAPD markers provides a simple and rapid technique to assess the overall success of a gynogenesis treatment. Carter et al. (1991) and Volckaert et al. (1994) used DNA fingerprinting to identify gynogens in tilapia, *Oreochromis aureus* and *O. niloticus*, and the African catfish, *Clarias gariepinus*, respectively. This is a lengthy procedure which requires the use of a Southern hybridization. In this study only 7 RAPD primers were necessary to determine that gynogens had been produced in all four experiments. By examining the transmission of dominant maternal and paternal-specific RAPD bands in ten diploid progeny of a given cross, it was possible to determine the zygosity of the marker in the parent. If a band appears in all 10 progeny then this marker is in the homozygous state in the parent ($P = 1-(0.5)^{10} = 0.999$). Ideally all markers would be in the homozygous state in one parent, meaning that all offspring which received a copy of that parental genome would inherit the marker. Only one homozygous marker specific to just one of the parents in this study was found (the dam-specific OPC7 900-bp band pictured in lanes 20 through 25 of Figure 2). The paucity of unique homozygous markers may be explained by the fact that an outbred broodstock population was being used.

Heterozygous sire-specific markers will be transmitted to 0.5 of his offspring, and will be absent in all true gynogenetic diploids. Heterozygous dam-specific markers will also be transmitted to one half of her normal offspring, and will be seen in 0.5-0.83 of the

gynogenetic diploids depending on the relationship of the marker gene to the centromere. For genes near the centromere, suppression of second polar body extrusion will result in predominantly homozygous progeny, 0.5 with, and 0.5 without the dominant marker. For genes segregating randomly in relation to the centromere, 66% of the progeny will be heterozygous (Nace et al. 1970) and carrying one copy of the dominant marker, and one half of the remaining homozygotes will carry two copies of the marker, so that 0.66 + (0.33/2) = 0.83 of gynogenetic diploids will inherit at least one copy of the marker.

Two to four dominant, heterozygous sire-specific RAPD markers were used in this experiment to identify paternal inheritance in putative gynogenetic treatment groups. There is a 0.25, 0.125, and 0.0625 probability that a normal (non-gynogenetic) individual will not be detected when two, three or four markers, respectively, are used to screen for paternal inheritance. Seven markers would be necessary to obtain a probability of greater than 0.99 that a given individual was a true gynogen. This emphasizes the advantage of a homozygous sire-specific marker as the absence of that one marker in the progeny confirms gynogenesis, at least for that one RAPD locus. In this study we were interested in screening a number of putative gynogens in different treatment groups. Although each individual was not unequivocally classified as a gynogen with the number of heterozygous sire-specific markers used, we can ascertain that gynogens were produced in all four experiments because of the large number of individuals that collectively did not inherit any sire-specific markers.

The preceding discussion assumes that complete gynogenesis was achieved. Incomplete gynogenesis (partial paternal transmission) has been most commonly recorded when ionizing radiation was used to inactivate sperm (Chourrout and Quillet 1982, Thorgaard

et al. 1985), although paternal DNA transmission has been reported following UV-irradiation of sperm (Carter et al. 1991, Volckaert et al. 1994). Partial paternal transmission may go undetected by examining only a few RAPD markers. If it is important to prove unequivocally that there has been no paternal inheritance, then a more exhaustive RAPD analysis would be required using numerous sire-specific markers.

Experimental treatments to induce gynogenesis and polyploidy were designed on the basis of two previous studies with sturgeon (Romashov et al. 1963, Vassetzky 1967), and one study with the closely related paddlefish *Polyodon spathula* (Shelton et al. 1995). The temperature shock was applied at either 12 or 15 minutes post-fertilization, equivalent to 0.21 or $0.26_{\tau 0}$ using the *A. gueldenstaedti* τ_0 value of 57.5 min at 16°C (Tau, τ_0, is an undimensional unit of developmental rate, (Dettlaff and Dettlaff 1961). The temperature shock was therefore most probably initiated during Anaphase II (Dettlaff et al. 1993). Application of the temperature shock was a little earlier than that used in trials with paddlefish (Shelton et al. 1995), and later than that reported by Vassetzky (1967) for Russian sturgeon.

When similar treatments were applied to different batches of eggs very different rates of second polar body retention were observed. The stage of egg development at induced spawning may differ between individual females (Dettlaff et al. 1993). A batch-specific treatment response was also seen with the male gametes. Identical UV-irradiation levels resulted in batches of sperm with differing abilities to initiate egg activation. The overall low number of gynogens produced in experiments 1-3 suggests that the sperm were unable to effect egg activation following UV-irradiation. This hypothesis is supported by the low rates of neurulation in the haploid control groups, and the observation that very few of the eggs

receiving UV-irradiated sperm underwent even the first cleavage indicating that fertilization did not take place. The UV-irradiated sperm in Experiment 4 were more effective at egg activation, as evidenced by the 19% and 26% neurulation rates in the haploid control and gynogen treatment groups, respectively. Although our experiments were not designed to determine the optimal parameters for the induction of meiotic gynogenesis and polyploidy in white sturgeon, their results suggest that the optimal conditions may be dependent upon the unique developmental stage of the specific batches of gametes involved in each cross.

In the course of this experiment most of the embryos in the haploid groups died at an early stage of development before neurulation, or they displayed features of haploid syndrome (abnormal body shape, open blastopore) and failed to hatch. The six mosaics with predominantly-haploid erythrocytes that were detected in our ploidy analysis seemed to be fully viable, normal-appearing fish. Their weight at four months of age ranged from 15.33 to 26.86 g which was within the range of weights observed for the diploid and triploid fish in their respective treatment groups. RAPD analysis revealed that four of these predominantly-haploid mosaics had no apparent paternal inheritance (no analysis was done on the other two fish). Other authors have reported the production of haploid larvae (Swarup 1959, Vassetzky 1967), but as these fish were killed at or before they were one day old, their viability was not assessed. The presence of a secondary population of diploid erythrocytes, and possibly the polyploid nature of white sturgeon (Birstein and Vasiliev 1987, Blacklidge and Bidwell 1993) may offer some explanation as to how these mosaic fish were able to tolerate the normally lethal gene dosage insufficiencies of the haploid condition. Five of the six mosaic fish with predominantly-haploid blood cells, and the three fish with tetraploid blood cells occurred in

cold shock treatments. Other authors have reported the production of haploids (Swarup 1959), and polyploid mosaics with cold shock treatments (Lemoine and Smith 1980, Refstie et al. 1982).

Measuring erythrocyte nuclear volume with a Coulter Counter and channelyzer is an indirect method to assess ploidy. The accuracy of Coulter Counter ploidy determinations for this species were verified by analyzing the blood from a subsample of fish by flow cytometry, a direct method of ploidy determination. The ploidy determination data of the Coulter Counter and flow cytometer were in complete agreement, although the flow cytometry estimate of the diploid DNA content, 10.7 pg, was larger than two previous estimates, 10.2 and 9.463 pg, that have been reported for white sturgeon (Hinegardner 1976, Blacklidge and Bidwell 1993). Overall the Coulter Counter and channelyzer was an inexpensive, rapid and accurate technique for ploidy determination in sturgeon.

Conclusions

In conclusion, the production of viable polyploid and gynogenetic white sturgeon was described. Gynogenetic progeny were found to include both males and females suggesting that a female heterogametic (ZW:WW) genetic sex determination system is in operation in white sturgeon. The proportion of males in the gynogenetic and triploid offspring suggested that the primary sex-determining element was segregating independently of the centromere. Although the effectiveness of the treatments designed to induce gynogenesis and second polar body retention was variable, the high percentage of gynogenetic and polyploid sturgeon in some progeny groups suggests that there is potential to use these techniques for further

research and applications to aquaculture. One application would be to investigate the possibility that a proportion of female gynogens are WW "super"females which could prove to be of use as broodstock for the production of outbred monosex female populations. A novel technique to rapidly screen putative gynogenetic progeny for the presence of paternal inheritance using RAPD markers was also described. This simple technique could be easily adapted to verify gynogenesis or androgenesis in any progeny group with known parentage.

Acknowledgments

The authors thank G. H. Thorgaard, S. D. Mims, J. G. Cloud and F. A. Chapman for helpful discussions in the planning stages of this study, A. B. Britt for the loan of the UVC-lamp and the radiometer, and D. H. Mistry for optimizing the protocol to allow PCR amplification of DNA derived from a sturgeon barbel. We also thank personnel from the Aquaculture and Fisheries Program, University of California, Davis for their assistance in rearing fish. This research was funded by the University of California Genetic Resource Conservation Program, a Jastro Research Scholarship, and in part by a grant from the National Sea Grant College Program, National Oceanic and Atmospheric Administration, U.S. Department of Commerce, under grant number NA36RG0537, project R/A-99 through the California Sea Grant College System, and in part by the California State Resources Agency. The views expressed herein are those of the authors and do not necessarily reflect the views of NOAA or any of its sub-agencies. The U.S. government is authorized to reproduce and distribute for governmental purposes.

TABLE 1. Neurulation and normal hatching frequencies, and ploidy analysis at 4 months of age by experimental treatment group in white sturgeon (*Acipenser transmontanus* Richardson).

Exp.[1] (No.)	Design[2]	EGG Treatment	(No.)	Neurulation (%)	Normal Hatch (%)	PLOIDY ANALYSIS DATA Total (No.)	Diploid (%)	Triploid (%)	Other (No.)
1	Diploid	Untreated	940	93	72	177	100		
	Haploid	Untreated	703	1	0	0			
	Triploid	34°C, 1 min	836	93	45	142	55	44	1 (DT)[3]
		34°C, 2 min	770	93	30	131		100	
		34°C, 5 min	845	77	9	40	20	80	
	Gynogen	34°C, 1 min	758	8	< 1	⌜			
		34°C, 2 min	792	6	3	20[4]	100		
		34°C, 5 min	977	8	< 1	⌞			
2	Diploid	Untreated	1489	88	74	50	100		
	Haploid	Untreated	1138	< 1	0	0			
	Triploid	3°C, 60 min	2244	43	17	218	41	57	4 (H)[5]
	Gynogen	3°C, 60 min	6405	< 1	< 1	15	100		
3	Diploid	Untreated	968	89	84	150	100		
	Haploid	Untreated	2588	< 1	0	0			
	Triploid	32°C, 2 min	1162	89	63	240	10	90	
		32°C, 3.5 min	1127	93	79	11[6]		100	
		32°C, 5 min	1095	92	69	229	3	97	
		3°C, 15 min	1146	90	82	179	98	1	1 (T)[7]
		3°C, 30 min	1103	87	70	255	64	35	2 (T)
		3°C, 60 min	1108	76	52	229	10	90	
		3°C, 180 min	1063	22	4	12	50	42	1 (H)
	Gynogen	32°C, 2 min	1410	2	< 1	⌜			
		32°C, 3.5 min	1475	2	2	14[8]	100		
		32°C, 5 min	1178	< 1	< 1	⌞			
		3°C, 15 min	1319	5	< 1	⌜			
		3°C, 30 min	1430	4	< 1	19[9]	100		
		3°C, 60 min	1290	6	4				
		3°C, 180 min	1274	< 1	< 1	⌞			
4	Diploid	Untreated	1574	87	74	60	100		
	Haploid	Untreated	1840	19	< 1	1	100		
	Triploid	34°C, 3 min	2255	87	53	227	29	71	
	Gynogen	34°C, 3 min	10631	26	21	50[10]	98		1 (H)

TABLE 1 (*continued*). Neurulation and normal hatching frequencies, and ploidy analysis at 4 months by experimental treatment group in white sturgeon (*Acipenser transmontanus* Richardson).

[1]Treatments within one experiment used ova from the same female.
[2] Design refers to the progeny type that the experimental treatments were devised to induce.
[3] (DT) = diploid-tetraploid mosaic.
[4] All surviving gynogens from experiment 1 were pooled.
[5] (H) = predominantly-haploid mosaic.
[6] The remainder of this group was accidentally killed by a larval rearing system failure.
[7] (T) = tetraploid.
[8] All surviving putative gynogens from experiment 3 heat shock treatments were pooled.
[9] All surviving putative gynogens from experiment 3 cold shock treatments were pooled.
[10] A subset of 50 putative gynogens from experiment 4 were analyzed for ploidy.

TABLE 2. Sex ratios and mean body weight of 23-24 month old gynogenetic, triploid, and diploid white sturgeon *(Acipenser transmontanus* Richardson).

Exp. (No.)	Group	N	Males (%)	Females (%)	Mean Body Weight (kg) (± sd) Male	Female
1	Gynogen	12	50	50	1.53 (.93)	1.50 (.66)
2	Gynogen	15	33	67	2.02 (.82)	2.20 (.67)
3	Gynogen	23	26	74	2.23 (.62)	2.06 (.48)
4	Gynogen	123	18	82	2.04 (.81)	2.11 (.64)
	Triploid	123	14	86	2.20 (.74)	2.26 (.60)
	Diploid	85	48	52	2.95 (.99)	2.64 (.70)

TABLE 3. Possible sex ratios of gynogenetic and triploid progeny groups from species with female heterogamety, and the sex determination system inferred by each set of ratios.

GYNOGENS		TRIPLOIDS	INFERENCE
Male	Female		
—	All ♀ (ZW)	All ♀ (ZZW)	Obligate crossover between sex-determining element and centromere, Dominant "W" or autosomal balance, 2Z/3A = female
		All intersex (ZZW)	Obligate crossover between sex-determining element and centromere, Autosomal balance, 2Z/3A = intersex
		All ♂ (ZZW)	Obligate crossover between sex-determining element and centromere, Autosomal balance, 2Z/3A = male
All ♂ (ZZ)	—	All ♂ (ZZZ)	Sex-determining element tightly linked to centromere, WW and ZWW inviable
		1/2 ♂ (ZZZ) 1/2 ♀ (ZWW)	Sex-determining element tightly linked to centromere, WW inviable, Dominant "W" or autosomal balance, 1Z/3A = female
1/2 ♂ (ZZ)	1/2 ♀ (WW)	1/2 ♂ (ZZZ) 1/2 ♀ (ZWW)	Sex-determining element tightly linked to centromere, WW viable, Dominant "W" or autosomal balance, 1Z/3A = female
1/6 ♂ (ZZ)	5/6 ♀ (WW:4ZW)	1/6 ♂ (ZZZ) 5/6 ♀ (ZWW:4ZZW)	Sex-determining element segregating independently of centromere, WW viable, Dominant "W" or autosomal balance, Z/A < 0.67 = female
		5/6 ♂ (ZZZ:4ZZW) 1/6 ♀ (ZWW)	Sex-determining element segregating independently of centromere, WW viable, Autosomal balance, 2Z/3A = male
		1/6 ♂ (ZZZ) 2/3 intersex (4ZZW) 1/6 ♀ (ZWW)	Sex-determining element segregating independently of centromere, WW viable, Autosomal balance, 2Z/3A = intersex, 1Z/3A = female
1/5 ♂ (ZZ)	4/5 ♀ (ZW)	1/5 ♂ (ZZZ) 4/5 ♀ (ZZW) OR 1/6 ♂ (ZZZ) 5/6 ♀ (ZWW:4ZZW)	Sex-determining element segregating independently of centromere, WW inviable, Dominant "W" or autosomal balance, 2Z/3A = female, 1Z/3A = inviable or female

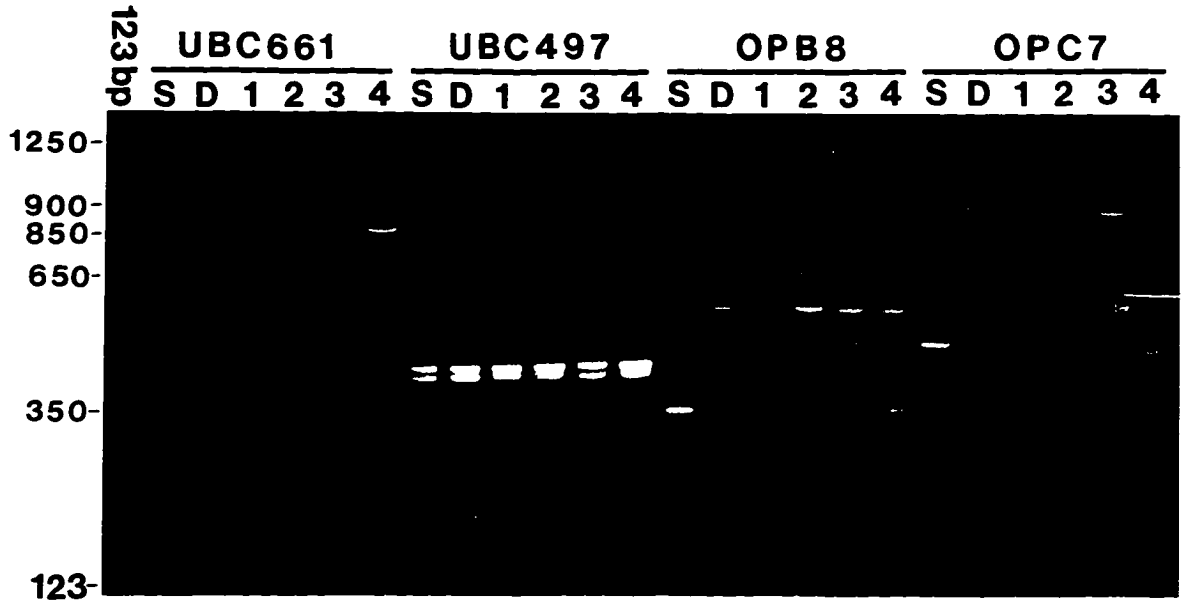


FIGURE 1. Agarose gel electrophoresis of RAPD PCR amplification products. Lane 1 = 123-bp ladder. Four RAPD primers (UBC661, UBC497, OPB8, OPC7) were used to amplify DNA from a sire (S), dam (D), and four of their putative gynogenetic progeny (1, 2, 3, 4).

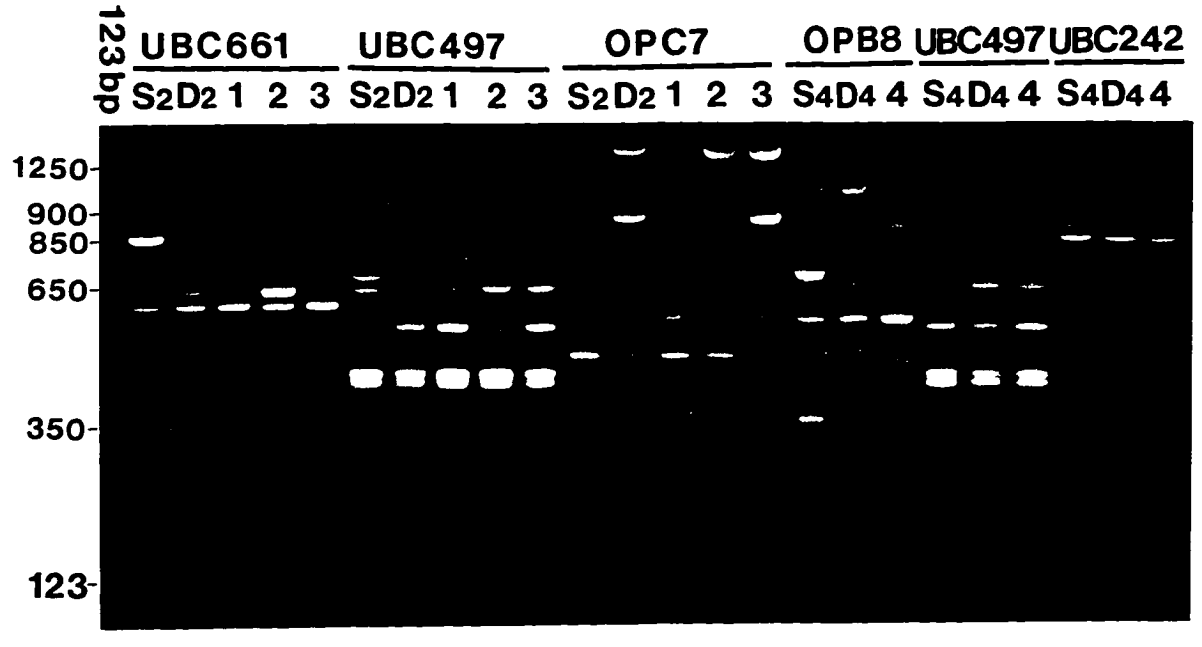


FIGURE 2. Agarose gel electrophoresis of RAPD PCR amplification products. Lane 1 = 123-bp ladder. Five RAPD primers (UBC661, UBC497, OPC7, and OPB8, UBC497, UBC242) were used to amplify DNA from the sire and dam of experiment 2 (S_2, D_2), and 4 (S_4, D_4), and their predominantly-haploid progeny 1, 2, 3, and 4, respectively.

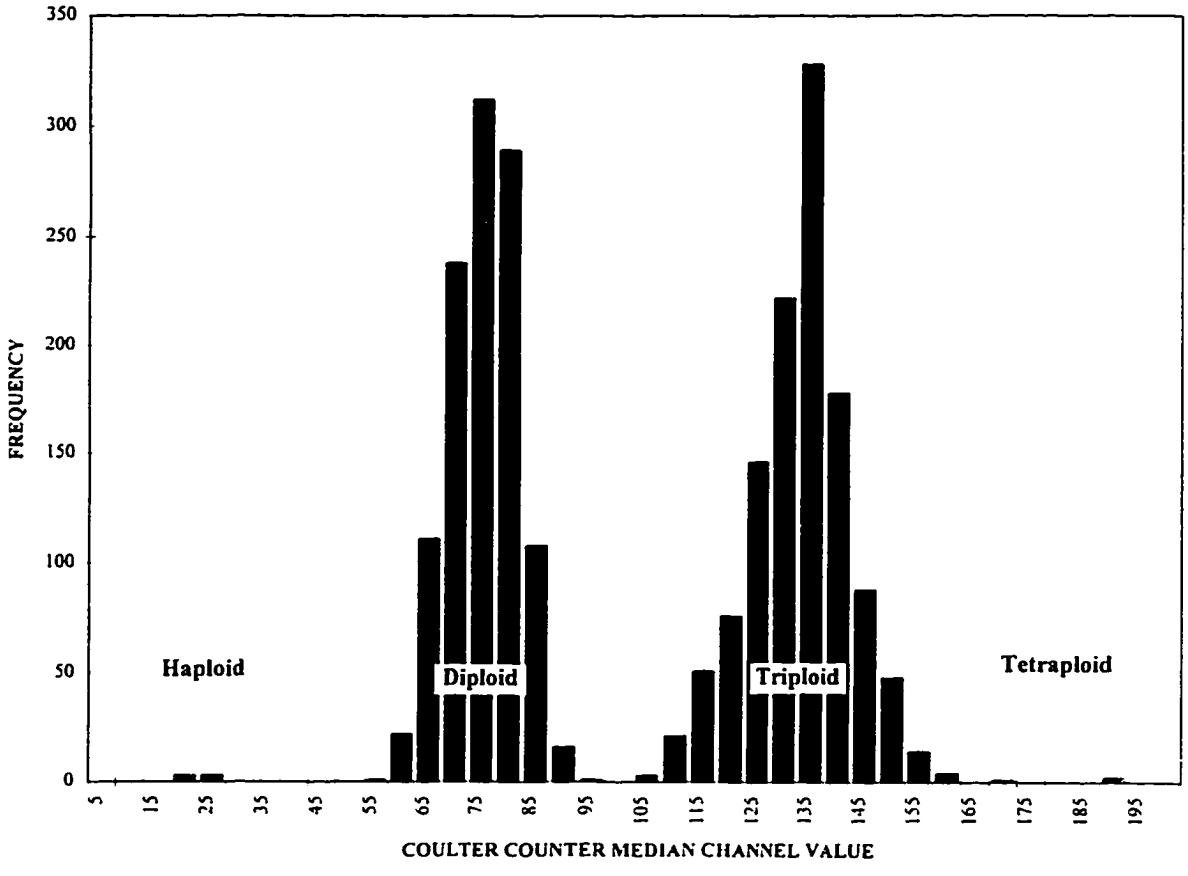


FIGURE 3. Median Coulter Counter channel values of erythrocytes collected from predominantly-haploid mosaic (n=6), diploid (n=1127), triploid (n=1263) and tetraploid (n=3) sturgeon at 4 months. The mean (± s.d.) haploid 20.50 (± 1.38), diploid 73.14 (± 6.32), triploid 130.72 (± 9.01), and tetraploid 182.67 (± 8.99) channel values correspond to approximate nuclear volumes of 23, 47, 74, and 98 μm^3, respectively.

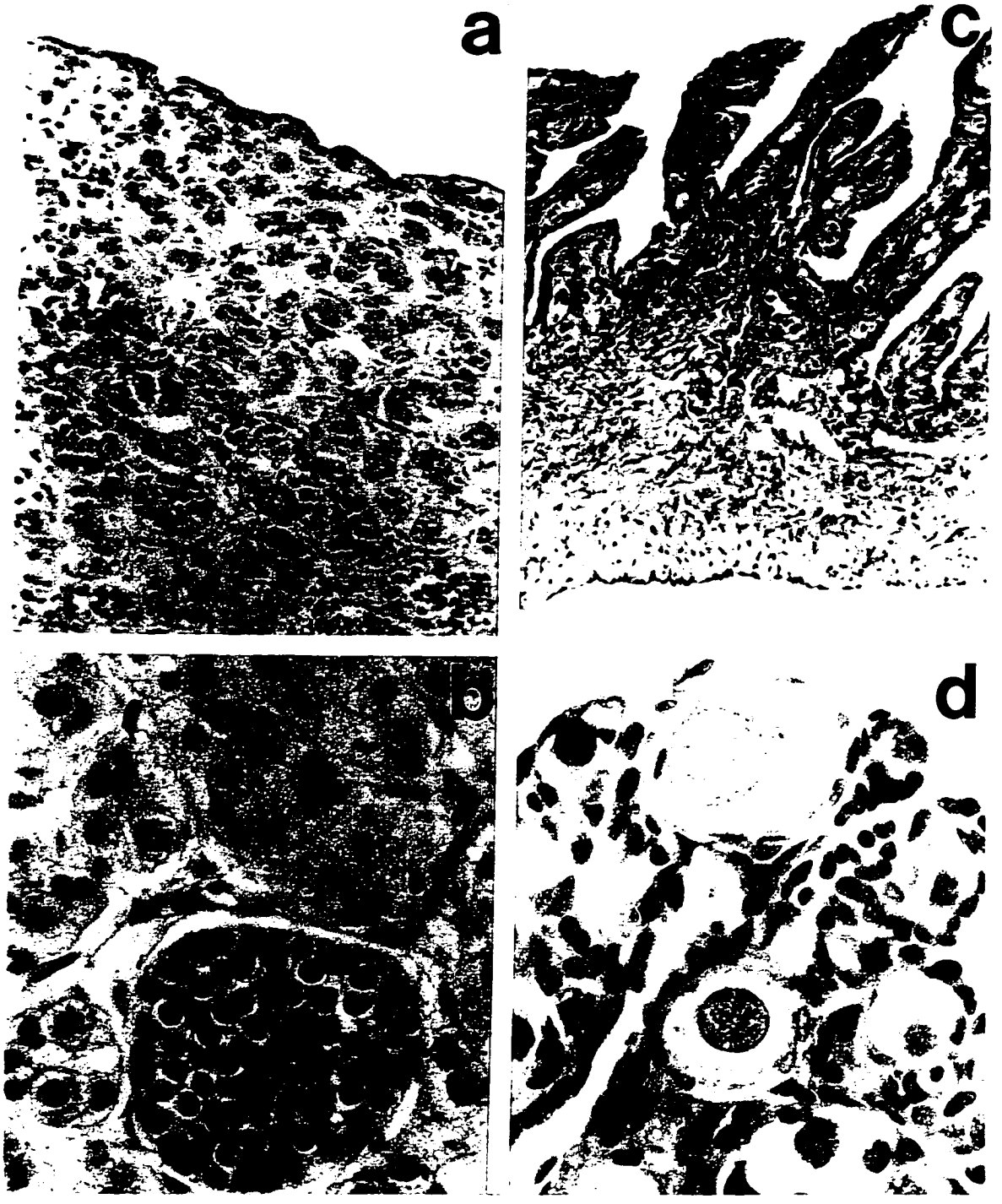


FIGURE 4. Sections of representative male and female gonads from 23-24 month old white sturgeon (*Acipenser transmontanus*). Testis (a,b) showing a smooth epithelial layer, incomplete testicular cyst formation and a few clusters of primary spermatocytes. Ovary (c,d) showing well-defined ovigerous folds on the lateral side and clusters of oogonia and primary oocytes adjacent to the epithelial layer. a,c x 175. b,d x 525.

CHAPTER 3. EXPERIMENTAL APPROACHES USED IN AN ATTEMPT TO ISOLATE MOLECULAR GENETIC MARKERS FOR THE IDENTIFICATION OF SEX IN WHITE STURGEON (*Acipenser transmontanus* RICHARDSON)

Alison L. Van Eenennaam and Juan F. Medrano

Introduction

The genetic sex of many species cannot be deduced by external morphology - a problem that is usually exacerbated when dealing with embryonic or juvenile forms. One effective solution to this problem is to use DNA markers to diagnose sex. Such markers will be present in species where one sex possesses an unique chromosome or DNA sequence (Griffiths and Tiwari 1993), and may not be present in species with XX:XO (ZO:ZZ), multilocus, or environmental sex determination systems. White sturgeon are sexually monomorphic and the current diagnostic technique for sex identification requires a surgical biopsy of sexually-differentiated gonads (Conte et al. 1988). This procedure requires the fish to be of 7-8 kg body weight and of at least three years of age. The availability of an age-independent, DNA-based sex identification procedure would significantly enhance the economic feasibility of domestic caviar production systems. Sexed males could be culled and/or used for flesh production at a young age while immature females could be maintained for the production of valuable caviar at 6-10 years of age (Peykani 1993, Logan et al. 1995).

Sturgeon are bony fish (Class Osteichthyes) which belong to the monophyletic ancestral group of ray-finned fish, Order Acipenseriformes, Infraclass Chondrostei. No

hermaphrodites have been recorded in domestic white sturgeon, although very rare cases of rudimentary hermaphroditism have been reported in wild chondrosteans (Atz and Smith 1976, Chapman et al. 1996). The sex ratio in adult populations of white sturgeon from the San Francisco Bay was 1♂:1♀ (N = 855) (Chapman et al. 1996). These observations suggest that this species has a gonochoristic type of sexuality and that sex is inherited genetically in white sturgeon. Environmental sex determination produces variations in sex ratios when there are systematic fluctuations in the environmental factors influencing sex (Bull 1983). Observations on natural populations of other chondrosteans suggest similar patterns of gonochorism (Zubova 1971, Holčík 1986). No obvious morphological difference has been found between any specific chromosome pair to indicate the presence of sex chromosomes in chondrostean species (Fontana and Colombo 1974, Holčík 1986) and therefore it is not possible to identify or confirm cytologically the existence of a heterogametic sex chromosome in white sturgeon. A preliminary study using restriction enzymes and a ZFY probe from humans failed to identify sex-specific sequences in white sturgeon (Ferreiro et al. 1989).

Several techniques have been employed for the isolation of sex-associated molecular markers in different species. One of the first techniques to be used was subtractive hybridization (Lamar and Palmer 1984). A typical subtraction experiment involves the addition of a large excess of DNA from one sample (driver) which lacks the trait of interest to a small amount of DNA from the other sample (tester) which possesses the trait of interest. The DNA mixture is then denatured and complementary strands are allowed to reanneal. Tester DNA fragments predominately form hybrids with the vast excess of driver DNA fragments. The hybrid DNA complexes, composed of sequences that are common to both the

tester and the driver, are then either removed by physical means, or are designed to be more difficult to clone than the complementary DNA complexes derived from sequences found only in the tester. Several improvements in this basic technique have been devised and have been used to clone sex-associated DNA sequences from a variety of animal species (Lamar and Palmer 1984, Kunkel et al. 1985, Devlin et al. 1991, Fletcher et al. 1993, Nakayama et al. 1994). Another fairly new technique that also has been used for the isolation of molecular genetic markers for the identification of sex (Drew and Brindley 1995, Navin et al. 1996, Wigger et al. 1996) is representational difference analysis (RDA) (Lisitsyn et al. 1993). RDA is a modification of the subtractive hybridization process which utilizes the polymerase chain reaction (PCR) to kinetically enrich for sequences that are uniquely found in tester DNA.

Subtractive hybridization methodologies require some knowledge of the mechanism of sex determination to allow the DNA from each sex to be designated as either driver (homogametic sex) or tester (heterogametic sex). A methodology for identifying sex-linked markers that requires no prior knowledge as to which is the heterogametic sex is bulked segregant analysis (BSA). The underlying principle of BSA is the grouping together of individuals that share a common trait so that the genomic regions that are associated with that trait can be studied against a randomized background of unlinked loci (Michelmore et al. 1991). Bulked segregant analysis has been used in conjunction with the randomly amplified polymorphic DNA (RAPD) assay (Welsh and McClelland. 1990, Williams et al. 1990) to successfully identify sex-associated markers in several different species (Mulcahy et al. 1992, Griffiths and Tiwari 1993, Wardell et al. 1993, Hormaza et al. 1994, Cushwa et al. 1996). Given a sufficient number of different decamer primers, it should be possible to isolate a

RAPD sex-specific marker for any organism where one sex possesses an unique chromosome or chromosomal segment (Griffiths and Tiwari 1993). In this study we employ subtractive hybridization, RDA and BSA methodologies to screen for genetic markers associated with sex in white sturgeon.

Materials and Methods

Genetic Stocks

Three genetically distinct groups of fish, referred to as the "unrelated", "full-sib" and "hybrid" groups, were used during the course of this research. The "unrelated" group consisted of 10 unrelated adult white sturgeon of each sex from the domestic broodstock collection of the University of California, Davis and a commercial sturgeon farm (Stolt Sea Farm, California, LLC). Females ranged from 9 to 14 years of age, and males ranged from 4 to 11 years of age. The "full-sib" group of fish consisted of 10 male and 10 female white sturgeon full-siblings. These fish were hatched in March 1994 after crossing a 9 year old domestic broodstock male with a 14 year old domestic broodstock female. The "full-sibs" were raised until sexual differentiation and sex was identified at 21 months of age (December 1995) using the methods detailed in Chapter 2. The "hybrid" group of fish consisted of 10 male and 10 female hybrid full-siblings. Hybrids were produced (May 1995) after using semen from a wild-caught lake sturgeon (*Acipenser fulvescens*) male of unknown age to fertilize ova from a 12 year old white sturgeon domestic broodstock female (Figure 1). Hybrids were raised until sexual differentiation which occurred at an earlier age than in purebred white

sturgeon. Sex was identified in the "hybrid" group of fish at 18½ months of age (November 1996), compared to 21 months of age in the purebred white sturgeon.

DNA Extraction

Several methods were tried during the development of an optimum protocol for the extraction of DNA from sturgeon. Typical methods for extracting DNA require the use of the organic compounds phenol and chloroform. We also included methodologies that did not require the use of these toxic solvents in our attempts to optimize a protocol for the extraction of sturgeon DNA (Medrano et al. 1990, Cummings and Thorgaard. 1994). We initially tried using blood as the DNA source tissue, as it can be easily obtained from live animals. We were unable to develop a method that consistently yielded good quality DNA from sturgeon blood. Purity of the DNA was compromised by what appeared to be a protein mass with a clear gelatinous appearance which coprecipitated with the DNA. This problem seemed to be exacerbated in sexually-mature fish. We found that DNA extracted from blood was difficult to quantify with a spectrophotometer and was incompletely digested with restriction endonucleases. PCR appeared to be unaffected by the impurities present in blood-derived DNA. We found that excellent quality DNA was obtained using spleen as the source tissue and a modified phenol/chloroform-based extraction protocol (Appendix 1A). This method has the disadvantage of requiring invasive tissue collection but it yields DNA of very high purity. This method was used to obtain DNA for all of the following experiments except for those involving the "unrelated" group of broodstock fish as we could not collect spleen tissue from these valuable animals. Blood-derived DNA was therefore used in experiments involving the "unrelated" group of fish.

Subtractive Hybridization

Methods used were based on those of Fletcher et. al. (1993) and are outlined in the diagram on the following page. Appendix 1B details the methodology used in this experiment with DNA from 10 white sturgeon full-sibs of each sex. Equal amounts of genomic DNA from females were combined and randomly sheared by sonication. An excess of sheared female DNA was biotin labeled and combined with *Sau*3AI partially-digested male tester DNA. The mixture was heat denatured and allowed to slowly reanneal. Female-specific fragments and hybrid duplexes, containing sequences present in the DNA of both sexes, were removed by streptavidin-coated paramagnetic particle capture. The remaining fragments, enriched for male-specific sequences, were ligated into a pBluescript vector (Stratagene, La Jolla, CA) which had been digested with *Bam*HI to generate *Sau*3AI compatible ends and subsequently dephosphorylated with alkaline phosphatase. Following transformation in competent *Escherichia coli*, 40 putative recombinant clones (white colonies) were selected, transferred to nitrocellulose membranes, and hybridized to ^{32}P-labeled male and female white sturgeon genomic DNA. Inserts that showed a greater hybridization affinity for the male genomic DNA were themselves PCR ^{32}P-labeled, and hybridized to Southern blots of *Bam*HI, *Bgl*II, and *Sau*3AI digested white sturgeon male and female genomic DNA. The plasmid inserts from recombinant clones that did not produce a hybridization signal in both sexes were sequenced using an automated Applied Biosystems, Inherit (Perkin Elmer Cetus, Norwalk, CT) sequencer. Specific PCR primers derived from these sequences were used in PCR reactions with either male or female sturgeon DNA template.

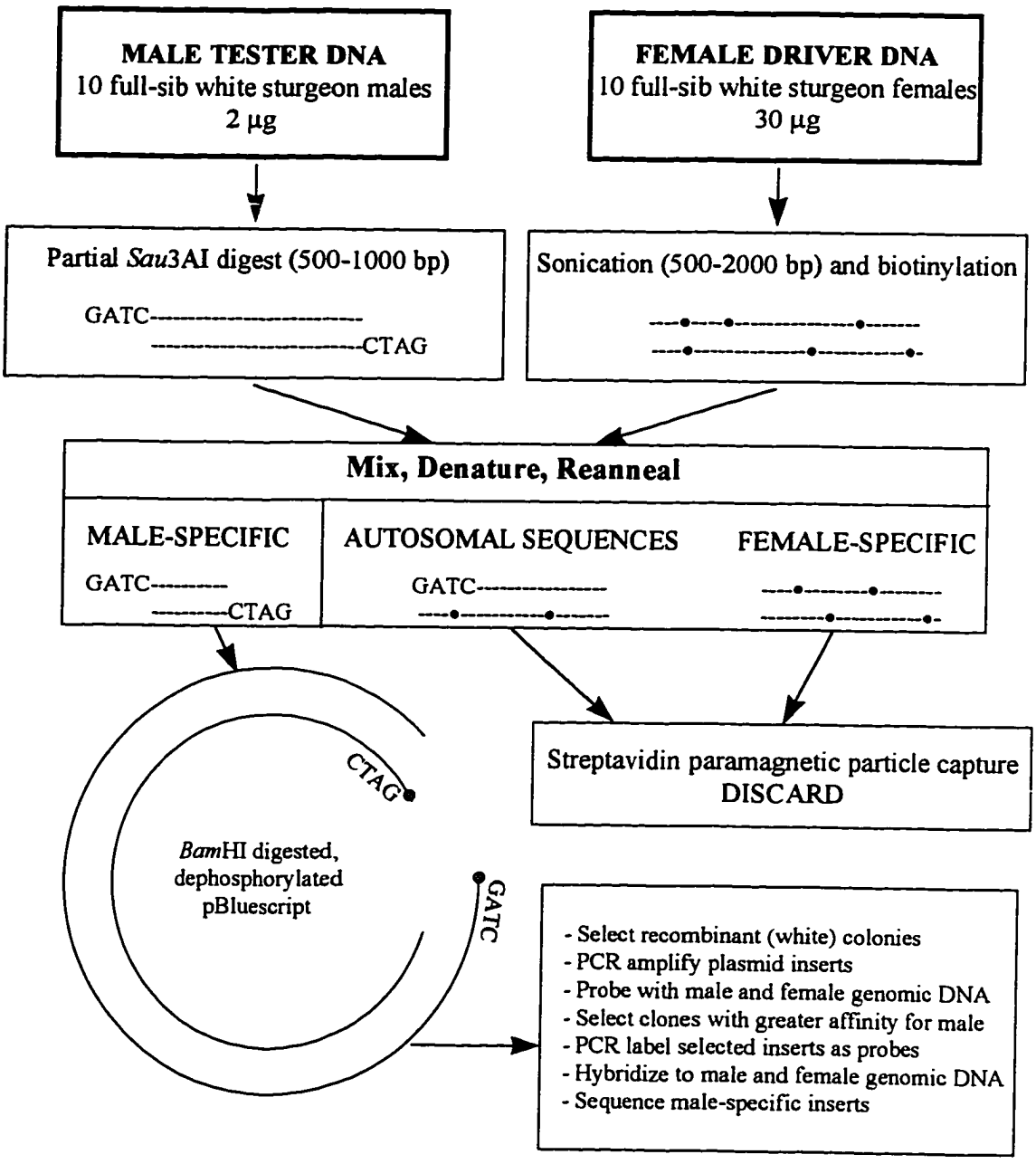


DIAGRAM 1. Schematic of the subtractive hybridization procedure.

Representational Difference Analyses

Methods used were based on those of Lisitsyn et al. (1993, 1994) and are outlined in the diagram below.

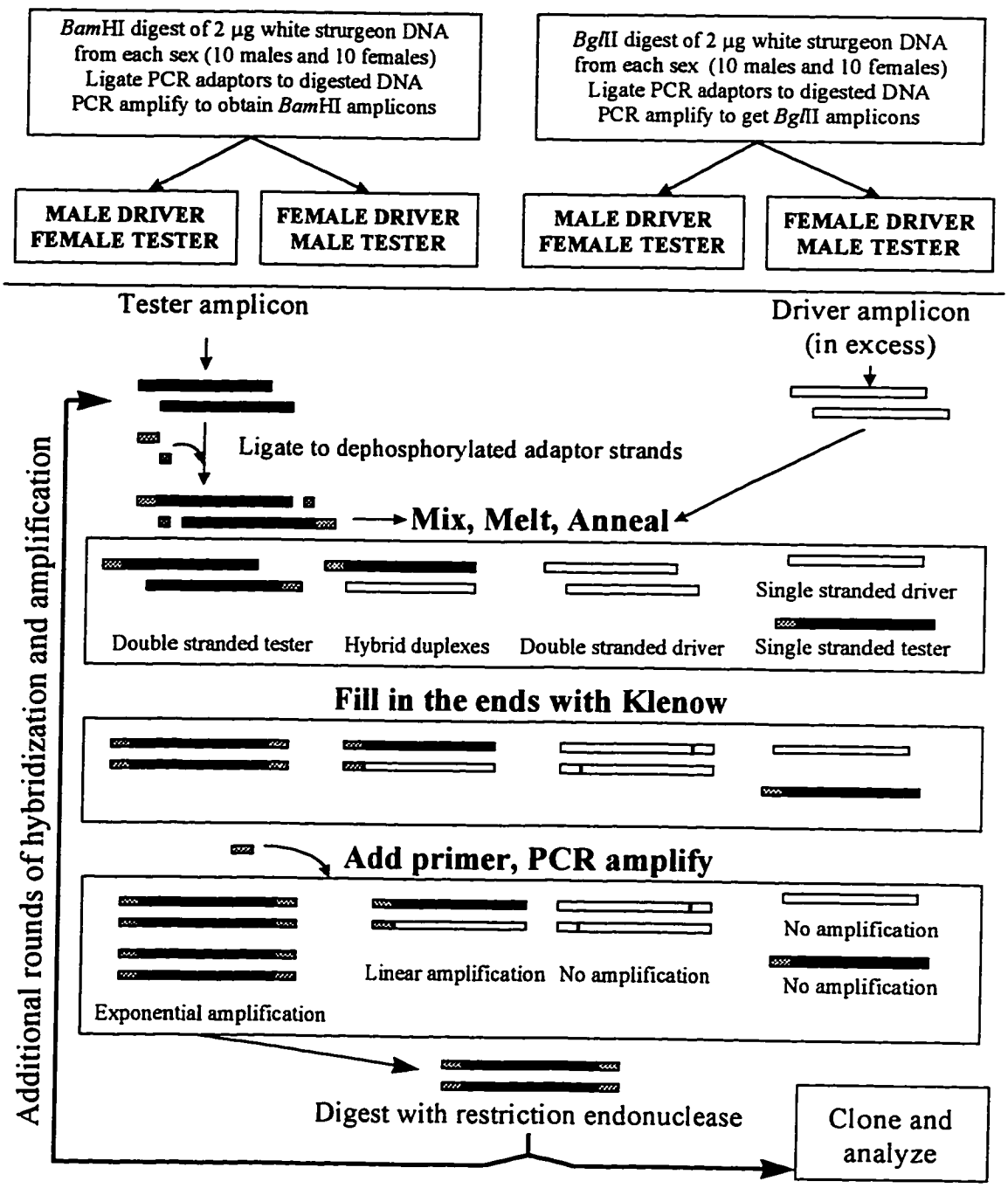


DIAGRAM 2. Schematic of the representational difference analysis procedure.

Appendix 1C contains a detailed protocol of the RDA methodology used in this study with equal amounts of DNA from 10 white sturgeon full-sibs of each sex. Two distinct representations were made using *Bgl*II and *Bam*HI restriction enzymes and their corresponding anchor primers (Operon Tech., Alameda, CA). Initially male DNA was used as the tester and female DNA was used as the driver, and then this order was reversed. Therefore a total of four RDA were performed: *Bgl*II representations (1) male tester with female driver, and (2) female tester with male driver; and *Bam*HI representations (3) male tester with female driver, and (4) female tester with male driver. Each RDA involved three rounds of competitive hybridization. In the absence of a third-round difference product 8-20 additional cycles of PCR were performed following the addition of 3 µl of fresh AmpliTaq DNA polymerase (Promega, Madison, WI). Difference products were separated on agarose gels and purified by excising the band and placing it in 200 µl TE at 4°C overnight to allow for the elution of the DNA. Difference products were then PCR ^{32}P-labeled using a specific PCR primer (primer J-24, Appendix 1C), and hybridized to Southern blots of *Bam*HI, *Bgl*II, and *Sau*3AI digested white sturgeon male and female genomic DNA.

Bulked Segregant Analyses

Three genetically distinct bulked segregant analysis (BSA) each using 1200 different decamer primers were performed using DNA bulks (male and female) that were created by combining equal amounts of genomic DNA from 10 fish of each sex. The first BSA used DNA from the "unrelated" group of fish, the second from the "full-sib" fish, and the third from the "hybrid" fish. RAPD reactions were performed in a 12 µl volume using 20 ng of bulk DNA (5 ng/µl) and the reaction conditions described in Chapter 2 for OP (Operon) primers,

while for UBC (University of British Columbia) primers the primer concentration was increased to .58 μM. A total of 1200 RAPD primers (400 OP and 800 UBC) were used to screen the bulks. The UBC primers included sets one to eight (100 primers/set) and the OP primers included sets A to P, R, S, W, and AD (20 primers/set). RAPD polymorphisms detected between the bulks were reproduced at least one time. If a bulk polymorphism was reproducible, the individual DNA samples used to create both bulks were screened using the same RAPD primer and reaction conditions.

RAPD fragments that were found in individuals of only one sex were gel purified and PCR reamplified using the RAPD primer that originally amplified the band and AmpliTaq DNA polymerase (Promega) in the place of AmpliTaq DNA polymerase Stoffel fragment (Perkin Elmer Cetus). This change of enzyme was necessary to allow for the success of the TA cloning system. The reamplification products were run on a 2% agarose gel, purified using GENECLEAN II (Bio 101, La Jolla, CA), and cloned using the TA Cloning System (Invitrogen, Carlsbad, CA). Plasmid DNA from overnight cultures of putative recombinant plasmids (white colonies) was prepared using the QIAquick Spin Plasmid Miniprep Kit (Qiagen, Chatsworth, CA). Restriction enzyme analysis was used to confirm that the correct fragments had been cloned. Both ends of the cloned RAPD fragments were sequenced using Sequenase (USB, Cleveland, OH), and the 10 bp RAPD primers were converted into pairs of strand-specific 20-22 bp sequence characterized amplified region (SCAR) primers (Paran and Michelmore 1993). These SCAR primers were used in PCR reactions with either male or female sturgeon DNA template.

Results

Subtractive Hybridization

A control plate prepared from cells that were transformed only with dephosphorylated *Bam*HI digested pBluescript vector contained a single white colony out of a total of 20. One half of the colonies on plates prepared from cells transformed with "male-enriched" DNA were white (recombinant). Forty recombinant clones containing 100-400 bp inserts were analyzed for the presence of male-specific inserts. Dot blots prepared from minipreps of the 40 recombinant plasmids probed with genomic DNA derived from male and female sturgeon showed no difference in the hybridization patterns between the two sexes (Figure 2). There appeared to be some background hybridization of the sturgeon genomic DNA to the nonrecombinant pBluescript plasmid (G1 and H1, Figure 2). This experiment was repeated using Southern blots of the PCR-amplified inserts separated on an agarose gel and probed with labeled male and female genomic DNA (Figure 3). No clones hybridized specifically to the male probe. Five of the clones (A5, A12, A13, A14, A15) did appear to give a stronger signal when probed with the male DNA suggesting a sex-associated differential hybridization affinity. Inserts from these five clones were PCR-labeled and hybridized to Southern blots of digested male and female genomic DNA. Four of these clones appeared to be multicopy DNA present in both sexes, and hybridized strongly to small *Sau*3AI restriction fragments (Figure 4). One clone (A12) appeared to be a single copy sequence as there was no detectable signal produced after hybridization of this insert to genomic DNA from either sex, even after exposing the Southern blot to autoradiographic film for several days. This clone was sequenced (Sequence 1, Appendix 2), and specific PCR primer pairs were derived from this

sequence. Using these primers a band of the same size was amplified with either male or female sturgeon DNA template (data not shown), implying that the sequence was derived from sturgeon but that is was not sex-specific.

Representational Difference Analyses

Amplicons made from *Bgl*II and *Bam*HI representations were quite distinct with repetitive bands being present in both representations (Figure 5). As expected there was no obvious difference between the amplicons prepared from male and female DNA. No difference products were visible at the completion of the third round of amplification in any of the four RDA experiments (*Bgl*II representations: male tester with female driver, and female tester with male driver; *Bam*HI representations: male tester with female driver, and female tester with male driver). Additional cycles of PCR amplification did produce some small (100-200 bp) fragments which proved by Southern analysis to be repetitive, sex-independent sturgeon DNA sequences (data not shown).

Bulked Segregant Analyses

Table 1 summarizes the results of the three BSA experiments each using 1200 RAPD primers. Primers that produced either a RAPD band that was only present in one sex, or amplification products with the DNA from only one sex, or that generated only partial amplification products with the DNA from one sex were rescreened. In the "unrelated", "full-sib" and "hybrid" BSA experiments, 123, 106 and 127 primers were rescreened, respectively. Following reconfirmation of the bulk polymorphism, the individual DNA samples used to create both bulks were screened using primers OP: A8, C2, C7, C11, E6, F9, G2, G13, I14, J8, L9, O16, P5 and UBC: 169, 298, 580, 589, 661 for the "unrelated" group of fish; primers

OP: C7, I10, J18, M4, W15 and UBC: 271, 322, 516, 564 for the "full-sib" group of fish; and primers OP: C20, P14 and UBC: 100, 123, 167, 169, 326, 471, 689 for the "hybrid" group of fish. No primer in any of the three BSA was found to amplify a band that was found in all of the individuals of one sex, and was absent in all of the individuals of the other sex (Table 2).

The "unrelated" group screen identified 17 RAPD primers which generated reproducible bulk polymorphisms (Figure 6). Screening of the "unrelated" individuals that made up these bulks with these RAPD primers showed that the polymorphism often resulted from a band that amplified very strongly in one or two of the individuals that made up one of the bulks. Polymorphic bands produced by primers OP C7 and UBC 661 were found in 3 and 2 of the 10 animals from one sex, respectively, and none of the animals from the other sex (Figure 7). These bands were sequenced (Sequence 2 and 3, Appendix 2) and specific SCAR PCR primer pairs derived from these sequences amplified bands of the same size using either male or female sturgeon DNA template (SM, SF, Figure 7). The SCAR primer bands had the same restriction digest pattern as the bands amplified by the RAPD primers (data not shown). This suggests that the RAPD primers were amplifying rare autosomal alleles that were by chance found in 2 or 3 "unrelated" animals of only one sex in our sample group.

In a survey of 20 RAPD primers to look at parental polymorphisms in the "full-sib" and "hybrid" groups, we identified an average of one band that was polymorphic between the parents of the white sturgeon "full-sibs"and an average of six bands that differed between the parents of the "hybrid" group (Figure 8). Both the "full-sib" and the "hybrid" group screens identified only nine RAPD primers which produced reproducible bulk polymorphisms, and

none of these polymorphic bands proved to be sex-specific as the band was seen in at least one of the ten individual animals from either sex (Figure 9, and Table 2).

Discussion

None of the methods utilized in this study enabled us to successfully identify unique sex-associated DNA sequences in the white sturgeon. The first method we described was a subtractive hybridization technique using male DNA as the tester and an excess of biotinylated female DNA as the driver. Analysis of recombinant clones derived by this method failed to identify a DNA fragment that hybridized specifically to male DNA. This experimental design was based on the unproven assumption that male sturgeon carry sex-specific DNA sequences. We now know after sexing the gynogenetic progeny described in Chapter 2 that a female heterogametic genetic sex determination system is in operation in white sturgeon. The fragments that we did clone in this experiment turned out to be mainly repetitive sequences not directly associated with the sex of the animals. Subtractive hybridization has been found to be inefficient for the comparison of genomic DNAs from higher eukaryotes (Lisitsyn 1995). The high genomic complexity means that the concentration of each unique sequence in the tester-driver DNA mixture is low and the formation of hybrid DNA fragments does not go to completion. Even if the subtractive hybridization is repeated, as was done in this experiment, the maximum ratio of the copy number of a target sequence after subtractive hybridization to its initial copy number in the genomic DNA only reaches 100-fold (Wieland et al. 1990). This limits the success of this method to the purification of relatively long or relatively abundant sequences (representing 0.1-1% of the genome) (Lisitsyn 1995).

Fish sex chromosomes are less morphologically distinct than are those of mammals, and an argument for the recent divergence of fish sex chromosomes is that male and female heterogamety can be found in the same order, the same genus, and even in the same species (Kallman 1984). Subtractive hybridization techniques have been used to successfully isolate Y or W-chromosomal DNA sequences in only two species of fish, *Leporinus elongatus* (Nakayama et al. 1994), and chinook salmon (*Oncorhynchus tshawytscha*) (Devlin et al. 1991). In both of these experiments the sex-specific DNA sequences isolated were repetitive in nature. Subtractive hybridization was used unsuccessfully to isolate sex-specific sequences from rainbow trout (*Oncorhynchus mykiss*) (Nakayama et al. 1994). Aside from the possibility that male white sturgeon may lack sex-specific DNA, one of the reasons why our subtractive hybridization experiment may have been unsuccessful at isolating sex-specific sequences from white sturgeon is because there may be only a limited amount of repetitive DNA sequence accumulation or sequence divergence between the monomorphic white sturgeon sex chromosomes. These subtle polymorphisms may be beyond the detection limits of this methodology. Alternatively, sex-specific restriction fragments cut by *Sau*3AI may have been too large to successfully subclone into the pBluescript vector. Even if we had used female as the tester and male as the driver as would be intimated by the results of Chapter 2, the technical limitations of subtractive hybridization may have prevented the successful isolation of female-specific DNA sequences.

RDA was developed to overcome some of the limitations of subtractive hybridization techniques. It raises the efficiency of subtractive hybridization by combining it with two additional elements: representation and kinetic enrichment (Lisitsyn 1995). Specifically RDA

is designed to isolate restriction fragments within the size range of PCR amplification from the tester but not the driver. This technique can be applied to any trait whose presence implies homozygosity for a particular allele. To generate markers linked to a trait using RDA it is important to carefully select the source of the DNA used to make up the tester and driver. The driver needs to contain all of the alleles present in the tester DNA except in the region surrounded by the target gene (Lisitsyn et al. 1994). Sex-specific pools of DNA have been used in conjunction with RDA to isolate sex-specific repetitive DNA sequences from a dioecious trematode (*Schistosoma mansoni*) (Drew and Brindley 1995), cattle (Wigger et al. 1996), and mice (Navin et al. 1996). In our experiments we pooled DNA from 10 full-sibs of each sex to make up the driver and tester DNA. The probability of an allele unassociated with sex being present in the tester but not in the driver was at the most $(½)^{10}(1-½^{10}) < 0.001$. Unlike the subtractive hybridization experiment we alternated the sex of the driver in each RDA so as not to presuppose which was the homogametic sex.

The absence of third round difference products in the four RDAs may be explained by technical or theoretical considerations. Sex-specific DNA sequences may not have been included in the tester amplicons generated by *Bgl*II and *Bam*HI restriction endonucleases. For mammalian DNA, 2-15 % of the total genomic sequences are represented in amplicons made with different restriction endonucleases, generating driver and tester DNA samples with a complexity of 40-300 Mb (Lisitsyn 1995). Sex-specific repetitive sequences surrounding a sex-determining locus could conceivably lack both *Bgl*II and *Bam* HI recognition sites. Finally, there may have been a technical problem with the rather complex RDA methodology. The total absence of any visible third round difference products, and the fact that the small

(100-200 bp) fragments generated by additional cycles of PCR amplification were repetitive, sex-independent sturgeon DNA sequences, suggests that contamination was not a problem in our experiments. It would have been useful to include a positive control by intentionally adding some foreign DNA with a known *Bam*HI or *Bgl*II 150-1500 bp restriction fragment to the tester at the beginning of the experiment.

BSA involves screening for differences between two pooled DNA samples derived from a segregating population. BSA assumes that markers adjacent to the targeted gene will be in linkage disequilibrium. The length of the region that is in linkage disequilibrium with the targeted gene will depend upon the genetic constitution of the segregating population. Bulking individuals from multiple families will increase the probability that linkage equilibrium will obstruct BSA (Michelmore et al. 1991). In this study we performed three separate BSA experiments on genetically-distinct groups of fish and the expected outcome of each experiment differed based on the genetic structure of the population. In the first BSA with the "unrelated" group we expected to identify a sex-specific marker so closely linked to the sex-determining locus that it would be in linkage disequilibrium with the target gene in unrelated individuals. When this analysis was unsuccessful we repeated the procedure using groups of related animals to increase the overall level of linkage disequilibrium and therefore the length of the region in linkage disequilibrium with putative sex-determining genes. We expected that a sex-associated marker identified within the "full-sib" group may have proven to be sex-specific only for the "full-sib" group and their parents (i.e. not tightly linked to the sex-determining locus such that it would be in linkage equilibrium in the general population), but it would have provided a starting point to find markers more closely linked to the sex-

determining locus. Finally, when the "full-sib" analysis was unsuccessful we repeated the procedure with the "hybrid" group. The success of BSA when using related animals depends upon the genetic divergence between the parents in the region surrounding the target locus (Michelmore et al. 1991). The high level of genetic divergence between the parents of the hybrid cross, as evidenced by the increased number of polymorphic RAPD bands per primer (Figure 8), increased the number of loci screened and the probability that a locus in a region in linkage disequilibrium with sex-determining genes would be identified.

The fact that no specific sex-associated marker was identified in the three BSA experiments suggests that sex-specific DNA sequences may not exist in white sturgeon. Even if white sturgeon has a ZO:ZZ sex determination system, which seems unlikely given the sex ratios observed in Chapter 2, we would have expected to see sex-specific polymorphisms in the "hybrid" BSA as only males would have inherited the white sturgeon Z chromosome from the dam. In bulked segregant analyses with other species where the DNA bulks differed by the trait of sex, a range of only 20 to as many as 700 different RAPD primers were required to identify at least one sex-associated DNA marker (Mulcahy et al. 1992, Griffiths and Tiwari 1993, Hormaza et al. 1994, Cushwa et al. 1996). We used 1200 RAPD primers in each of our BSA experiments. It is possible to determine the theoretical number of primers that would have been expected to generate a sex-associated band by determining the value of the variables in the following equation: *size of window screened (cM)/genome size (cM) x number of RAPD primers screened x average number of polymorphic bands per primer between the parents of the progeny group.*

RAPD markers can conservatively identify a 15 cM window on either side of a targeted locus in BSA (Michelmore et al. 1991). No linkage map has ever been developed for a sturgeon species, and consequently no estimates are available as to the genome size. Extrapolating from the data available on the zebrafish (*Danio rerio*) which has a 1C value of 1.8 pg (Hinegardner and Rosen 1972) and a minimum genetic map distance of 2720 cM (Postlethwait et al. 1994), one map distance estimate for white sturgeon could be 2720 x (4.73/1.8) = 7148 cM (1C = 4.73 pg, Blacklidge and Bidwell 1993). This estimate may be erroneous due to the higher chromosome number of white sturgeon $2n = 271$ (Chapter 5) as compared to zebrafish $2n = 50$ (Sola et al. 1981), and the unknown level of recombination in sturgeon. The overall length of a genome in map units reflects more the number of chromosome arms per genome than the genome size in base pairs. Using chromosome number as a criteria for estimating the genetic map distance of white sturgeon, the length estimate may be increased to 271/50 x 2720 ≈ 14750 cM. Using this larger estimate for the size of the white sturgeon genome and the fact that each of the 1200 primers generated an average of one polymorphic band between the parents of the "full-sibs", the number of primers that would have been expected to generate a sex-specific polymorphism in the "full-sib" screen was 30/14750 x 1200 x 1 = 2.4. Approximately fifteen primers (30/14750 x 1200 x 6) would have been expected to generate a sex-specific polymorphic band in the "hybrid" cross.

Most of the primers which generated reproducible polymorphic bands between the bulks in all three experiments subsequently proved to be unassociated with sex by the analysis of the individuals that made up the bulks (Table 2). This can be explained in the case of the "unrelated" group by the fact that the primers were amplifying rare alleles that were by chance

found in unrelated animals of only one sex in our sample group (Figure 7). In the "full-sib" and "hybrid" groups sometimes over half of the animals that made up the bulk not displaying a given polymorphic band, displayed that band upon individual analysis (Table 2). Michelmore (1991) found that amplification could often be detected only when at least .2 - .4 of the DNA bulk mixture contained the amplified allele. He noted that RAPD analysis was unexpectedly insensitive at detecting rare alleles of certain loci. The fact that we had no false positives in these experiment (i.e. a band that was segregating faithfully according to sex in the 20 fish that made up the bulks, and was not sex-specific when tested on a larger group of fish with the same genetic background) suggests that pooling the DNA from ten individuals of each sex was sufficient to eliminate interference from unlinked loci. In fact we might even have predicted one false positive in the "full-sib" BSA ($(½)^{10}(1-½^{10})$ x 1200 x 1 = 1.2), and as many as seven false positives in the "hybrid" BSA ($(½)^{10}(1-½^{10})$ x 1200 x 6 = 7).

Conclusions

In conclusion, we were unable to identify genetic markers associated with sex in white sturgeon using subtractive hybridization, RDA and BSA methodologies. In the absence of unperceived technical difficulties, these results suggest that either: 1) there are no sex-specific sequences in sturgeon implying that they have some type of a multilocus sex determination system; or 2) the sex-specific DNA that is present in sturgeon may be comprised of unusual sequences which were not complementary to the 1200 random decamer primers or the recognition sequence of the restriction endonucleases used in this study; or 3) sex-specific sequences represent a very small portion (1-5 kb) of the genome.

Acknowledgments

The authors thank Stolt Sea Farm, California, LLC for assisting us with blood collection from their sturgeon broodstock, Fred Binkowski, University of Wisconsin, for providing lake sturgeon milt, and Joel P. Van Eenennaam for technical assistance with hybrid and full-sib production, gonad collection, and histological analyses. This research was funded by a grant from the National Sea Grant College Program, National Oceanic and Atmospheric Administration, U.S. Department of Commerce, under grant number NA36RG0537, project R/A-99 through the California Sea Grant College System, and in part by the California State Resources Agency. The views expressed herein are those of the authors and do not necessarily reflect the views of NOAA or any of its sub-agencies. The U.S. government is authorized to reproduce and distribute for governmental purposes. A. L. Van Eenennaam was supported by a Sea Grant traineeship.

TABLE 1. Outcome of polymerase chain reactions using 1200 randomly amplified polymorphic DNA primers (RAPD-PCR) in a bulked segregant analysis comparing two bulks (10 individuals/bulk) made up of male and female sturgeon DNA.

RAPD-PCR outcome	Origin of the DNA that made up the bulks		
	Unrelated[1]	Full-sib[2]	Hybrid[3]
No difference between the DNA from either sex	968	993	960
RAPD band present in only one sex	30	39	41
DNA from one of the sexes did not amplify	34	29	37
DNA from one of the sexes only partially amplified	59	38	49
No reaction products were detected	71	47	50
Reaction products were a smear	38	54	63

[1] Unrelated - Bulks consisted of DNA from 10 unrelated adult white sturgeon of each sex.
[2] Full-sib - Bulks consisted of DNA from 10 white sturgeon full-siblings of each sex.
[3] Hybrid - Bulks consisted of DNA from 10 hybrid (lake sturgeon (*Acipenser fulvescens)* male x white sturgeon female) full-siblings of each sex.

TABLE 2. Number of individual animals whose DNA generated the polymorphic RAPD-PCR band which had previously been identified in only one sex in a bulked segregant analysis comparing bulks (10 individuals/bulk) made up of male and female sturgeon DNA.

GROUP	RAPD PRIMER	MALE	FEMALE
Unrelated[1]			
	OP A8	1/8	2/8
	OP C2	5/10	1/10
	OP C7	0/10	3/10
	OP C11	1/10	4/10
	OP E6	1/8	2/8
	OP F9	3/8	4/8
	OP G2	1/8	3/8
	OP G13	0/8	1/8
	OP I14	band not apparent	band not apparent
	OP J8	1/8	3/8
	OP L9	0/8	1/8
	OP O16	3/8	2/8
	OP P5	5/5	4/5
	UBC 169	1/10	3/10
	UBC 298	0/10	2/10
	UBC 580	0/10	1/10
	UBC 589	1/10	2/10
	UBC 661	2/10	0/10

TABLE 2 *(continued)*. Number of individual animals whose DNA generated the polymorphic RAPD-PC band which had previously been identified in only one sex in a bulked segregant analysis comparing two bulks (10 individuals/bulk) made up of male and female sturgeon DNA.

GROUP	RAPD PRIMER	MALE	FEMALE
Full-sib[2]			
	OP C7	6/10	7/10
	OP I10	8/10	10/10
	OP J18	6/10	7/10
	OP M4	2/4	4/4
	OP W15	3/3	1/4
	UBC 271	1/3	1/4
	UBC 322	3/4	4/4
	UBC 516	3/4	4/4
	UBC 564	2/4	1/4
Hybrid[3]			
	OP C20	9/10	4/10
	OP P14	6/10	3/10
	UBC 100	3/10	6/10
	UBC 123	4/10	6/10
	UBC 167	6/10	1/10
	UBC 169	3/10	8/10
	UBC 326	6/10	2/10
	UBC 421	6/10	6/10
	UBC 689	3/10	9/10

[1] Unrelated - Bulks consisted of DNA from 10 unrelated adult white sturgeon of each sex.
[2] Full-sib - Bulks consisted of DNA from 10 white sturgeon full-siblings of each sex.
[3] Hybrid - Bulks consisted of DNA from 10 hybrid (lake sturgeon (*Acipenser fulvescens)* male x white sturgeon female) full-siblings of each sex.

FIGURE 1. External appearance of 22 month old lake (*Acipenser fulvescens*) x white (*A. transmontanus*) sturgeon hybrid (top), and purebred white sturgeon (bottom).

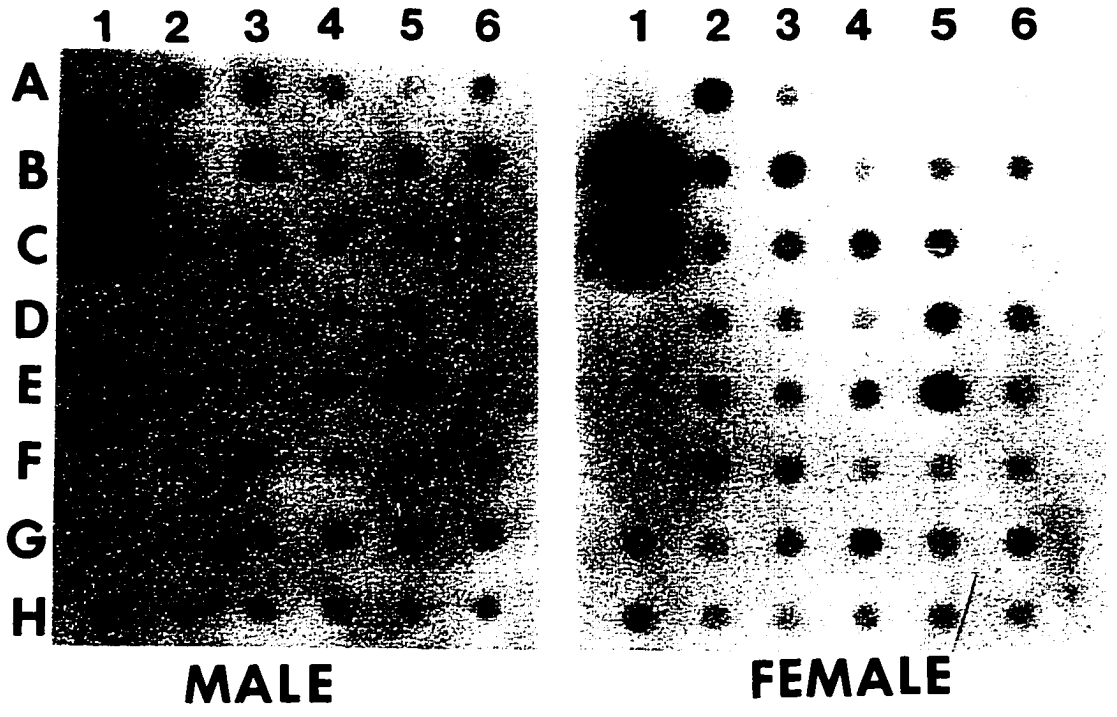


FIGURE 2. Hybridization of ^{32}P-labeled MALE and FEMALE genomic DNA to a Southern dot-blot of DNA (≈ 100 ng/well unless otherwise indicated) from white sturgeon, negative controls of bovine and mouse PCR products, pBluescript, and 40 recombinant pBluescript clones (A1-B20) containing sturgeon DNA inserts. Adjacent table shows the arrangement of the dot blots shown above.

	1	2	3	4	5	6
A	Buffer	A1	A2	A3	A4	A5
B	♂ sturgeon genomic DNA	A6	A7	A8	A9	A10
C	♀ sturgeon genomic DNA	A11	A12	A13	A14	A15
D	Bovine β-lactoglobulin	A16	A17	A18	A19	A20
E	Bovine κ-casein	B1	B2	B3	B4	B5
F	Murine ZFY	B6	B7	B8	B9	B10
G	100 ng pBluescript	B11	B12	B13	B14	B15
H	200 ng pBluescript	B16	B17	B18	B19	B20

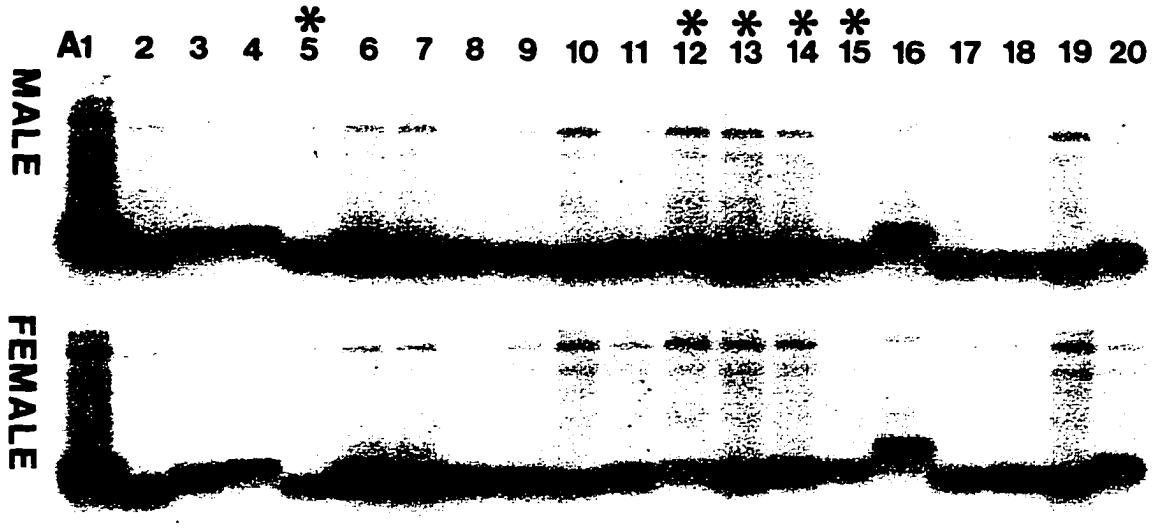


FIGURE 3. Hybridization of ^{32}P-labeled male and female genomic DNA to a Southern blot of white sturgeon DNA inserts which were PCR amplified from 20 recombinant pBluescript plasmids (A1- 20) and run on a 2% agarose gel. Asterisks indicate clones where the signal derived from hybridization with the male probe is darker than that derived from the female probe.

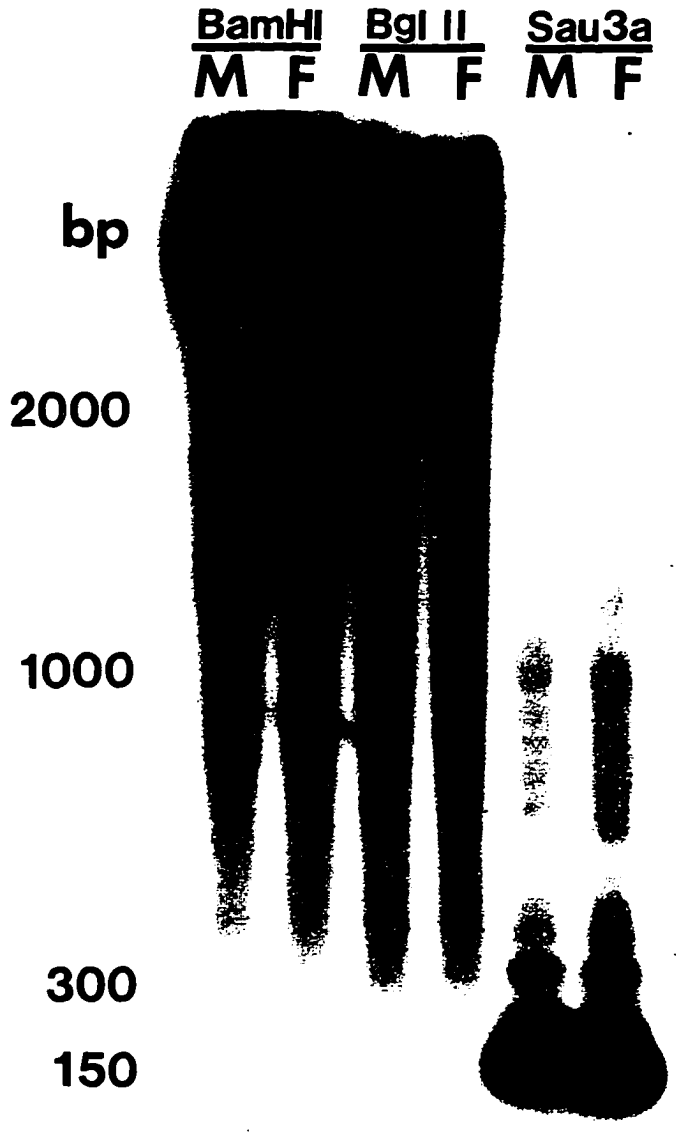


FIGURE 4. Southern blots of male (M) and female (F) white sturgeon DNA digested with three restriction enzymes (*Bam*HI, *Bgl*II, *Sau*3AI) and run on a 0.7% agarose gel. The probe was a ^{32}P-labeled white sturgeon DNA insert which was PCR amplified from a recombinant pBluescript plasmid (A13).

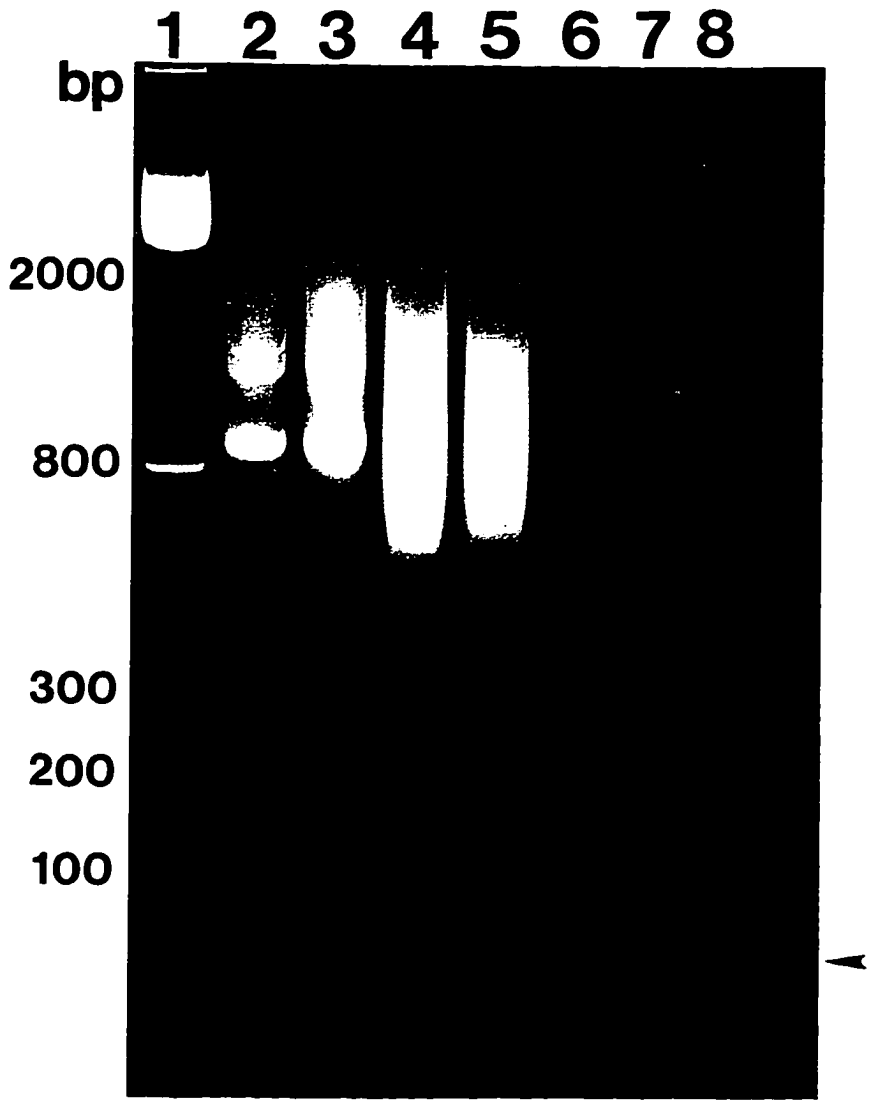


FIGURE 5. Agarose gel electrophoresis (2%) of representational difference analysis products using DNA from 10 white sturgeon full-sibs of each sex. Lane 1 = 100 bp ladder, lane 2 = *Bam*HI male amplicon, lane 3 = *Bam*HI female amplicon, lane 4 = *Bgl*II male amplicon, lane 5 = *Bgl*II female amplicon, and lanes 6-8 are the first, second and third round difference products, respectively, from *Bgl*II representations with female DNA as the driver and male DNA as the tester. Arrowhead indicates the faint smear of PCR primers evident in lane 8.

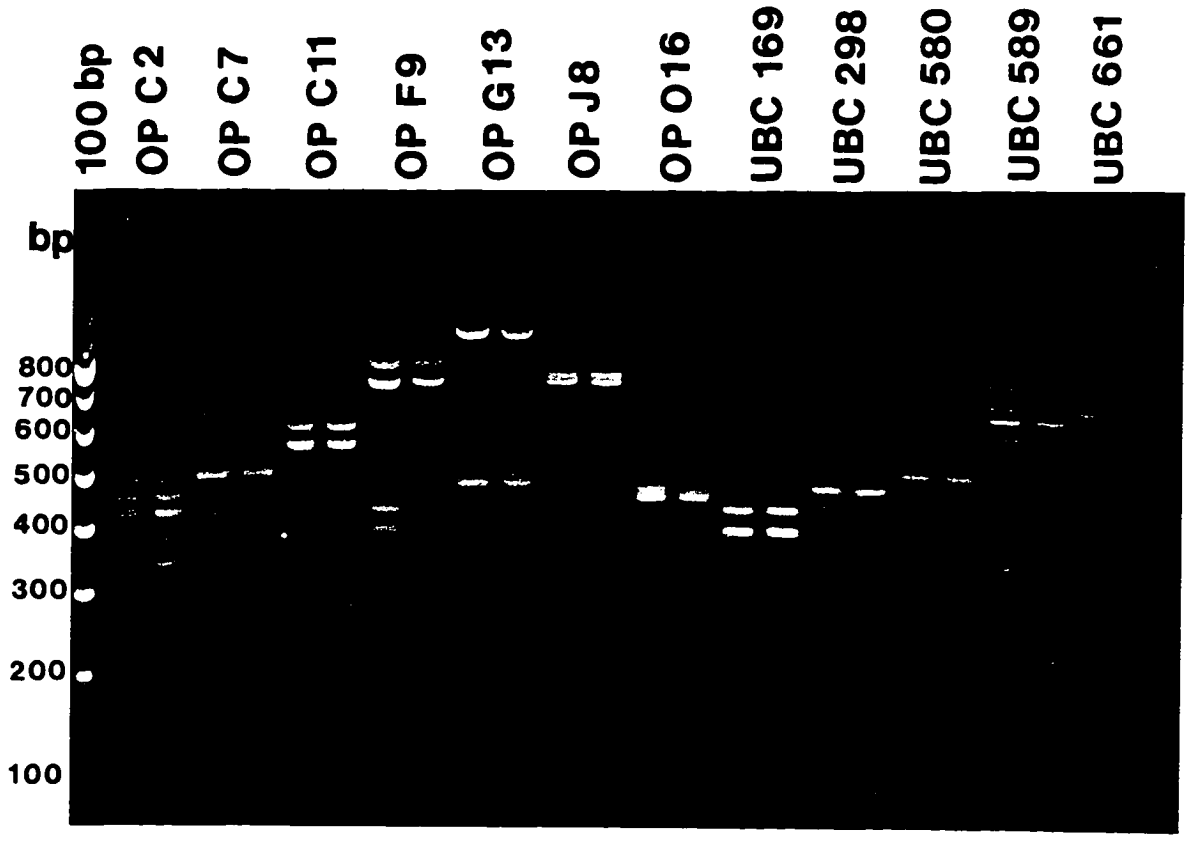


FIGURE 6. Agarose gel electrophoresis (2%) of RAPD PCR amplification products. Lane 1 = 100-bp ladder. Example of 12 RAPD primers (OP C2, OP C7, OP C11, OP F9, OP G13, OP J8, OP O16, UBC 169, UBC 298, UBC 580, UBC 589, UBC 661) used to amplify combined DNA from 10 unrelated white sturgeon males (left lane), and 10 unrelated females (right lane), that showed sex-associated DNA bulk polymorphisms.

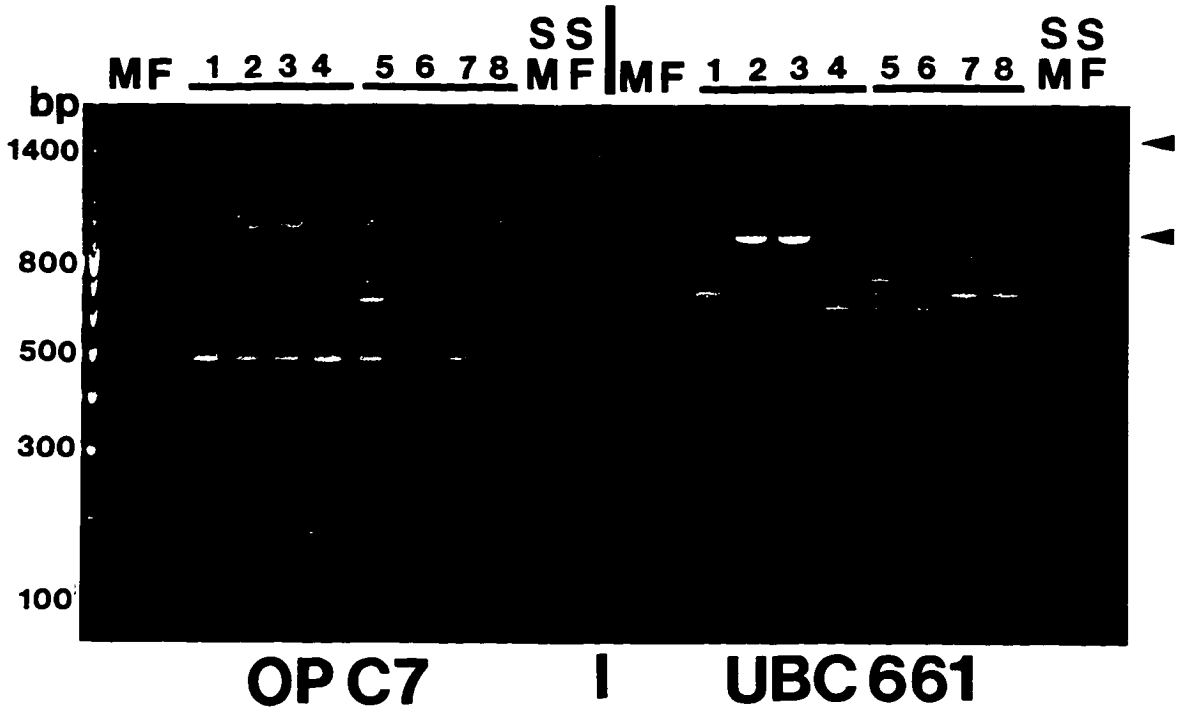


FIGURE 7. Agarose gel electrophoresis (2%) of RAPD PCR amplification products using primers OP C7 and UBC 661. Lane 1 = 100-bp ladder. M, combined DNA from 10 unrelated white sturgeon males; F, combined DNA from 10 unrelated white sturgeon females; 1-4, DNA from 4 of the 10 individuals in the combined male DNA bulk; 5-8, DNA from 4 of the 10 individuals in the combined female DNA bulk; SM and SF, male and female amplification products (arrowheads) respectively, using SCAR primers designed from the sequence of the polymorphic RAPD bands.

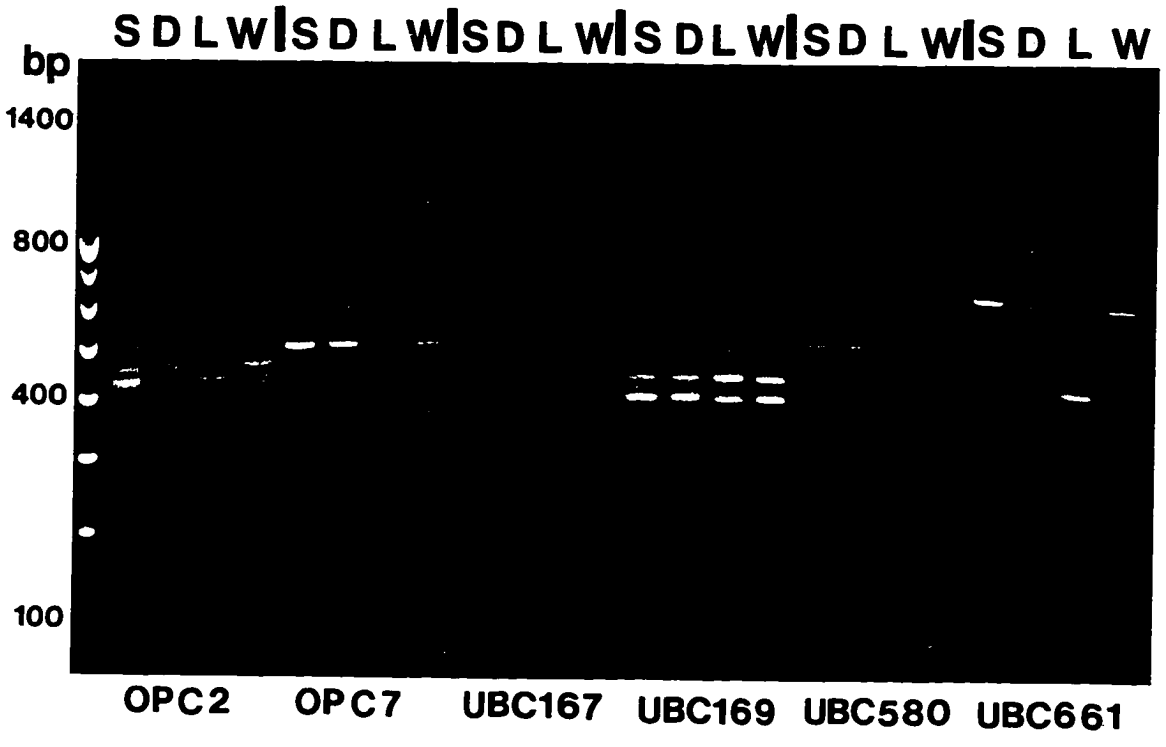


FIGURE 8. Agarose gel electrophoresis (2%) of RAPD PCR amplification products. Lane 1 = 100-bp ladder. Example of 6 RAPD primers (OP C2, OP C7, UBC 167, UBC 169, UBC 580, UBC 661) used to amplify DNA from the white sturgeon sire of the "full-sib" progeny group (S), the white sturgeon dam of the "full-sib" progeny group (D), the lake sturgeon sire of the "hybrid" progeny group (L), and the white sturgeon dam of the "hybrid" progeny group (W).

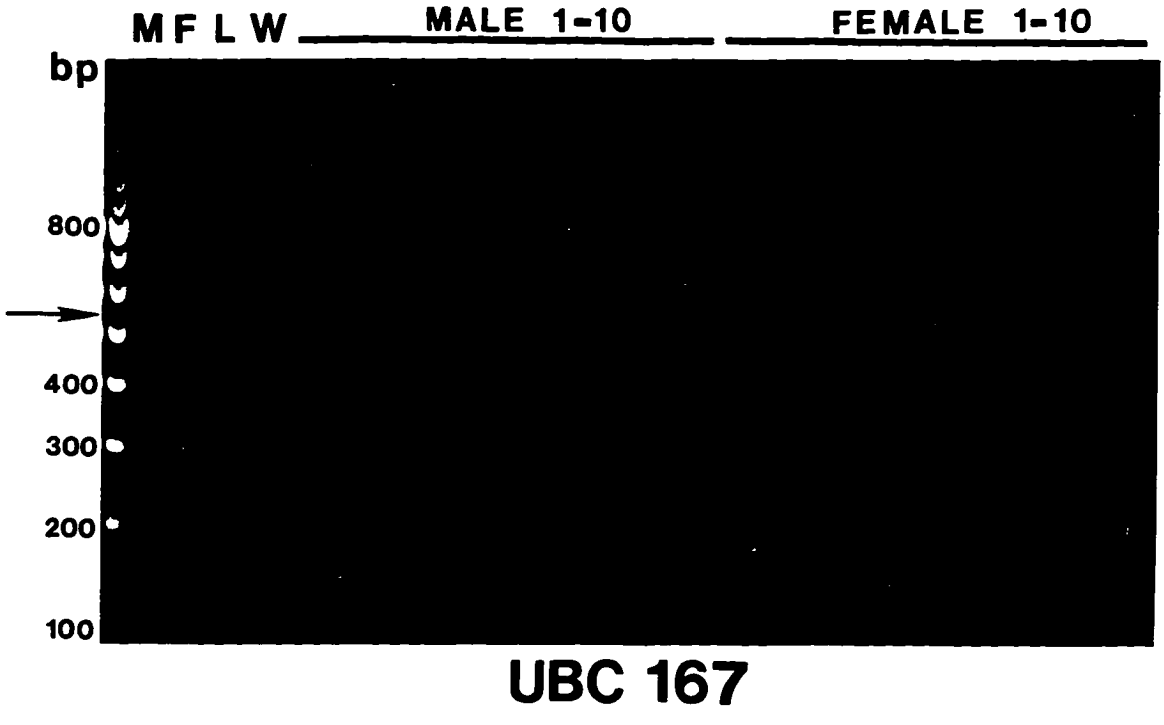


FIGURE 9. Agarose gel electrophoresis (2%) of RAPD PCR amplification products using RAPD primer UBC 167. Lane 1 = 100-bp ladder. M, DNA combined from 10 hybrid male progeny; F, DNA combined from 10 hybrid female progeny; L, DNA from the lake sturgeon sire; W, DNA from the white sturgeon dam; MALE 1-10, DNA from the 10 individuals in the combined male DNA bulk; FEMALE 1-10, DNA from the 10 individuals in the combined female DNA bulk; arrow indicates a polymorphic 550 bp band that was seen in M, W, six individual males and one female.

CHAPTER 4. SYNAPTONEMAL COMPLEX ANALYSIS IN SPERMATOCYTES OF WHITE STURGEON (*Acipenser transmontanus* RICHARDSON).

Alison L. Van Eenennaam, James D. Murray, and Juan F. Medrano

Submitted in part to Genome

Introduction

White sturgeon (Class Osteichthyes) belong to the phylogenetically unique ancestral group of ray-finned fish, Order Acipenseriformes. Karyotypes of extant Acipenseriformes can be divided into two groups, both of which are characterized by a very large number of chromosomes, about half of which are microchromosomes. The first group has a chromosome number of approximately 120 and a mean genome size (1C) of 1.6-2.5 pg, and the second group has a chromosome number of 240-250 and a genome size of 4.0-4.8 pg (Birstein et al. 1993, Blacklidge and Bidwell 1993). It is considered that the contemporary Acipenseriformes have a polyploid origin with the $2n \approx 120$ group being of a tetraploid (4N) origin, and the $2n \approx 240$-250 group being of an octoploid (8N) origin (Ohno et al. 1969, Dingerkus and Howell 1976, Birstein and Vasiliev 1987, Birstein et al. 1993, Blacklidge and Bidwell 1993); although on the basis of nucleolus organizer region (NOR) location Fontana (1994) contends that species with 120 chromosomes should in fact be considered diploid, and that species with 240-250 chromosomes are of a tetraploid origin.

Synaptonemal complex (SC) analyses have been used in cytogenetic studies of plants and animals to investigate the process of meiotic chromosome synapsis because of the high-resolution permitted by the electron microscope. Two techniques for the preparation of

meiocytes, three-dimensional reconstruction of serial sections (reviewed in Gillies 1975) and two-dimensional surface-tension spreading (Counce and Meyer 1973), offer alternative approaches for SC analyses with each providing different analytical strengths. The major advantage of the surface-tension spreading technique is the comparative ease of rapidly producing and analyzing large numbers of nuclei (Moses 1980, Greenbaum et al. 1986).

Heteromorphism for sex chromosomes is displayed at the level of SCs as heteromorphic synaptic configurations (Moses 1980). In many vertebrate species, especially those with strongly dimorphic sex chromosomes, the SCs of heteromorphic sex bivalents are readily distinguished from those of the autosomes (Solari 1974, Moses 1977, Solari 1977, Hale and Greenbaum 1986, Dollin et al. 1989, Hedin et al. 1990, Reed et al. 1990, Amaral and Jorge 1994). Sex chromosomes typically pair later than the autosomes and may not initiate synapsis until all of the autosomal bivalents have become fully paired (Moses 1980). In those species in which the sex chromosomes do not fully synapse, the unpaired regions typically exhibit heteropycnosis with conspicuous condensation and thickening. The actual sex chromosome synaptic configuration seen in the heterogametic sex will depend upon the gonosomal system operating in the species, the degree of homology between the heterologous sex chromosomes, and the stage of meiotic prophase being examined.

Fish represent an extremely heterogeneous group with regard to their mechanism of sex determination. Social, environmental and multiple genetic factors can all act to determine or change the sex of adult fish in certain species. A variety of genetic sex determination systems have been reported in fish including XY-XX; ZZ-ZW; XX-XO; ZO-ZZ and multiple gonosomal systems (reviewed in Solari 1994). Conventional karyological examination of most

species reveals that the sex chromosomes are morphologically undifferentiated suggesting that the majority of fish exhibit a primitive condition of sex-chromosome evolution (Bull 1983, Devlin et al. 1991). Recently, synaptonemal complex surface-spreading techniques have been used to address questions related to fish cytogenetics and have permitted the direct identification of heteromorphic sex chromosomes in three species of fish (Yajuan and Qixing 1991, Foresti et al. 1993, Oliveira et al. 1995a).

Synaptonemal complex analyses have not been previously reported for any sturgeon species. The one published mitotic karyotype of white sturgeon ($2n = 248 \pm 8$) is divided into 52 meta- and submetacentric pairs, and 74 pairs of acrocentrics and microchromosomes (Fontana 1994). The numerous small microchromosomes make it difficult to determine the exact chromosome number. Conventional mitotic studies have not revealed sex chromosomes in sturgeon species (Fontana and Colombo 1974, Holčík 1986), and it is not known what type of sex-chromosome system exists in white sturgeon. In this study, surface-spread SC preparations of testes material from white sturgeon were examined to: (i) develop a meiotic karyotype for this species including the first detailed morphology of the microchromosomes; (ii) examine the meiotic pairing behavior of this ancient polyploid-derived species; and (iii) determine if the pairing behavior and/or morphology of the synaptonemal complexes allows for the direct identification of sex chromosomes.

Materials and Methods

The SC analysis of spermatocytes from white sturgeon was carried out using modifications of the surface spreading technique (Wise and Nail. 1987, Oliveira et al. 1995a).

Testes from 52 domestically-reared males (3-4 years of age, 5-7 kg) raised at high stocking densities in indoor tanks with 20°C semi-recirculated water were obtained from a commercial white sturgeon farm, "Stolt Sea Farm, California, LLC". A cross section of a testis from each male was fixed in 10% buffered formalin for histology and light microscopy to determine the proportion of cysts with different types of germ cell (spermatogonia, spermatocytes, spermatids, and spermatozoa) (Chapman et al. 1996). The remainder of each testis were placed in ice cold Hank's saline solution (HSS) and minced to form a cell suspension. This suspension was allowed to settle and the pink layer just below the supernatant was pipetted off in a small quantity in HSS. One drop of the cell suspension, two drops of unbuffered .2M sucrose, and two drops of .2% "Lipsol" alkaline laboratory cleaning detergent, pH 8.5 (LIP, West Yorkshire, England) were mixed gently and allowed to settle for 3 min on a microscope slide coated with a thin layer of plastic. Ten drops of fixative (4% w/v paraformaldehyde, pH 8.5) were then added and after 15 min excess fluid was removed from the slide by tilting it on top of a paper towel. Slides were gently rinsed in a 0.4% (v/v) Kodak Photo-Flo 200 solution and left to air dry in a vertical position overnight in a dust-free box. Slides were then washed in distilled water, silver stained with 50% silver nitrate in a moist chamber at 60°C (Kodama et al. 1980), rinsed, and air dried.

Prior to use microscope slides were coated with a thin layer of plastic. Slides were first dipped in 1N HCl for 10 min, rinsed in distilled water, dipped in absolute alcohol for 10 min, and polished dry with a lint-free cloth. Three drops of Lipsol were pipetted onto the upper surface of each slide, smeared over it, and gently polished off to leave a fine layer of detergent which promotes the floating off of the plastic film. After air drying the slide was

dipped for 3 seconds in a solution of 0.75% (w/v) Falcon-brand Optilux tissue culture petri dish dissolved in reagent-grade chloroform. The dipped slide was held in the vapor phase above the plastic solution until the solution stopped dripping off the slide. After drying, the edges of the plastic film on the top surface of the slide were sealed down with a narrow strip of nail polish to prevent the film from lifting during the fixing and staining steps.

Slides were examined with a light microscope and areas with a high concentration of well-spread SC structures were marked on the underside with a black marker. A diamond slide marker was then used to cut out the appropriate plastic areas on the upper surface of the slide. These plastic fragments were floated off the slide in warm tap water and picked up with 50-mesh copper electron microscope grids. The position of any well-spread, discrete spermatocytes on each grid was mapped using light microscopy, by very carefully placing the grid on a clean microscope slide. Finally the grids were examined at 75kV with a Hitachi H-600 electron microscope. The multiple-micrographs (2000-4000X) from a subset of 12 well-spread nuclei with few if any stretched or overlapping SCs were scanned and reconstructed into a collage using Adobe Photoshop software (Adobe Systems Inc, Mountain View, CA). The SCs from each nuclei were counted, and arranged according to length to produce a meiotic karyotype and to allow for the measurement of the total SC length.

Results

Slides were prepared from the testes of 52 males and examined under a light microscope. The majority of the slides contained very few or no SC spreads, suggesting that most of the gonads had few cells at the primary spermatocyte stage. On slides from some

males the overwhelming majority of cells were spermatozoa, while on slides from other males there was no evidence of either spermatozoa or spermatocytes. There was no obvious correlation between the gross appearance (white, cream, pink) and texture (soft, hard) of the testis tissue and the proportion of primary spermatocytes that were evident on slide preparations. Good SC spreads were obtained on slides prepared from the gonadal tissue of six males and further SC analyses concentrated on electron micrographs of spermatocytes from these six fish. Histological analyses of testes from these males revealed that at least 30% of the gonadal cysts contained spermatocytes (Table 1). A representative section of an ideal testes for white sturgeon SC analysis (Fish # 27 - 85% spermatocytes) is shown in Figure 1.

Electron micrographs of 6 zygotene and 41 pachytene nuclei were obtained. Due to the large number of chromosomes in this species it was exceedingly difficult to locate complete, well-spread nuclei for analysis. The entire SC complement was present in 26 of the 47 nuclei examined, with the remainder having only partial spreads available for analysis due to overlapping nuclei, spermatozoa, cellular debris, or grid bars. Nuclei were assigned to meiotic substage based on the degree of synapsis and the morphology of the lateral elements (LEs) of the SCs, and nucleoli (Greenbaum et al. 1986, Dollin et al. 1989, Jiménez et al. 1990). The overt characteristics of SCs in spermatocytes from white sturgeon were similar to those described for other fish (Yajuan and Qixing 1991, Foresti et al. 1993, Oliveira et al. 1995a, Rodionova et al. 1996). Chromosomal axes represented by the LEs of the SCs were well resolved, and centromeric regions appeared to overlap and span the LEs of paired bivalents in most of the nuclei. Attachment plaques were rarely observed and there was no

obvious polarization of the LEs. The SC data provided excellent resolution of the microchromosomes.

A classical leptotene with complete but still unsynapsed LEs was not found in our SC preparations of white sturgeon. Zygotene nuclei (Figure 2) were scarce and characterized by incomplete synapsis, unpaired univalents, interlocks, and diffuse lightly-stained nucleoli. The progression of synapsis was different among the chromosomes. The microchromosomes often showed complete SCs before the macrochromosomal LEs had fully formed (Figure 2a). By late zygotene this asynchronism resulted in the presence of a large number of short, complete SCs alongside completely formed but unsynapsed interstitial loops of macrochromosomal LEs (Figure 2b,c). Macrochromosomal SCs usually exhibited a bidirectional synapsis initiated from both sets of telomeres (Figure 2c), often before the interstitial LEs had completed formation. The interstitial region of the macrochromosomes was frequently the last to complete synapsis. However, unsynapsed terminal segments were sometimes seen in zygotene nuclei suggesting that synapsis was not invariably initiated at the telomeres (Figure 2d).

The formation of SCs was complete at pachytene. Unpaired univalents became increasingly heteropycnotic as pachytene progressed. Most pachytene nuclei (Figure 3a) had prominent darkly staining nucleoli which were usually detached from the SCs so it was not possible to identify the chromosomes bearing NORs. Generally we found no sign of alignment or pairing involving more than two chromosomes in zygotene or pachytene nuclei, however, numerous pairing irregularities were observed during the course of this analysis (Figure 3b-d, Figure 4). One common pachytene SC configuration that we called the gap configuration (G, Figure 3b, 4a-d) was seen in 13 of the 47 nuclei from five of the males. This structure was one

of the longer SCs in the complement and it often appeared to be bicentric. Possible examples of the "G" SC configuration in zygotene (G?, Figure 2b-c) suggest that one of the short LEs may pair with the long bicentric LE before the other. Two males had a large univalent (A, Figure 4e) that was evident in all of the nuclei from those particular individuals suggesting that they were aneuploid. The univalent illustrated in Figure 4e was the longest LE in the complement. Only one obvious trivalent (T, Figure 4i) was observed in a single nuclei from a male that exhibited normal pairing in all other nuclei, suggesting that an isolated chromosomal rearrangement had taken place.

Pachytene nuclei from all males had varying numbers of very small often heteropycnotic univalents (H, Figure 3c-d, Figure 4h), and/or apparently self-paired U-shaped foldback elements with no obvious centromeric region (U, Figure 3d, Figure 4f-h). The smallest SC elements were often heteropycnotic univalents which did not appear to self-pair although they were often seen in a relaxed U-shaped configuration. What appeared to be attachment plaques were sometimes evident on the telomeres of these tiny heteropycnotic microchromosomes (Figure 3d). In many pachytene SCs it appeared that the LEs were not evenly spaced and that they often converged towards each other in the absence of obvious twisting (Figure 4h).

Twelve well-spread nuclei from four of the males were used to ascertain the SC number and length (Table 2). To obtain a SC count we tallied every separate SC component including univalents and foldback elements. There was both between and within animal variation in the pachytene SC count (139 ± 3.4) for this species, due in part to the various pairing irregularities. The length of the SC complement for these 12 nuclei was 481.8 ± 55.7

μm. The length of the foldback and U-shaped elements was measured from the top of the loop to the telomeres, while the length of straight unpaired univalents was measured from telomere to telomere. The SC length and mean packing density (pg DNA/μm SC) are compared to values obtained for other bony fish (Osteichthyes) and representative species from other vertebrate classes in Table 3.

A representative meiotic karyotype for this species aligned in order of decreasing SC length is presented in Figure 5. The largest SCs are all metacentric or submetacentric with the first acrocentric SC occurring at SC # 20 in this karyotype. There is no obvious dividing line that can be used to separate the macrochromosomes and the microchromosomes as the length of the SCs decreases gradually. This karyotype includes a "G" SC configuration (G, SC # 21), five self-paired foldback elements (U, SC # 135-139), and two very small univalents (H, SC # 140-141), the smaller of which is markedly heteropycnotic. The "G" SC configuration was seen in 8 of the 12 nuclei for which the SC length was measured, and it ranged from the 18th to the 26th longest SC in the karyotype. This range in karyotype position may be due to the fact that the continuous bicentric LE of this SC often appeared to be stretched. When the two centromeric regions were discernable in the "G" SCs, they were positioned at .16-.28 of the total length of the bicentric LE from the telomeres (Figure 4a-c). Overall, we found no distinguishable bivalent that consistently exhibited asynaptic behavior or that had axes of unequal length which could be associated with heteromorphic sex chromosomes.

Discussion

Studies of synaptonemal complexes have rapidly become a major focus of research in the area of meiotic cell biology. Numerous investigators have substaged the temporal sequence of meiotic prophase I events by identifying the characteristic features displayed during the various stages of prophase (Greenbaum et al. 1986, Dollin et al. 1989, Jiménez et al. 1990, Villagómez 1993). Common parameters used for substaging prophase are the appearance and degree of pairing of the LEs, and the morphology of the sex chromosome bivalent and nucleoli.

Determining the criteria for substaging prophase I was beyond the scope of this study, but it was of some importance to know the approximate stage of prophase of each cell analyzed due to the process of synaptic adjustment. Synaptic adjustment, first described by Moses and Poorman (1981), is a mechanism which acts during pachytene to reduce the amount of unpaired chromatin through heterosynapsis. In zygotene, structurally heterologous homologs first synapse with each other, forming distinctive configurations such as inversion loops and duplication/deletion buckles. This homosynapsis is followed by localized desynapsis and heterosynapsis which produces a straight-paired bivalent by the end of pachytene. Heterosynapsis has been described not only for heteromorphic autosomes but also for the sex chromosomes in a variety of species (Solari 1977, Ashley 1987, Hedin et al. 1990, Reed et al. 1990, Hogan et al. 1992, Foresti et al. 1993, Rodionova et al. 1996). Length differences and pairing defects between potentially heteromorphic sex chromosomes should therefore be looked for and analyzed at early pachytene, prior to the onset of synaptic adjustment and heterosynapsis (Moses 1980).

Zygotene nuclei in this study were characterized by incomplete synapsis and unpaired univalents. The onset of pachytene in many species is equated with the completion of autosomal synapsis, but prior to sex chromosome pairing (Greenbaum et al. 1986, Hogan et al. 1992). This was not a criterion that could be used in this study as there was no one chromosome pair that was consistently observed to be undergoing delayed synapsis. The onset of pachytene had to therefore be judged by the appearance of the SCs and the extent of synapsis. The obvious difficulty in using this criterion to stage pachytene when looking for pairing defects between potential sex chromosomes is that by the time all the bivalents are judged to be fully paired, synaptic adjustment may have already occurred and sex chromosomes may therefore appear as a straight-paired bivalent despite having displayed earlier pairing irregularities. The problem lies in determining whether univalents or pairing defects are present in a nuclei because autosomal pairing has not yet gone to completion, or if they are present as the result of a sex chromosome-related delayed synapsis. It could be that the atypical heteropycnotic univalents or the "G" SC configuration represent sex chromosomes, and that they are not seen in all pachytene nuclei because they are only conspicuous during the early stages of pachytene, prior to synaptic adjustment and/or heteromorphic pairing. Heteromorphic pairing was used to explain the absence of SC length differences or pairing defects in the heterogametic (male) sex of the guppy (Rodionova et al. 1996), and synaptic adjustment was observed to equalize the LEs of indistinct sex chromosomes in reptiles (Reed et al. 1990). In species lacking strongly dimorphic sex chromosomes care must be made before concluding the absence of sex chromosomes on the basis of homomorphic synaptic configurations in pachytene nuclei.

It is not clear what type of sex-chromosome system exists in white sturgeon. Mitotic studies have revealed no morphological difference between any specific chromosome pair, although the resolution of the microchromosomes in mitotic preparations is such that morphology is indistinct and the presence of very small microchromosomes (for example Figure 5, SC # 140-141) may be easily overlooked. It may be that one of the unpaired or self-paired univalents observed in many nuclei in this study represent an X chromosome in an XO sex-chromosome system. In surface-spread preparations of the XO grasshopper, the X chromosome appeared as a single unpaired axis (Santos et al. 1993). Likewise the "G" SC configuration (Figure 4a) could represent a X_1X_2Y trivalent association as was demonstrated for the ground skink (Hedin et al. 1990). For this later model to be feasible, one of the centromeric regions on the bicentric LE would have to be inactive to allow for normal disjunction. It is difficult to confirm or refute any proposed sex-chromosome system in this species due to the numerous pairing irregularities and chromosome number variations which have now been observed in both mitotic and meiotic studies. We can conclude that there was no consistent evidence for the existence of an obviously dimorphic sex chromosome pair in SC analyses of spermatocytes from white sturgeon. Given that the sex of the heterogamete in white sturgeon has not yet been proven, it would be of interest to examine the SCs in oocytes of this species as was recently done for rainbow trout (Oliveira et al. 1995a).

The application of the surface-spread technique for the visualization of whole-cell complements of SCs has allowed for the examination of meiotic pairing behavior in polyploid-derived species (Gillies 1989, Loidl and Schweizer 1992, Oliveira et al. 1995a), and for the accurate determination of microchromosomal morphology (Hale et al. 1988, Hedin et al.

1990, Reed et al. 1990). We found no evidence of alignment or pairing involving more than two LEs in white sturgeon, other than a single trivalent (Figure 4i) in one cell of one individual, suggesting that the process of diploidization is now complete in this ancient polyploid. A similar finding was reported for *Xenopus laevis*, a species of probably tetraploid origin, but not for the autotetraploid-derived rainbow trout which showed the presence of irregular synapses involving segments of three or four LEs in 3.4% of spermatocytes and oocytes from mid to late zygotene (Oliveira et al. 1995a). This trout study examined many more zygotene nuclei than the current study. Interestingly, irregular synapsis was absent in pachytene nuclei from rainbow trout suggesting correction of the multivalent zygotene synapses. Given that crossing over occurs during pachytene when only bivalents are present, it is interesting to consider and explain how residual tetrasomic inheritance (Allendorf and Thorgaard 1984) is occurring in rainbow trout.

Microchomosomal morphology is detailed for the first time in sturgeon species (Figure 5). Both metacentric and acrocentric microchromosomes were observed and the majority of them appeared to have normal SCs. There were varying numbers of apparently self-paired foldback elements of microchromosomal size in many nuclei. A centromere was not always evident on these structures and they did not appear heteropycnotic in pachytene. It may be that these chromosomes are accessory chromosomes. Accessory chromosomes have been reported in other animals (Switonsky et al. 1987, Fletcher and Hewitt 1988, Del Cerro et al. 1994), including fish (Salvador and Moreira-Filho 1992, Andreata et al. 1993), and they often form foldback hairpin loops during prophase I of meiosis (Khazanehdari and Jones 1996). On the basis of the variability in chromosome number found in mitotic studies some authors have

suggested that other sturgeon species may be polymorphic in microchromosomal number (Vasiliev et al. 1980, Birstein and Vasiliev 1987), although the technical difficulties involved in working with so many microchromosomes may also explain this variability. In a preliminary C-banding study of white sturgeon (Sola et al. 1994), a variable number of small macrochromosomes (1-7) were found to be entirely heterochromatic and the authors considered them to be accessory chromosomes. The unfolded length of the self-paired elements found in this study could be consistent with the size of small macrochromosomes.

The diploid number of a white sturgeon population in Italy has been reported to be 248 ± 8 (Fontana 1994), and so we expected to see approximately 124 SC components in pachytene nuclei. The fact that we saw an average of 139 may be partially explained by the presence of univalents and foldback elements which were each counted as one SC component, the undercounting of microchromosomes in mitotic spreads, the possibility of accessory chromosomes in this genome, and perhaps by chromosome number variations between California white sturgeon and the white sturgeon population in Italy. The gradual decrease in SC size in the karyotype and the absence of unique identifying features made it impossible to determine which specific SCs were associated with the variation in SC counts between nuclei. It is not possible to determine the microchromosomal morphology from the mitotic karyotype reported by Fontana (1994), but it does appear that the largest acrocentric chromosome pair in the mitotic karyotype (approximately the seventh largest pair) should have been represented within the first 10 SCs of the meiotic karyotype. The fact that the first acrocentric SC does not appear until SC # 20 in the meiotic karyotype (Figure 5), and that no acrocentric chromosome was found in the first 10 SCs in any of the nuclei that were

analyzed, suggests that there may be karyotypic differences between the strains of white sturgeon in Italy (Fontana 1994) and in California. There was no obvious periodicity in the meiotic karyotype which might have been expected from a polyploid-derived genome and which was reported in mitotic studies on the North American paddlefish, *Polyodon spatula*, ($2n$ = 120, Acipenseriformes) (Dingerkus and Howell 1976).The total SC length (481.8 ± 55.7 μm) was high for the class Osteichthyes, but the mean packing density (pg DNA/μm SC) was not exceptional and was similar to that found in other vertebrate classes (Table 3).

Some unexpected complications of this analysis were the presence of macrochromosomal univalents in two of the six males examined and our inability to obtain SC spreads from the majority of the testes that we collected. The domestic fish used in this study were raised at high stocking densities in indoor tanks with semi-recirculated water held at a constant temperature (20°C) and it may be that these artificial environmental conditions affect meiosis. These production males have not been used as broodstock and so their fertility has not been appraised. Sexual immaturity and the absence of the appropriate developmental stimuli (i.e. temperature and daylight fluctuations) may explain why testes from only a few of the fish contained significant proportions of primary spermatocytes. Another interesting feature of this analysis was the uneven spacing of the LEs in many SCs in which it appeared that the LEs often approached each other in the absence of twisting or other perturbations (Figure 4h). Overall SC analysis of this species revealed a very large SC complement with numerous pairing irregularities and unusual features. The vast macro- and microchromosome number combined with the unusual attributes that were observed during the course of this

study, make sturgeon both a challenging and an interesting candidate for further SC investigations.

Conclusions

Synaptonemal complex (SC) analysis of white sturgeon spermatocytes allowed for the development of a meiotic karyotype for this species. The SC number was 139 ± 3.4 with both between and within animal variation being observed. Metacentric, submetacentric and acrocentric macro- and microchromosomes were present in the karyotype. Numerous atypical pairing configurations were observed during the course of this analysis. Pachytene nuclei had varying numbers of univalents, self-paired foldback elements with no obvious centromeric region, and SC peculiarities. No consistent evidence of alignment or pairing involving more than two lateral elements was found indicating that the process of diploidization is now complete in this ancient polyploid. We found no bivalent that consistently exhibited asynaptic behavior or that had axes of unequal length suggesting that heteromorphic sex chromosomes are not present in white sturgeon spermatocytes. However, the possibility of synaptic adjustment and heteromorphic pairing in combination with the pairing irregularities and chromosome number variation make it difficult to draw any firm conclusions from this analysis regarding the presence or type of sex chromosomes in the males of this species.

Acknowledgments

The authors thank Luis A. Perez Carrasco for helpful discussions in the planning stages of this study, "Stolt Sea Farm, California, LLC" for assisting us with gonad collection, and Joel P. Van Eenennaam for technical assistance with sample collection and histological analyses. This research was funded by a grant from the National Sea Grant College Program, National Oceanic and Atmospheric Administration, U.S. Department of Commerce, under grant number NA36RG0537, project R/A-99 through the California Sea Grant College System, and in part by the California State Resources Agency. The views expressed herein are those of the authors and do not necessarily reflect the views of NOAA or any of its sub-agencies. The U.S. government is authorized to reproduce and distribute for governmental purposes. A. L. Van Eenennaam was supported by a Sea Grant traineeship.

TABLE 1. Percentage of cysts with different types of germ cell from the testes of males used in a synaptonemal complex analysis of spermatocytes from *Acipenser transmontanus*.

Fish #	Gonial	Spermatocytes	Spermatids	Late spermiogenesis[1]	Spermatozoa
11	50	30	20	--	--
18	5	70	15	5	5
27	--	85	5	5	5
34	--	50	20	20	10
37	5	60	15	10	10
51	--	50	30	10	10

[1] Developing spermatozoa with incomplete flagella development.

TABLE 2. Synaptonemal complex (SC) count, length and features from twelve nuclei examined in a SC analysis of spermatocytes from *Acipenser transmontanus*.

Fish #	SC count	Length (μm)	Prophase I substage	Macro-chromosome univalent	Microchromosomal SCs		Position of "G" SC[3]
					Self-paired foldback elements[1]	Hetero-pycnotic univalents[2]	
18	137	420	Pachytene	1	4	-	23
27	141	452	Pachytene	-	5	2	21
27	141	528	Pachytene	-	1	2	-
27	139	571	Pachytene	-	4	2	26
27	136	424	Pachytene	-	2	2 (3?)	18
27	140	447	Pachytene	-	3	2	-
27	143	426	Pachytene	-	3	2	-
27	132	457	Pachytene	-	2	1	19
34	140	463	Pachytene	-	5	2	20
37	138	540	Late-zygotene	-	6	-	23
37	145	567	Pachytene	-	7	-	-
37	137	487	Pachytene	-	5	2	21

[1] Self-paired foldback SCs with no obvious heteropycnosis or centromeric region (U).

[2] Very small often heteropycnotic univalents which do not self-pair although they often appear in a relaxed U-shaped configuration (H).

[3] Position of the commonly seen bicentric "G" SC configuration (see text) in the descending-length ordered meiotic karyotype of the nuclei.

TABLE 3. Total synaptonemal complex (SC) length and mean packing density (pg DNA/μm) for Osteichthyes and representative species from Amphibia, Reptilia, Aves and Mammalia.

Class/species	Sex	SC length (μm) Mean ± SD	CV[1]	N[2]	1C (pg)	Packing density (pg DNA/μm)	Reference
Osteichthyes							
Acipenser transmontanus	M	482 ± 56	11.6	12	4.73[3]	9.8×10^{-3}	This paper
Pomoxis annularis	M	127 ± 13	10.5	9	1.04	8.2×10^{-3}	Peterson (1994)
Lepomis macrochirus	M	130 ± 7	5.6	3	1.01	7.8×10^{-3}	Peterson (1994)
Micropterus salmoides	M	143 ± 18	12.6	10	1.02	7.1×10^{-3}	Peterson (1994)
Dorosoma cepedianum	M	78 ± 11	14.2	5	0.99	12.7×10^{-3}	Peterson (1994)
Oncorhynchus mykiss	M	144 ± 16	11.1	10	2.75	19.1×10^{-3}	Peterson (1994)
	M	259 ± 49	18.9	10	2.33	9.0×10^{-3}	Oliveira (1995)
	F	229 ± 34	14.8	2	2.33	10.2×10^{-3}	Oliveira (1995)
Amphibia							
Xenopus laevis	M	559 ± 28	5.0	7	3.1	5.5×10^{-3}	Loidl (1992)
	F	900 ± 71	7.9	2	3.1	3.4×10^{-3}	Loidl (1992)
Reptilia							
Anolis carolinensis	M	133 ± 14	10.5	10	2.2	16.5×10^{-3}	Peterson (1994)
Aves							
Gallus domesticus	M	211 ± 35	16.6	10	1.3	6.16×10^{-3}	Peterson (1994)
Mammalia							
Mus domesticus	M	168 ± 24	14.3	10	3.4	20.2×10^{-3}	Peterson (1994)

[1] Coefficient of variation = (standard deviation/mean) x 100%
[2] Number of complete sets of SCs measured
[3] Blacklidge and Bidwell (1993)

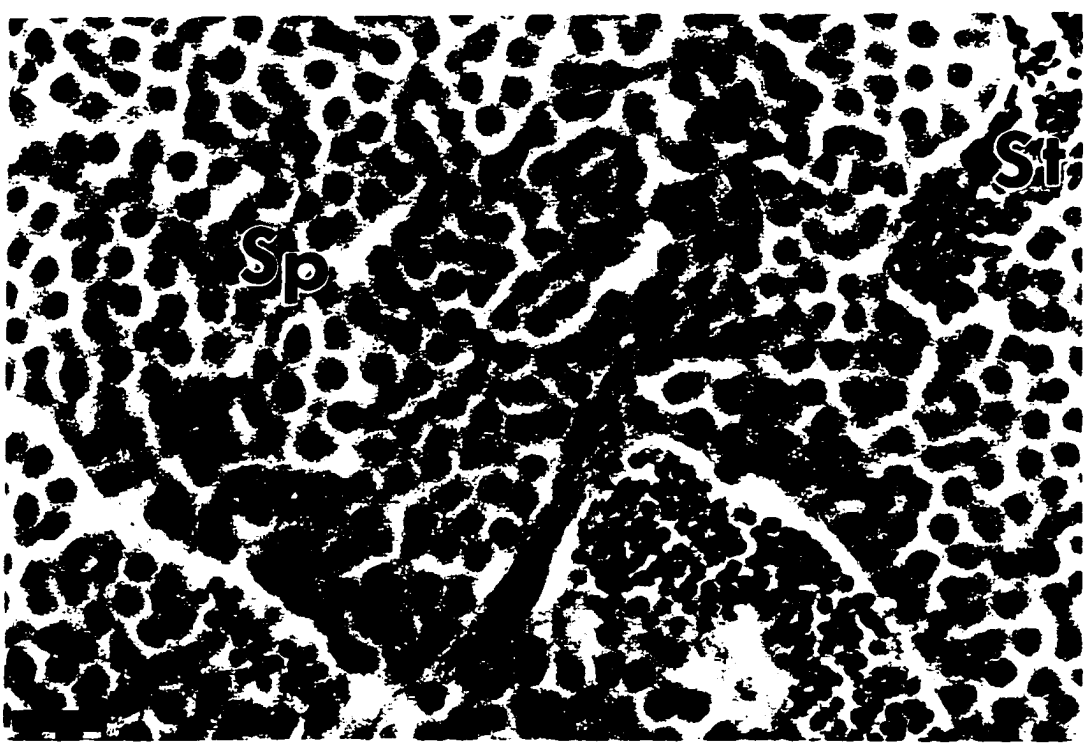


FIGURE 1. Section of a white sturgeon (*Acipenser transmontanus*) testis suitable for synaptonemal complex analysis showing testicular cysts containing primarily spermatocytes. Sp = spermatocytes, St = spermatids. Bar = 20 μm.

FIGURE 2. Electron micrographs of zygotene nuclei of male white sturgeon, *Acipenser transmontanus*. **a** Early zygotene (Fish # 18). **b** Late zygotene (Fish # 37). **c** Unsynapsed interstitial loop (Fish # 34). **d** Unsynapsed terminal segments (Fish # 37). Arrowheads indicate unsynapsed macrochromosomal lateral elements. N = nucleolus, I = unresolved interlock, G? = possible zygotene appearance of the "G" SC configuration (see text). Bar = 1 μm.

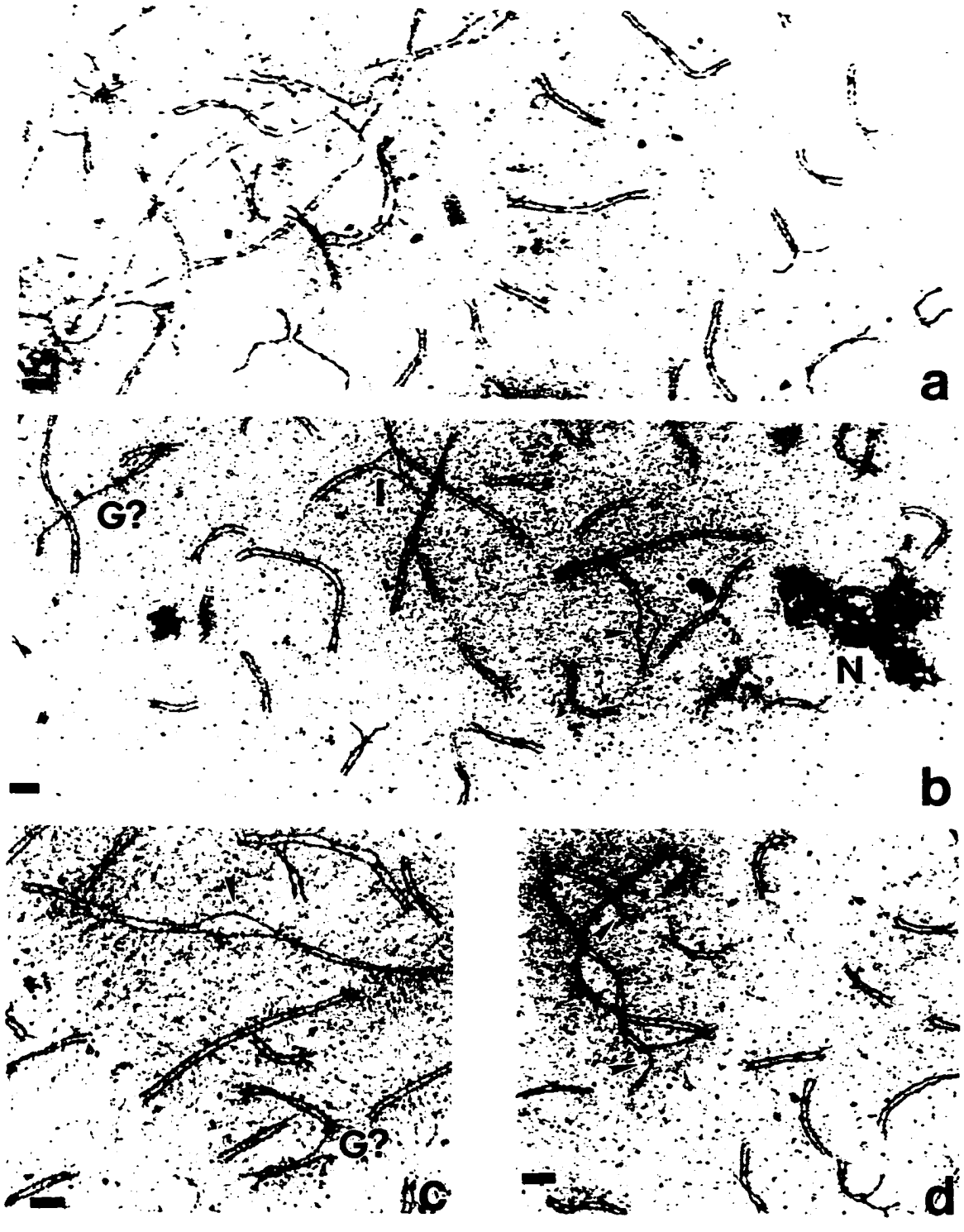


FIGURE 3. Electron micrograph of a complete pachytene nucleus from a male white sturgeon, *Acipenser transmontanus* (Fish # 34). **a** Nuclei with 140 SC elements. **b-d** Close up of the areas outlined in **a**. Arrows indicate the position of centromeres. N = nucleolus, G = "G" SC configuration, H = small heteropycnotic univalent, U = self-paired foldback element with no obvious centromeric region. Bar = 1 μm.

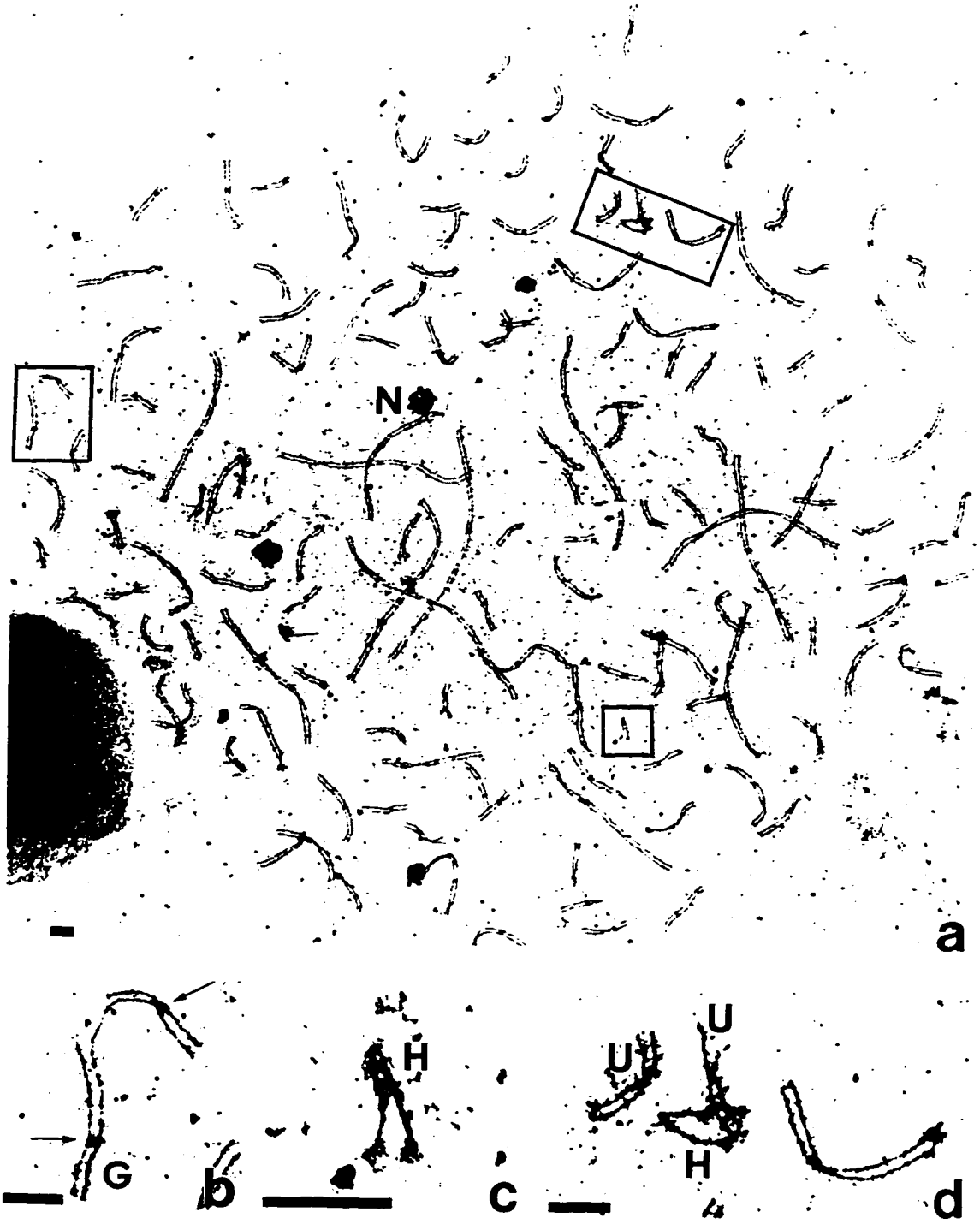


FIGURE 4. Electron micrographs of representative examples of pairing irregularities found in pachytene nuclei of male white sturgeon, *Acipenser transmontanus*. **a-d** "G" SC configuration (G). Arrowheads indicate location of discernable centromeres, and numbers are the proportion of the length of the bicentric lateral element that the centromeres are located from the closest telomere (Fish # 27, 34, 27, and 18, respectively). **e** Macrochromosome univalent (A) (Fish # 18). **f-g** Self-paired foldback elements with no obvious centromeric region (U) (Fish # 27 and 18, respectively). **h** Unevenly spaced lateral elements (arrows), small heteropycnotic univalent (H) (Fish # 37). **i** Trivalent association (T) (Fish # 51). Bar = 1 μm.

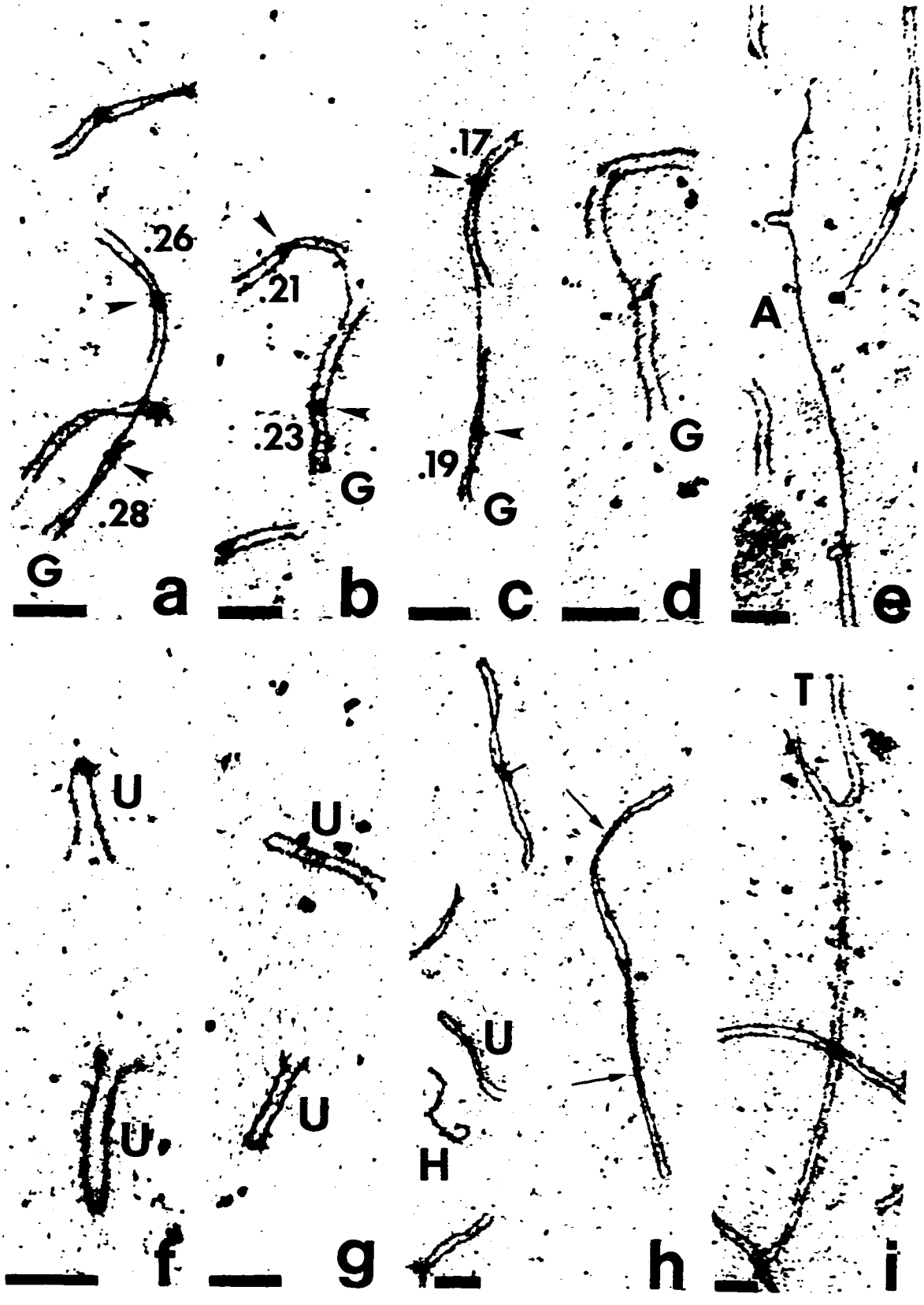


FIGURE 5. Meiotic SC karyotype of white sturgeon (*Acipenser transmontanus)* male with 141 SC elements arranged in order of decreasing size (Fish # 27). Arrowheads indicate apparent location of centromeres. G = "G" SC configuration, U = self-paired foldback elements with no obvious centromeric region, H = small heteropycnotic univalents. Bar = 1 μm.

CHAPTER 5. MITOTIC ANALYSES OF THE NORTH AMERICAN WHITE STURGEON (*Acipenser transmontanus* RICHARDSON).

Alison L. Van Eenennaam, James D. Murray, and Juan F. Medrano

To be submitted in part to Genome

Introduction

Some of the most ancient living ray-finned fish, sturgeon and paddlefish, belong to the order Acipenseriformes. Karyotypes of the sturgeon and paddlefish are characterized by a very large number of chromosomes, about half of which are microchromosomes (Table 3, Chapter 1). The order can be divided into two groups; the first group has a chromosome number of approximately 120 and a mean genome size (1C) of 1.6-2.5 pg and the second group has a chromosome number of 240-250 with a genome size of 4.0-4.8 pg (Birstein et al. 1993, Blacklidge and Bidwell 1993). Various authors have claimed that the first group is of a tetraploid origin and the second group is of an octoploid origin (Ohno et al. 1969, Burtzev et al. 1976, Dingerkus and Howell 1976, Birstein and Vasiliev 1987). No extant Acipenseriformes species has been found to have a diploid chromosome number of 60. Early Russian papers suggested that certain sturgeon species have a chromosome number of 60 (Serebryakova 1969, Burtzev et al. 1976), but it seems that the microchromosomes were not included in these chromosome counts. Conventional mitotic studies have not revealed heteromorphic sex chromosomes in any sturgeon species (Fontana and Colombo 1974, Holčík 1986).

There is often considerable variation (± 8) in the chromosome number reported for each species (Table 3, Chapter 1). This has been variously attributed to chromosome loss or fragmentation during slide preparation due to the strong hypotonic shock needed to avoid superimposition, the inclusion of cellular fragments or artifacts resembling microchromosomes in chromosome counts, overlapping of chromosomes leading to undercounting, difficulty in resolving the numerous dot- like microchromosomes, and actual counting errors (Dingerkus and Howell 1976, Vasiliev et al. 1980, Fontana et al. 1996, Gorshkova et al. 1996). A few authors have postulated that some of the chromosome number variation may be attributed to the presence of accessory B chromosomes (Vasiliev et al. 1980, Birstein and Vasiliev 1987, Sola et al. 1994). C-banding has been reported for only three sturgeon species including a preliminary study on white sturgeon which found that a variable number of small macrochromosomes in each metaphase spread were entirely heterochromatic (Sola et al. 1994, Fontana et al. 1996, Rab et al. 1996).

The diploid chromosome number of white sturgeon exported from North America to Italy during the 1980s has been reported to be 248 ± 8 (Fontana 1994), with the karyotype including 52 meta-centric and submeta-centric pairs and 74 pairs of acrocentrics and microchromosomes. This chromosome number of 248 ± 8 contrasts with that which might be expected based on the meiotic synaptonemal complex (SC) count of 139 ± 3.4 for a California white sturgeon population (Chapter 4). Here we report a mitotic chromosome study that was undertaken to: (i) determine the chromosome number and develop a mitotic karyotype for a California white sturgeon population, (ii) examine for the presence of entirely heterochromatic (C-positive heterochromatin) chromosomes in metaphase spreads, and (iii)

critically appraise metaphase spreads from both sexes for the presence of a heteromorphic sex chromosome pair. An improved and rapid C-positive heterochromatin staining technique for this species is also described.

Materials and Methods

Metaphase chromosomes were prepared from synchronized lymphocyte cultures (Appendix 1D) derived from eight California domestic white sturgeon of known sex (4 ♂, 4♀). The preparation of chromosome slides was carried out following standard procedures. For conventional karyotyping the slides were stained for 20-30 min with a 4% Giemsa solution (pH 6.8; Bio/medical Specialities Inc., Santa Monica, CA). Staining of constitutive heterochromatin (C-banding) was achieved according to the method of Sumner (1972) using slides that had been dessicated for at least 3 days. Slides were first incubated in 0.2N HCl for 1h at room temperature, rinsed in distilled H_2O and allowed to air dry. Slides were then incubated in a 5% $Ba(OH)_2$ at 50°C for 15-30 s and rinsed in 0.2N HCl and distilled H_2O. They were then either incubated in 2X SSC at 60°C for 1h, rinsed in distilled H_2O, allow to air dry, stained with a 4% Giemsa solution for 60-90 min, and examined with a light microscope (Olympus BH-2); or they were stained for 10 min in propidium iodide (400 ng/ml in 2X SSC), rinsed for 2 min in 2X SSC, mounted in 2 drops of DAPCO antifade stock (2.3% 1,4 diazabicyclo-(2,2,2 octane) in glycerol), and observed under the same microscope equipped for epifluorescence microscopy. Chromosomes recorded in photographs of discrete well-spread cells were counted to determine the chromosome number and good quality spreads were scanned and arranged according to length and morphology using Adobe

Photoshop software (Adobe Systems Inc, Mountain View, CA) to produce a mitotic karyotype.

Results

Photographs of 75 discrete spreads (2-14 per fish) were counted to determine the chromosome number (Table 1). We found the diploid chromosome number of white sturgeon to be 271 ± 2.5. Figure 1 shows a representative metaphase spread ($2n$ = 274) from a female fish, and Figure 2 shows a mitotic karyotype of a different cell derived from the same fish. This karyotype consists of 132 meta- and submetacentric chromosomes, 44 acrocentric chromosomes, and 98 microchromosomes. These numbers are somewhat arbitrary as it is difficult to differentiate between small macrochromosomes and microchromosomes. The size of the chromosomes decreases in a rather continuous pattern and approaches the resolution limits of the light microscope. Karyotypes of male and female fish did not differ from each other and there was no evidence of a heteromorphic sex chromosome pair.

C-banded metaphase spreads from a male and female white sturgeon stained with either Giemsa or propidium iodide are shown in Figure 3. We observed that the C-banding pattern was the same irrespective of which stain was used, however it was easier to identify the C-positive heterochromatin when using the propidium iodide staining method. Twenty C-banded, propidium iodide stained cells from each fish were examined to determine the number of entirely heterochromatic chromosomes (Table 1). There were a variable number (2-7) of heterochromatic chromosomes in the eight fish examined in this experiment. In three of the fish one of the heterochromatic chromosomes was slightly less brightly staining than the

others and it could most clearly be classified as heterochromatic in spreads where the chromosomes were somewhat condensed. Therefore a range of values is listed in Table 1 for the number of heterochromatic chromosomes found in these three fish. This should not be interpreted to mean that we observed within animal variation in the number of heterochromatic chromosomes. It merely reflects the fact that one of the heterochromosomes in these fish sometimes stained less intensely depending upon the degree of chromosome condensation.

Discussion

The white sturgeon chromosome number that we observed, 271 (± 2.5) is significantly higher than the previously published value of 248 (± 8) reported for a white sturgeon population in Italy (Fontana 1994). In the latter study there were 52 meta-centric and submeta-centric pairs, and 74 pairs of acrocentrics and microchromosomes in the karyotype. Using the same categorization, the present study found 66 meta- and submeta-centric pairs, and 71 pairs of acrocentrics and microchromosomes. This difference can be partially attributed to the subjective classification that is required to categorize small meta-, submeta-, and acro-centric macrochromosomes and microchromosomes. It was not possible to karyotypically identify the position of the additional pairs of chromosomes that were found in the present study.

One very clear difference between the karyotypes presented in the two studies is in the size of the largest acrocentric chromosome pair. The largest acrocentric chromosome pair reported by Fontana (1994) was approximately the 7th largest pair in the karyotype, whereas

in the present study it was approximately the 20th largest pair. Our data agrees with the meiotic SC karyotype for California white sturgeon in which we found the first acrocentric SC to be approximately the 20th element (Chapter 4). The chromosome number found in this study also better agrees with the chromosome number that might be predicted (139 SC x 2 = 278) from the average white sturgeon SC number found in Chapter 4. A variable number of the SCs included in the meiotic count (3-7) were univalents implying that this value of 278 is actually a slight (3-7) overestimate of the true chromosome number of the California white sturgeon.

Overall these data strongly suggest that there are chromosome number and karyotypic differences between the California white sturgeon populations and those animals sampled by Fontana (1994) in Italy. Juvenile white sturgeon native to the west coast of North America were first imported into Italy in 1981[2]. Fish from both the San Francisco Bay (California) and the Columbia River (Washington/Oregon) white sturgeon populations were exported to Italy. The fish examined in the present study ($2n \approx 271$) were domestic white sturgeon derived from wild San Francisco Bay broodfish. One possible explanation for the significant difference in karyotypes between the two studies may be the origin of the population founders. It may be that the specimens examined in the Italian study ($2n \approx 248$) were derived from wild broodfish spawned at the Columbia River hatchery, and that they constitute a different chromosomal race or even species to the San Francisco Bay population, but this needs to be examined.

Counting a large number of chromosomes in discrete cells from each individual allowed us to detect chromosome number variation between animals (Table 1). Part of this

[2] Ken Beer, The Fishery, Galt, California. Pers. comm.

variation can be explained by the variable number (2-7) of entirely heterochromatic chromosomes found in different individuals. Within each individual the number of heterochromatic chromosomes was consistent, whereas there was clear between animal variation. The heterochromatic chromosomes were most commonly small metacentric chromosomes (Figure 3). Accessory B chromosomes tend to be entirely heterochromatic in most organisms (Jones and Rees 1982), and it is tempting to speculate that the heterochromatic chromosomes seen in this study are B chromosomes. Accessory chromosomes have been reported in other animals (Switonsky et al. 1987, Fletcher and Hewitt 1988, Del Cerro et al. 1994), including fish (Salvador and Moreira-Filho 1992, Andreata et al. 1993). It may be that these heterochromatic chromosomes were the source of the variable number of univalents seen in meiotic SC spreads of this species (Chapter 4). It would be interesting to examine the metaphase I behavior of these chromosomes.

We consistently obtained good quality spreads using the lymphocyte culture protocol outlined in Appendix 1D. We had difficulty staining C-positive heterochromatin with Giemsa and found that the use of propidium iodide as the stain allowed us to better visualize the C-banding patterns (Figure 3). Aside from the entirely heterochromatic chromosomes, C-band positive heterochromatin was located mainly in the centromeric region of small to medium macrochromosomes and microchromosomes. Large metacentric chromosomes exhibited weak or no C-banding in the centromeric region. We found that when using propidium iodide as the stain the hot salt solution incubation following denaturation (1h, 2X SSC, 60°C) could be omitted, and so the length of time it took to complete the C-banding procedure was reduced. AT-enhancing fluorochromes (quinacrine, DAPI) have been used to resolve C-bands in

salmonids, a poeciliid, and several European percids; and a GC-enhancing fluorochrome (CMA) has been used to resolve C-bands in a North American percid (Haaf and Schmid 1984, Amemiya and Gold 1986, Mayr et al. 1987, Phillips and Hartley 1988). Gold et al. (1990a) state that fluorochromes may be used to resolve C-bands provided the heterochromatic regions are differentially rich in AT- or CG- base pairs relative to the remainder of the chromatin. In this study we resolved C-bands with propidium iodide which does not preferentially bind to either GC- or AT- rich DNAs (Saitoh and Laemmli 1994). It seems likely that the propidium iodide was fluorescing more brightly in regions of DNA which had not been fully denatured by treatment with barium hydroxide irrespective of the base pair content. A similar use of propidium iodide staining to detect nucleolus organizer regions in fish chromosomes was recently reported (Rab 1996).

C-banding and karyotypic analysis did not reveal a heteromorphic sex chromosome pair or any sex-related chromosomal polymorphism in white sturgeon. Similarly, in fluorescence *in situ* hybridization (FISH) experiments where labeled male and female sturgeon genomic DNA was hybridized to metaphase chromosome spreads of each sex (Appendix 3), no chromosome or chromosome arm was seen to be specifically hybridizing only to genomic DNA of the same sex. These results suggest that if sex chromosomes do exist in white sturgeon, then they are at a very early stage of differentiation such that they appear homomorphic and consequently cannot be distinguished by mitotic analyses.

Conclusions

The average chromosome number of 8 California white sturgeon was found to be 271 (range 265-276). This number is significantly higher than previous estimates for this species. A representative karyotype was found to consist of 132 meta- and submeta-centric chromosomes, 44 acrocentric chromosomes, and 98 microchromosomes. An improved C-banding technique revealed variation between animals in the number (2-7) of entirely heterochromatic metacentric chromosomes. These heterochromatic chromosomes may represent accessory B chromosomes. There was no evidence of a heteromorphic sex chromosome pair or any sex-related chromosomal polymorphism in this species.

Acknowledgments

The authors thank L. V. Millon and F. Fontana for helpful suggestions regarding sturgeon lymphocyte culture, and J. P. Van Eenennaam for technical assistance with sample collection. This research was funded by a grant from the National Sea Grant College Program, National Oceanic and Atmospheric Administration, U.S. Department of Commerce, under grant number NA36RG0537, project R/A-99 through the California Sea Grant College System, and in part by the California State Resources Agency. The views expressed herein are those of the authors and do not necessarily reflect the views of NOAA or any of its sub-agencies. The U.S. government is authorized to reproduce and distribute for governmental purposes. A. L. Van Eenennaam was supported by a Sea Grant traineeship.

TABLE 1. Individual fish and average chromosome number (including varying numbers of entirely heterochromatic chromosomes) obtained from 8 California white sturgeon (*Acipenser transmontanus* Richardson).

Sex	N[1]	Mean Chromosome number	S.D.	Range	N	Heterochromosome[2] number
Male	6	270	2.3	267-272	20	3-4
Male[3]	14	271	1.6	268-273	20	3
Male	11	271	1.7	268-273	20	5
Male	2	273	0.7	272-273	20	6-7
Female	13	267	1.1	265-269	20	2[4]
Female	12	271	2.0	268-275	20	2
Female	3	272	1.2	271-273	20	5-6
Female[5]	14	274	1.2	272-276	20	6
TOTAL	75	271	2.5	265-276	160	2[4]-7

[1] N = number of metaphase spreads counted to determine chromosome or heterochromosome number.
[2] Entirely heterochromatic (C-band positive) metacentric chromosome.
[3] Male shown in Figure 3a,b.
[4] One of the two heterochromatic chromosomes had only one heterochromatic arm.
[5] Female shown in Figures 1, 2, 3c,d.

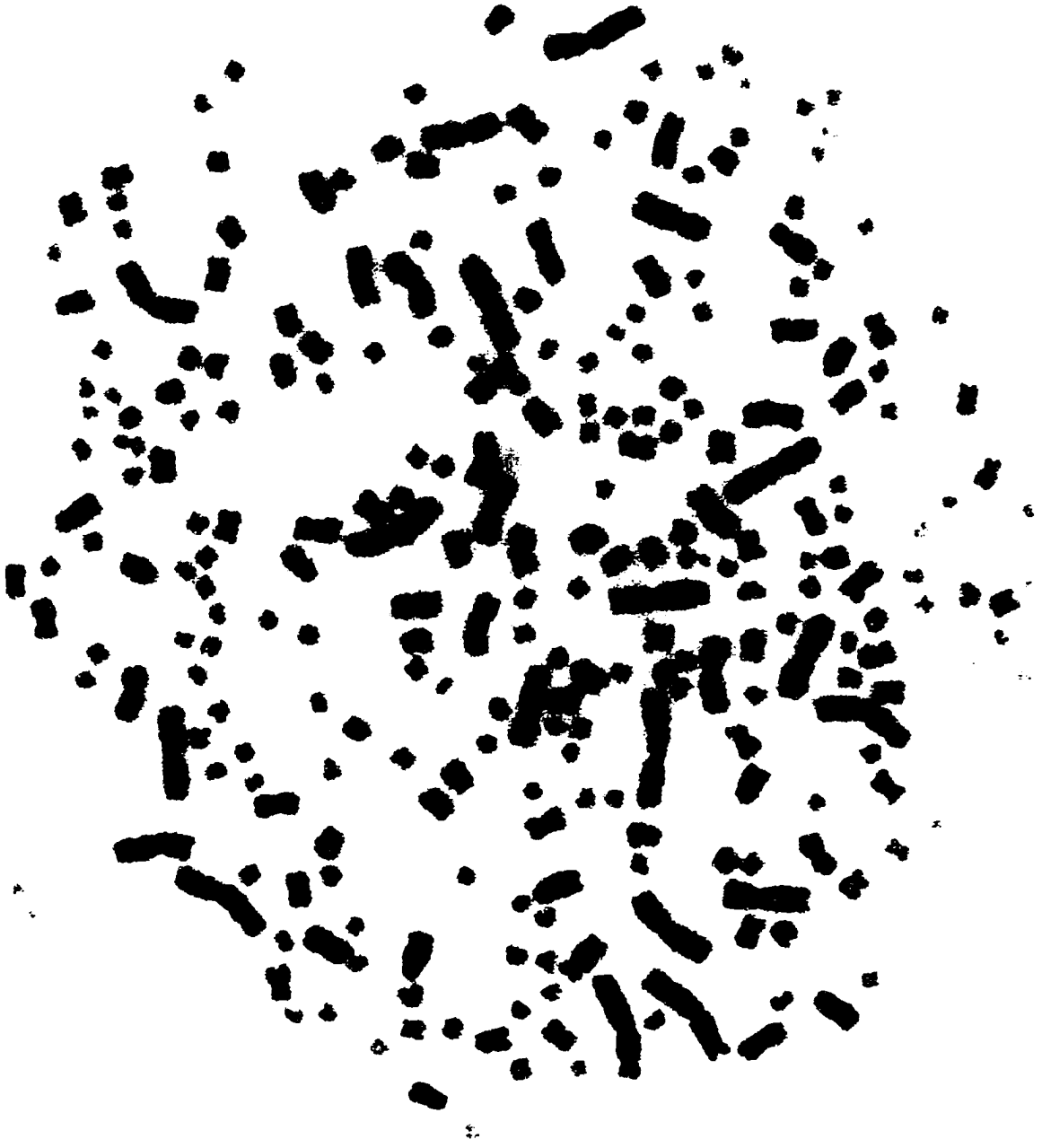


FIGURE 1. California white sturgeon (*Acipenser transmontanus)* metaphase spread ($2n$ = 274). x 2500.

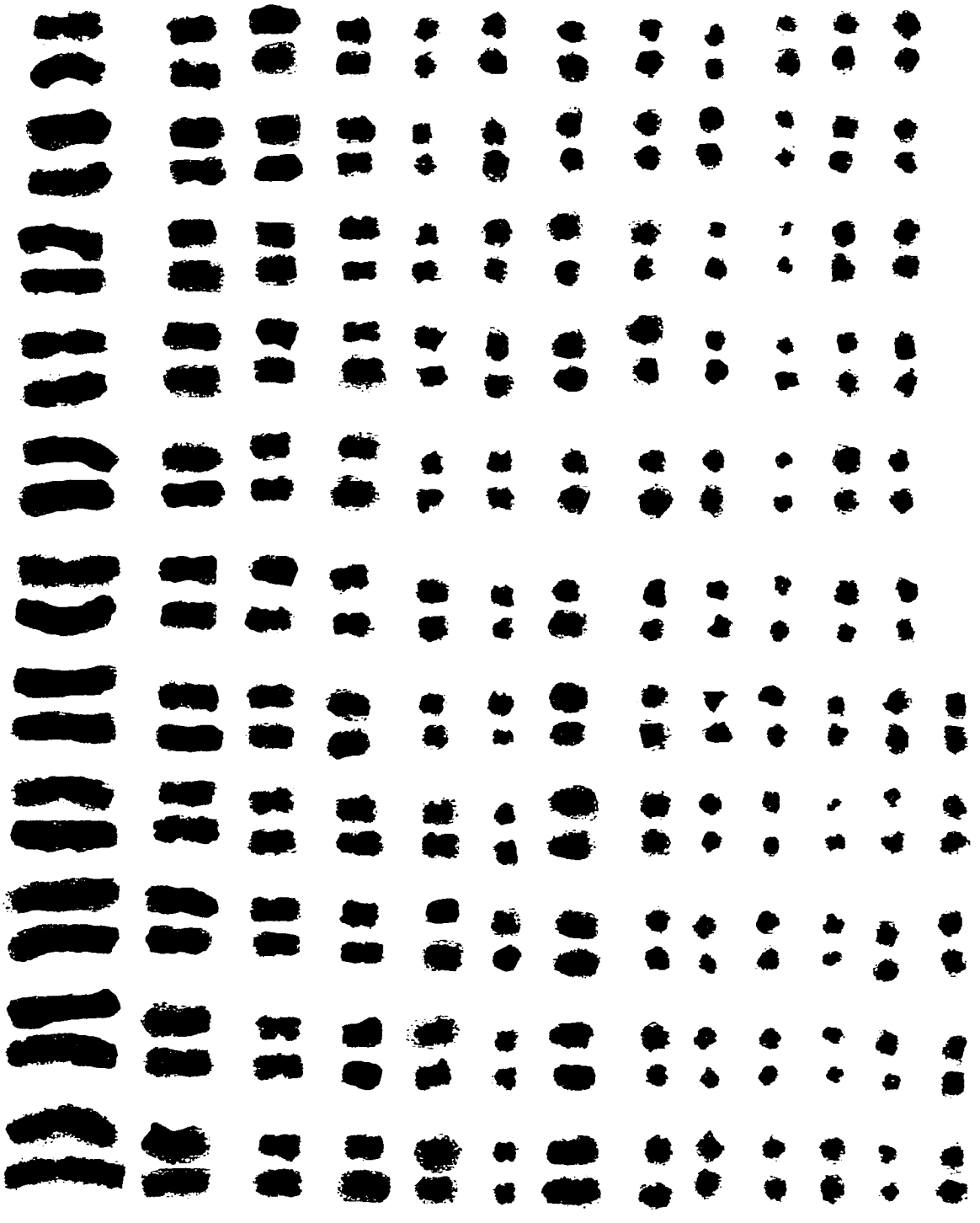


FIGURE 2. California white sturgeon (*Acipenser transmontanus*) karyotype ($2n = 274$). The meta- and submetacentric chromosomes were aligned in order of declining size, followed by the acrocentrics and microchromosomes. x 3000.

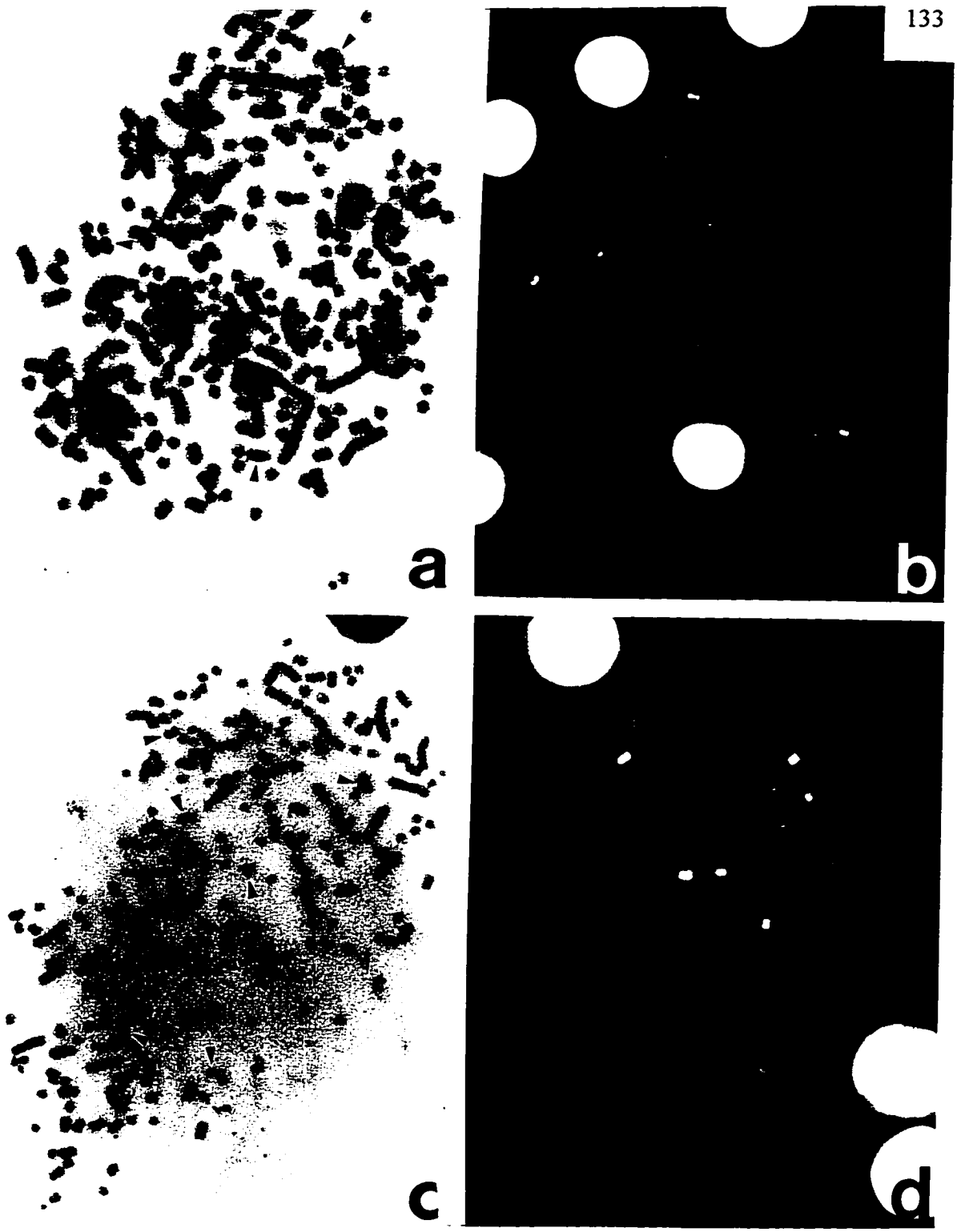


FIGURE 3. C-banded metaphase spreads from male (a, b) and female (c,d) white sturgeon (*Acipenser transmontanus)* stained with Giemsa (a, c) or propidium iodide (b, d). This male had 3 entirely heterochromatic chromosomes (arrow heads), and the female had 6. a x 1700, b x 1000, c x 975, d x 1250.

GENERAL DISCUSSION

Background

The fast-growing white sturgeon, *Acipenser transmontanus* Richardson, is an increasingly important species to California aquaculture. The current diagnostic technique for sex identification in this sexually-monomorphic species requires a surgical biopsy of sexually-differentiated gonads. The availability of monosex female populations or an age-independent, DNA-based sex identification procedure would significantly enhance the economic feasibility of domestic sturgeon caviar production systems. An understanding of the basic mechanism of sex determination operating in chondrostean species is required before techniques to identify sex using molecular markers or to create monosex female sturgeon populations can be developed.

The overall aim of this project was to understand the mode of sex determination in white sturgeon and to provide the first evidence as to what type of sex determination mechanism operates in chondrostean species. The following hypotheses were tested:

i) white sturgeon has a genetic sex determination system,

ii) one of the sexes in sturgeon is heterogametic, and

iii) sex-specific DNA polymorphisms exist between the sexes.

Several experimental approaches derived from the fields of transmission, molecular, and cytogenetics were used to examine the nature of the sex determination process. This discussion summarizes the results and conclusions of this research and proposes future directions for research in this field.

Experimental Findings

Gonadal sex of gynogens and triploids: The first series of experiments (Chapter 2) provided critical support for the hypotheses that white sturgeon has a genetic sex determination system, and that one of the sexes in sturgeon is heterogametic. The finding of a 1♂:1♀ sex ratio in diploid progeny and a significantly different ratio in triploid (1♂:6♀) and gynogenetic progeny (1♂:4.5♀) derived from the same batch of eggs supports a genetic sex determination system. These ratios in combination with the fact that males were present in gynogenetic progeny groups derived from 4 different females provide strong evidence in favor of a ZW:ZZ female heterogametic sex determination system. The sex ratios found in the diploid, triploid and gynogenetic progeny groups from the same female most closely match those ratios expected when the sex-determining element is segregating independently of the centromere.

Search for sex-specific DNA sequences: Subtractive hybridization, representational difference analysis, and bulked segregant analysis (BSA) methodologies were used in an unsuccessful attempt to identify sex-associated DNA sequences in white sturgeon. The fact that no specific sex-associated marker was identified in any of the three BSA experiments using different genetic stocks was unexpected given the findings of Chapter 2. Even if white sturgeon has a ZO:ZZ sex determination system such that there is no sex-specific DNA, which seems unlikely given the findings of Chapter 2, we would have expected to see sex-specific polymorphisms in the "hybrid" (lake sturgeon sire x white sturgeon dam) BSA as only male hybrids would have inherited the white sturgeon Z chromosome from the dam. It seems improbable that the "hybrid" BSA would have failed to identify an entire chromosome that was present in only one sex.

The combination of the results of chapters 2 and 3 suggest that: (i) a ZW:ZZ female heterogametic genetic sex determination system is in operation in white sturgeon; (ii) the sex-determining loci and sex-specific DNA sequences located on the W-chromosome are distal to the centromere and may represent a very small proportion of the genome (1-5 kb) or be comprised of unusual sequences which have no complementarity to the 1200 random decamer primers or the recognition sequence of the restriction endonucleases used in the experiments detailed in Chapter 3; and (iii) the Z and W chromosomes undergo recombination and are probably at an early stage of sex chromosome evolution and structural divergence. Results from Chapters 4 and 5 support the these findings.

Synaptonemal complex analysis of spermatocytes: No bivalent exhibited the atypical pairing behavior which is often characteristically associated with heteromorphic sex chromosomes in the meiotic analyses of white sturgeon spermatocytes supporting the hypothesis of male homogamety in this species. Chapter 4 also revealed that: (i) white sturgeon appear to have completed the process of diploidization as no multivalents were observed in pachytene nuclei; (ii) the meiotic karyotype is significantly different in number (139 ± 3.4) and chromosome order to the published mitotic karyotype; and (iii) pachytene nuclei had varying numbers of univalents (0-3), self-paired foldback elements with no obvious centromeric region (1-7), and other SC peculiarities. These results suggest that there may be accessory chromosomes in the white sturgeon karyotype, and that variations exist between the chromosome number and karyotype of California white sturgeon and those reported in the only published white sturgeon mitotic study which was performed in Italy (Fontana 1994).

Mitotic analyses of metaphase spreads: Mitotic analyses further supports the idea that the accumulation of sex chromosomal rearrangements is insufficient to be visible at the cytogenetic level. The average chromosome number from metaphase spreads of 8 California white sturgeon was $2n = 271$ (range 265-276). The mitotic chromosome number and karyotype agreed with the meiotic data presented in Chapter 4. An improved C-banding technique using propidium iodide and epifluorescence microscopy revealed between animal variation (2-7) in the number of entirely heterochromatic metacentric chromosomes. This may explain some of the variation that is observed in chromosome number estimates for sturgeon species. It is also tempting to speculate that these heterochromatic chromosomes represent accessory B chromosomes and that they appear as univalents or self-paired foldback elements in meiotic pachytene spreads. The large and variable chromosome number of this species makes it impossible to cytologically distinguish between a ZW:ZZ and a ZO:ZZ sex chromosome system on the basis of chromosome number alone.

Future Directions

Sex determination system of white sturgeon: There are two technically difficult but conceptually simple experiments that would further advance this field of research. The first would be undertaking long term progeny tests with the female gynogens produced in Chapter 2 to see if any of them are WW "super"females as would be evidenced by the production of all female offspring when crossed to a normal male. If such animals exist they would have an obvious value as the broodstock for commercial caviar production facilities. The absence of gynogens producing all female populations in such progeny tests would suggest that the

"WW" phenotype is inviable, despite the apparent morphological similarity of the W and the Z chromosomes in this species.

The second experiment would involve performing synaptonemal complex analyses on white sturgeon oocytes. Obtaining primary oocytes undergoing meiotic prophase could prove to be difficult although it has been successfully done in rainbow trout (Oliveira et al. 1995a). The completion of the first meiotic division in the oocytes takes place at ovulation and so it would be necessary to collect the eggs well prior to this time. Synaptonemal complex analysis of oocytes may reveal a ZW bivalent with the atypical pairing behavior characteristically associated with heteromorphic sex chromosomes. There is always the possibility that there is so little structural divergence between the Z and the W chromosomes that they will pair normally during pachytene and thus be indistinguishable from regular autosomes. If a heteromorphic sex chromosome pair is evident then a technique such as chromosome scraping (Ponce de León 1996) could potentially be used to isolate sex-chromosome specific DNA sequences from the region of the chromosomes (most likely distal from the centromere) that is undergoing abnormal pairing. These sequences could then be used to develop a molecular marker that could be used distinguish between the ZW females and ZZ males and possibly the WW "super"females.

All of the data presented in this research cannot unequivocally rule out ZO:ZZ female heterogamety. It seems unlikely that this system, which relies on an autosomal balance genetic switch mechanism, would be present in a polyploid-derived species because of the variation in sex chromosome:autosome ratios that polyploidy generates. However a system such as this may have developed subsequent to the process of diploidization in white sturgeon. The sex

ratios of the gynogens and triploids observed in experiment 2 provide strong evidence in favor of ZW:ZZ female heterogamety. It is very difficult to entirely dismiss the possibility of ZO:ZZ female heterogamety in the absence of either sex-specific DNA or invariant sex-specific chromosome numbers.

Cytogenetic analysis of white sturgeon: The cytogenetic data strongly suggest that there are chromosome number and karyotypic differences between the California and Italian white sturgeon populations. Given that these differences may be related to the fact that some of the white sturgeon exported to Italy were derived from wild Columbia River broodstock, it would be interesting to examine the karyotypes of known Columbia River white sturgeon to determine if they constitute a different chromosomal race, or even species, to the California San Francisco Bay population. Meiotic pachytene spreads and mitotic metaphase spreads both revealed the presence of apparent accessory chromosomes in the white sturgeon karyotype. It would also be interesting to examine the behavior of these accessory chromosomes during metaphase I and anaphase I of meiosis using light microscopy.

General Implications

This research has provided a lot information that is of interest and value to sturgeon researchers and aquaculturists. Although attempts to obtain sex-specific DNA sequences and monosex populations were unsuccessful, an unique insight regarding the basic sex determination mechanism of white sturgeon was obtained, and this may help to achieve these commercially-important goals in the future. The effectiveness of the treatments designed to induce gynogenesis and second polar body retention was variable (Chapter 2), however the

high percentage of gynogenetic and polyploid sturgeon produced in some experiments suggests that there is a potential to use these techniques for further research and applications to aquaculture. Commercial efforts to produce monosex female sturgeon populations should now focus on proving the existence and viability of WW "super"female gynogens. The isolation of sex-specific DNA sequences would assist this effort, but may require the use of more complex techniques (e.g. chromosome scraping) than were used in these studies.

The chromosome number variations and karyotypic differences that were found between the California and Italian white sturgeon populations may be related to the origin of the population founders. Further cytogenetic study is required to determine whether the Columbia River sturgeon constitute a different chromosomal race or even species to the California San Francisco Bay population. If these two populations do prove to be unique chromosomal races then this would have profound implications for the management of wild sturgeon stocks from these two locations.

Summary

Data support the hypothesis that white sturgeon has a ZW:ZZ female heterogametic genetic sex determination system. They further suggest that the W chromosome undergoes recombination with the Z chromosome and that the primary sex-determining element segregates independently of the centromere. Attempts to isolate sex-specific DNA sequences from white sturgeon of either sex were unsuccessful suggesting that sex-specific DNA may comprise a very small portion of the genome. White sturgeon sex chromosomes appear to be at a very early stage of sex chromosome differentiation and evolution.

REFERENCES

Agnèse, J.-F., Oberdorff, T., and Ozouf-Costaz, C. 1990. Karyotypic study of some species of family Mochokidae (Pisces, Siluriformes): evidence of female heterogamety. J. Fish Biol., **37**: 375-381.

Allendorf, F.W., and Thorgaard, G.H. 1984. Tetraploidy and the evolution of Salmonid fishes. *In* Evolutionary genetics of fishes. *Edited by* B. J. Turner. Plenum Press, New York. pp. 1-53.

Almeida-Toledo, L.F., Foresti, F., and Toledo-Filho, S.A. 1984. Complex sex-chromosome system in *Eigenmannia sp.* (Pisces, Gymnotiformes). Genetica (The Hague), **64**: 165-169.

Almeida-Toledo, L.F., Viegas-Péquignot, Foresti, F., and Toledo-Filho, S.A. 1988. BrdU replication patterns demonstrating chromosome homeologies in two fish species, genus *Eigenmannia*. Cytogenet. Cell Genet., **48**: 117-120.

Amaral, M.E.J., and Jorge, W. 1994. Synaptonemal complex analysis of the sex bivalent in goats. Genet. Sel. Evol., **26**: 369-375.

Amemiya, C.T., and Gold, J.R. 1986. Chromomycin A_3 stains nucleolus organizer regions of fish chromosomes. Copeia, **1986**: 226-231.

Andreata, A.A., Almeida-Toledo, L.F., Oliveira, C., and Toledo-Filho, S.A. 1993. Chromosome studies in Hypoptopomatinae (Pisces, Siluriformes, Loricariidae) II. ZZ/ZW sex-chromosome system, B chromosomes, and constitutive heterochromatin differentiation in *Microlepidogaster leucofrenatus*. Cytogenet. Cell Genet., **63**: 215-220.

Antoniou, E., Oulmouden, A., Skidmore, C.J., and Urdaci, M. 1996. Isolation of a Y-specific bovine DNA fragment using minisatellite-related PCR primers. Animal Genetics, **27**: 214-215.

Arai, R., and Swander, Y. 1974. Chromosomes of Japanese gobioid fish. (I). Bull. Nat. Sci. Mus. Tokyo, **17**: 97-102.

Arefjev, V.A. 1983. Polykaryogrammic analysis of ship, *Acipenser nudiventris*, Lovetsky (Acipenseridae, Chondrostei). (In Russian). Vopr. Ikhtiol., **23**: 209-218.

Arefjev, V.A. 1989. Karyotype variability in successive generations after hybridization between the great sturgeon, *Huso huso* (L.), and the sterlet, *Acipenser ruthenus* L. J. Fish Biol., **35**: 819 - 828.

Arefjev, V.A., and Nikolaev, A.I. 1991. Cytological analysis of the reciprocal hybrids between low- and high-chromosome acipenserids, the great sturgeon, *Huso huso* L., and the Russian sturgeon, *Acipenser gueldenstaedti* Brandt. Cytologia, **56**: 495-502.

Arlati, G., Belysheva, L.A., and Kaidanova, T.I. 1995. Karyological analysis of *Acipenser naccarii* (Bonaparte). *In* International symposium on sturgeons. *Edited by* A. D. Gersonovich and T. I. J. Smith. VNIRO Publishing, Moscow, Russia. pp. 119-123.

Ashley, T. 1987. Nonhomologous synapsis of the XY during early pachynema in In(X)1H male mice. Genetica (The Hague), **72**: 81-84.

Atz, J.W., and Smith, C.L. 1976. Hermaphroditism and gonadal teratoma-like growths in sturgeon (Acipenser). Bull. Southern California Acad. Sci., **75**: 119-126.

Avtalion, R.R., and Don, J. 1990. Sex determining genes in tilapia: a model of genetic recombination emerging from sex ratio results of three generations of diploid gynogenetic *Oreochromis aureus*. J. Fish Biol., **37**: 167-173.

Beçak, M.L., Beçak, W., and Rabello, M.N. 1966. Cytological evidence of constant tetraploidy in the bisexual South American frog, *Odontophrynus americanus*. Chromosoma, **19**: 188-193.

Bertollo, L.A.C., and Cavallaro, Z.I. 1992. A highly differentiated ZZ/ZW sex chromosome system in Characidae fish, *Triportheus guentheri*. Cytogenet. Cell Genet., **60**: 60-63.

Birstein, V.J. 1993. Sturgeons and paddlefishes: Threatened fishes in need of conservation. Conservation Biology, 7: 773-787.

Birstein, V.J., Poletaev, A.I., and Goncharov., B.F. 1993. DNA content in Eurasian sturgeon species determined by flow cytometry. Cytometry, **14**: 377-383.

Birstein, V.J., and Vasiliev, V.P. 1987. Tetraploid-octoploid relationships and karyological evolution in the order Acipenseriformes (Pisces): karyotypes, nucleoli, and nucleolus-organizer regions in four acipenserid species. Genetica (The Hague), **72**: 3-12.

Blacklidge, K.H., and Bidwell, C.A. 1993. Three ploidy levels indicated by genome quantification in Acipenseriformes of North America. J. Hered., **84**: 427-430.

Bridges, C.B. 1921. Triploid intersexes in *Drosophila melanogaster*. Science, **54**: 252-254.

Bull, J.J. 1983. Evolution of sex determining mechanisms. Benjamin Cummings, Menlo Park, Ca.

Burtzev, I.A., Nikoljukin, N.I., and Serebryakova., E.V. 1976. Karyology of the Acipenseridae family in relation to the hybridization and taxonomy problems. Ichthyol., **8**: 27-34.

Campos, H.H. 1972. Karyology of three galaxiid fishes, *Galaxias maculatus*, *G. platei*, and *Brachigalaxias vullocki*. Copeia, **1972**: 368-370.

Capriglione, T., Morescalchi, A., Olmo, E., Rocco, L., Stingo, V., and Manzo, S. 1994. Satellite DNAs, heterochromatin, and sex chromosomes in *Chionodraco hamatus* (Channichthyidae, Perciformes). Polar Biol., **14**: 285-290.

Carlson, D.M., Kettler, M.K., Fisher, S.E., and Whitt., G.S. 1982. Low genetic variability in paddlefish populations. Copeia, **1982**: 721-725.

Carter, R.E., Mair, G.C., Skibinski, D.O.F., Parkin, D.T., and Beardmore, J.A. 1991. The application of DNA fingerprinting in the analysis of gynogenesis in tilapia. Aquaculture, **95**: 41-52.

Cavalier-Smith, T. 1978. Nuclear volume control by nucleoskeletal DNA, selection for cell volume and cell growth rate, and the solution to the C-value paradox. J. Cell Sci., **34**: 247-278.

Chapman, F.A., Van Eenennaam, J.P., and Doroshov, S.I. 1996. The reproductive condition of white sturgeon, *Acipenser transmontanus*, in San Francisco Bay, California. Fish. Bull., **94**: 628-634.

Charlesworth, B. 1991. The evolution of sex chromosomes. Science, **251**: 1030-1033.

Chen, T.R. 1969. Karyological heterogamety of deep-sea fishes. Postilla, **130**: 1-29.

Chen, T.R., and Ebeling, A.W. 1968. Karyological evidence of female heterogamety in the mosquitofish, *Gambussia affinis*. Copeia, **1968**: 70-75.

Chourrout, D. 1986. Genetic manipulations in fish: Review of methods. *In* Proc. world symposium on selection, hybridization, and genetic engineering in aquaculture, Bordeaux, France, 27-30 May, 1986. Vol. II. *Edited by* K. Tiews. Heenenmann, Berlin. pp. 111-126.

Chourrout, D., and Quillet, E. 1982. Induced gynogenesis in the rainbow trout: Sex and survival of the progenies. Production of all-triploid populations. Theor. Appl. Genet., **63**: 201-205.

Conte, F.S., Doroshov, S.I., Lutes, P.B., and Strange., E.M. 1988. Hatchery manual for the white sturgeon (*Acipenser transmontanus* Richardson): with application to other North American acipenseridae. UC Cooperative Extension, Oakland, Ca.

Counce, S.J., and Meyer, G.F. 1973. Differentiation of the synaptonemal complex and the kinetochore in *Locusta* spermatocytes studied by whole mount electron microscopy. Chromosoma, **44**: 231-253.

Cummings, S.A., and Thorgaard., G.H. 1994. Extraction of DNA from fish blood and sperm. BioTechniques, **17**: 427-429.

Cushwa, W.T., Dodds, K.G., Crawford, A.M., and Medrano, J.F. 1996. Identification and genetic mapping of random amplified polymorphic DNA (RAPD) markers to the sheep genome. Mamm. Genome, 7: 580-585.

Davis, K.B., Simco, B.A., Goudie, C.A., Parker, N.C., Cauldwell, W., and Snellgrove, R. 1990. Hormonal sex manipulation and evidence for female homogamety in channel catfish. Gen. Comp. Endocrinol., **78**: 218-223.

Del Cerro, A.L., Fernández, A., and Santos, J.L. 1994. Spreading synaptonemal complexes of B isochromosomes in the grasshopper *Omocestus burri*. Genome, **37**: 1035-1040.

Dettlaff, T.A., and Dettlaff, A.A. 1961. On relative dimensionless characteristics of the development duration in embryology. Arch. Biol., **72**: 1-16.

Dettlaff, T.A., Ginsburg, A.S., and Schmalhausen, O.I. 1993. Sturgeon Fishes: Developmental Biology and Aquaculture. Springer-Verlag, New York.

Devlin, R.H., McNeil, B.K., Groves, T.D.D., and Donaldson, E.M. 1991. Isolation of a Y-chromosomal DNA probe capable of determining genetic sex in chinook salmon (*Oncorhynchus tshawytscha*). Can. J. Fish. Aquat. Sci., **48**: 1606-1612.

Dingerkus, G., and Howell, W.M. 1976. Karyotypic analysis and evidence of tetraploidy in the North American Paddlefish, *Polyodon spathula*. Science, **194**: 842-844.

Dollin, A.E., Murray, J.D., and Gillies, C.B. 1989. Synaptonemal complex analysis of hybrid cattle. I. Pachytene substaging and the normal full bloods. Genome, **32**: 856-864.

Drew, A.C., and Brindley, P.J. 1995. Female-specific sequences isolated from *Schistosoma mansoni* by representational difference analysis. Mol. Biochem. Parasitol., **71**: 173-181.

Du, S.J., Devlin, R.H., and Hew, C.L. 1993. Genomic structure of growth hormone genes in chinook salmon (*Oncorhynchus tshawytscha*): presence of two functional genes, GH-I and GH-II, and a male-specific pseudogene, GH-ψ. DNA Cell Biol., **12**: 739-751.

Ebeling, A.W., and Chen, T.R. 1970. Heterogamety in teleostean fishes. Trans. Am. Fish Soc., **99**: 131-138.

Ferreiro, C., Medrano, J.F., and Gall, G.A.E. 1989. Genome analysis of rainbow trout and sturgeon with restriction enzymes and hybridization with a ZFY gene derived probe to identify sex. Aquaculture, **81**: 245-251.

Fletcher, H.L., and Hewitt, G.M. 1988. Synaptonemal complexes of univalent B chromosomes in the grasshoppers *Euthystira brachyptera* and *Myrmeleotettix maculatus*. Heredity, **60**: 383-386.

Fletcher, S., Darragh, D., Fan, Y., Grounds, M.D., Fisher, C.J., and Beilharz, M.W. 1993. Specific cloning of DNA fragments unique to the dog Y chromosome. GATA, **10**: 77-83.

Fontana, F. 1994. Chromosomal nucleolar organizing regions in four sturgeon species as markers of karyotype evolution in Acipenseriformes (Pisces). Genome, **37**: 888-892.

Fontana, F., Lanfredi, M., Rossi, R., Bronzi, P., and Arlati, G. 1996. Karyotypic characterization of *Acipenser gueldenstaedti* with C-, $AgNO_3$, and fluorescence banding techniques. Ital. J. Zool., **63**: 113-118.

Fontana, F., and Colombo, G. 1974. The chromosomes of Italian sturgeons. Experientia, **30**: 739-742.

Fontana, F., Jankovic, D., and Zivkovic, S. 1975. Somatic chromosomes of *Acipenser ruthenus* L. Arch. biol. nauka, Beograd, **27**: 33-35.

Fontana, F., Lanfredi, M., Rossi, R., Bronzi, P., and Arlati, G. 1995. Established cell lines from three sturgeon species. Sturgeon Quart., **3**: 6-7.

Foresti, F., Oliveira, C., Galetti, P.M., Jr., and Almeida-Toledo, L.F. 1993. Synaptonemal complex analysis in spermatocytes of tilapia, *Oreochromis niloticus* (Pisces, Cichlidae). Genome, **36**: 1124-1128.

Galetti, P.M., Jr., and Foresti, F. 1986. Evolution of the ZZ/ZW system in *Leporinus* (Pisces, Anostomidae). Role of constitutive heterochromatin. Cytogenet. Cell Genet., **43**: 43-46.

Galetti, P.M., Jr., Foresti, F., Bertollo, L.A.C., and Filho, O.M. 1981. Heteromorphic sex chromosomes in three species of the genus *Leporinus* (Pisces, Anostomidae). Cytogenet. Cell Genet., **29**: 138-142.

Gillies, C.B. 1975. Synaptonemal complex and chromosome structure. Ann. Rev. Genet., **9**: 91-109.

Gillies, C.B. 1989. Chromosome pairing and fertility in polyploids. *In* Fertility and chromosome pairing: Recent studies in plants and animals. *Edited by* C. B. Gillies. CRC Press, Boca Raton, Fl. pp. 137-176.

Gold, J.R., Li, Y.C., Shipley, N.S., and Powers, P.K. 1990a. Improved methods for working with fish chromosomes with a review of metaphase chromosome banding. J. Fish Biol., **37**: 563-575.

Gold, J.R., Ragland, C.J., and Schliesing, L.J. 1990b. Genome size variation and evolution in North American cyprinid fishes. Genet. Sel. Evol., **22**: 11-27.

Goldstein, P., and Triantaphyllou, A.C. 1980. Karyotype analysis of the plant-parasitic nematode *Heterodera glycines* by electron microscopy. II. The tetraploid and an aneuploid hybrid. J. Cell Sci., **43**: 225-237.

Goldstein, P., and Triantaphyllou, A.C. 1981. Pachytene karyotype analysis of tetraploid *Meloidogyne hapla* females by electron microscopy. Chromosoma, **84**: 405-412.

Gorshkova, G., Gorshkov, S., Gordin, H., and Knibb, W. 1996. Karyological studies in hybrids of beluga *Huso huso* (L.) and the Russian sturgeon *Acipenser güldenstädti* Brandt. The Israeli Journal of Aquaculture, **48**: 35-39.

Greenbaum, I.F., Hale, D.W., and Fuxa, K.P. 1986. The mechanism of autosomal synapsis and the substaging of zygonema and pachynema from deer mouse spermatocytes. Chromosoma, **93**: 203-212.

Griffiths, R., and Tiwari, B. 1993. The isolation of molecular genetic markers for the identification of sex. Proc. Natl. Acad. Sci. USA, **90**: 8324-8326.

Grunina, A.S., and Neifakh, A.A. 1991. Induction of diploid androgenesis in the Siberian sturgeon *Acipenser baeri* Brandt. Ontogeny, **22**: 53-56.

Haaf, T., and Schmid, M. 1984. An early stage of ZW/ZZ sex chromosome differentiation in *Poecilia sphenops* var. *melanistica* (Poeciliidae, Cyprinodontiformes). Chromosoma, **89**: 37-41.

Hagerup, O. 1927. *Empetrum hermaphroditum* (Lge) Hagerup. A new tetraploid, bisexual species. Dansk Botanisk Arkiv., **5**: 1-17.

Hale, D.W., and Greenbaum, I.F. 1986. The behavior and morphology of the X and Y chromosomes during prophase I in the Sitka deer mouse (*Peromyscus sitkensis*). Chromosoma, **94**: 235-242.

Hale, D.W., Ryder, E.J., Sudman, P.D., and Greenbaum, I.F. 1988. Application of synaptonemal complex techniques for determination of diploid number and chromosomal morphology in birds. Auk, **105**: 776-779.

Hammerman, I.S., and Avtalion, R.R. 1979. The sex-ratio as a tool for the determination of genotype - a model of autosomal influence. Theor. Appl. Genet., **55**: 177-187.

Hartley, S.E. 1991. C, Q, and restriction enzyme banding of the chromosomes in brook trout (*Salvelinus fontinalis*) and Arctic charr (*Salvelinus alpinus*). Hereditas, **114**: 253-261.

Hedin, M.C., Sudman, P.D., Greenbaum, I.F., and Sites, J.W., Jr. 1990. Synaptonemal complex analysis of sex chromosome pairing in the common ground skink, *Scincella lateralis* (Sauria, Scincidae). Copeia, **1990**: 1114-1122.

Hedrick, R.P., McDowell, T.S., Rosemark, R., Aronstein, D., and Lannan, C.N. 1991. Two cell lines from white sturgeon. Trans. Am. Fish Soc., **120**: 528-534.

Hinegardner, R. 1976. The cellular DNA content of sharks and some other fishes. Comp. Biochem. Physiol., **55B**: 367-370.

Hinegardner, R., and Rosen, D.E. 1972. Cellular DNA content and the evolution of teleostean fishes. The American Naturalist, **106**: 621-644.

Hodgkin, J. 1992. Genetic sex determination mechanisms and evolution. Bioessays, **14**: 253-261.

Hogan, K.M., Jarrell, G.H., Ryder, E.J., and Greenbaum, I.F. 1992. Heterosynapsis and axial equalization of the sex chromosomes of the northern bobwhite quail. Cytogenet. Cell Genet., **60**: 131-134.

Holčík, J. 1986. General introduction to fishes. Acipenseriformes. The freshwater fishes of Europe. Vol. I. Part II. AULA-Verlag, Wiesbaden, Germany.

Hormaza, J.I., Dollo, L., and Polito, V.S. 1994. Identification of a RAPD marker linked to sex determination in *Pistacia vera* using bulked segregant analysis. Theor. Appl. Genet., **89**: 9-13.

Horvat, S., and Medrano, J.F. 1996. Fine genetic mapping of the region surrounding the *high growth* (*hg*) locus in mouse Chromosome 10: targeting random amplified polymorphic DNA (RAPD) markers. Mamm. Genome, **7**: 312-314.

Howell, B.R., Baynes, S.M., and Thompson, D. 1995. Progress towards the identification of the sex-determining mechanism of the sole, *Sole solea* (L.), by the induction of diploid gynogenesis. Aquaculture Res., **26**: 135-140.

Hunter, G.A., Donaldson, E.M., Goetz, F.W., and Edgell, P.R. 1982. Production of all-female and sterile coho salmon, and experimental evidence for male heterogamety. Trans. Am. Fish Soc., **111**: 367-372.

Hunter, G.A., Donaldson, E.M., Stoss, J., and Baker., I. 1983. Production of monosex females groups of chinook salmon (*Oncorhynchus tshawytscha*) by the fertilization of normal ova with sperm from sex-reversed females. Aquaculture, **33**: 355-364.

Ihssen, P.E., McKay, L.R., McMillan, I., and Phillips, R.B. 1990. Ploidy manipulation and gynogenesis in fishes: Cytogenetic and fisheries applications. Trans. Am. Fish Soc., **119**: 698-717.

Ijiri, K.-I., and Egami, N. 1980. Hertwig effect caused by UV-irradiation of sperm of *Oryzias latipes* (Teleost) and its photoreactivation. Mut. Res., **69**: 241-248.

Jiménez, R., Burgos, M., Sánchez, A., and Díaz de la Guardia, R. 1990. Synaptonemal complex analysis of spermatocytes of *Talpa occidentalis* (Insectivora, Mammalia): autosomal synapsis and substaging of zygonema and pachynema. Cytogenet. Cell Genet., **53**: 97-102.

Jones, K.W. 1989. Inactivation phenomena in the evolution and function of sex chromosomes. *In* Evolutionary mechanisms in sex determination. *Edited by* S. S. Wachtel. CRC Press, Boca Raton, Fl. pp. 69-90.

Jones, R.N., and Rees, H. 1982. B Chromosomes. Academic Press, London.

Kallman, K.D. 1965. Genetics and geography of sex determination in the poecilid fish, *Xiphophorus maculatus*. Zoologica, **50**: 151-190.

Kallman, K.D. 1984. A new look at sex determination in Poeciliid fishes. *In* Evolutionary genetics of fishes. *Edited by* B. J. Turner. Plenum Press, New York. pp. 95-171.

Kawamura, N., Sahara, K., and Iizuka, T. 1994. Preferential pairing of sex chromosomes in the tetraploid silkworms (*Bombyx mori*). Hereditas, **121**: 73-78.

Khazanehdari, K.A., and Jones, G.H. 1996. Meiotic synapsis of the *Allium porrum* B chromosome: evidence for a derived isochromosome origin. Genome, **39**: 1199-1204.

Khuda-Bukhsh, A.R., and Manna, G.K. 1974. Somatic chromosomes in seven species of teleostean fish. Chrom. Inf. Serv., **17**: 5-6.

Kirpichnikov, V.S. 1981. Genetic basis of fish selection. Springer, Berlin.

Kobel, H.R. 1985. Sex determination in polyploid *Xenopus*. South African J. Sci., **81**: 205-206.

Kodama, Y., Yoshida, M.C., and Sasaki, M. 1980. An improved silver staining technique for nucleolus organizer regions by using nylon cloth. Jpn. J. Human Genet., **25**: 229-233.

Koehler, M.R., Neuhaus, D., Engel, W., Schartl, M., and Schmid, M. 1995. Evidence for an unusual ZW/ZW'/ZZ sex-chromosome system in *Scardinius erythrophthalmus* (Pisces, Cyprinidae), as detected by cytogenetic and H-Y antigen analyses. Cytogenet. Cell Genet., **71**: 356-362.

Kowtal, G.V. 1986. Preliminary experiments in induction of polyploidy, gynogenesis, and androgenesis in the white sturgeon, *Acipenser transmontanus* Richardson. *In* Proc. world symposium on selection, hybridization, and genetic engineering in aquaculture, Bordeaux, France, 27-30 May, 1986. Vol. II. *Edited by* K. Tiews. Heenenmann, Berlin. pp. 317-324.

Kunkel, L.M., Monaco, A.P., Middlesworth, W., Ochs, H.D., and Latt, S.A. 1985. Specific cloning of DNA absent from the DNA of a male with an X chromosome deletion. Proc. Natl. Acad. Sci. USA, **82**: 4778-4782.

Kuz'min, Y.V. 1991. Comparative study of isozymes of muscle malate dehydrogenase of the Ob population of the Siberian sturgeon, *Acipenser baeri*, and the Don and Kama sterlet, *Acipenser ruthemus*. J. Ichthyol., **31**: 139-144.

Lamar, E.E., and Palmer, E. 1984. Y-encoded, species-specific DNA in mice: Evidence that the Y chromosome exists in two polymorphic forms in inbred strains. Cell, **37**: 171-177.

Le Grande, W.H. 1975. Karyology of six species of Louisiana flatfishes (Pleuronectiformes: Osteichthyes). Copeia, **1975**: 516-522.

Lemoine, H.L., Jr.,, and Smith, L.T. 1980. Polyploidy induced in brook trout by cold shock. Trans. Am. Fish Soc., **109**: 626-631.

Levin, I., Crittenden, L.B., and Dodgson, J.B. 1993. Genetic map of the chicken Z chromosome using random amplified polymorphic DNA (RAPD) markers. Genomics, **16**: 224-230.

Levy, M. 1976. Altered glycoflavone expression in induced autotetraploids of *Phlox drummondii*. Biochem. System. Ecol., **4**: 249.

Li, M.F., Marrayatt, V., Annand, C., and Odense, P. 1985. Fish cell culture: two newly developed cell lines from Atlantic sturgeon (*Acipenser oxyrhynchus*) and guppy (*Poecilia reticulata*). Can. J. Zool., **63**: 2867-2874.

Lieder, U. 1963. On presumptive sex chromosomes in Perca, Acerina and Anguilla. Biol. Zbl., **82**: 296-302.

Lisitsyn, N., Lisitsyn, N., and Wigler, M. 1993. Cloning the differences between two complex genomes. Science, **259**: 946-951.

Lisitsyn, N.A. 1995. Representational difference analysis: finding the differences between genomes. Trends Genet., **11**: 303-307.

Lisitsyn, N.A., Segre, J.A., Kusumi, K., Lisitsyn, N.M., Nadeau, J.H., Frankel, W.N., Wigler, M.H., and Lander, E.S. 1994. Direct isolation of polymorphic markers linked to a trait by genetically directed representational difference analysis. Nature Genet., **6**: 57-63.

Lloyd, M.A., Fields, M.J., and Thorgaard, G.H. 1989. Bkm minisatellite sequences are not sex associated but reveal DNA fingerprint polymorphisms in rainbow trout. Genome, **32**: 865-868.

Lloyd, M.A., and Thorgaard, G.H. 1988. Restriction endonuclease banding of rainbow trout chromosomes. Chromosoma, **96**: 171-177.

Logan, S.H., Johnston, W.E., and Doroshov, S.I. 1995. Economics of joint production of sturgeon (*Acipenser transmontanus* Richardson) and roe for caviar. Aquaculture, **130**: 299-316.

Loidl, J., and Schweizer, D. 1992. Synaptonemal complexes of *Xenopus laevis*. J. Hered., **83**: 307-309.

Mair, G.C., Scott, A.G., Penman, D.G., Skibinski, D.O.F., and Beardmore, J.A. 1991a. Sex determination in the genus *Oreochromis*. 2. Sex-reversal, hybridization, gynogenesis and triploidy in *O. aureus* Steindachner. Theor. Appl. Genet., **82**: 153-160.

Mair, G.C., Scott, A.G., Penman, D.G., Skibinski, D.O.F., and Beardmore, J.A. 1991b. Sex determination in the genus *Oreochromis*. 2. Sex reversal, hybridization, gynogenesis and triploidy in *O. niloticus* (L.). Theor. Appl. Genet., **82**: 144-152.

Markert, C.L. 1975. Genetics and Evolution. Isozymes. Vol. IV. Academic Press, New York.

Mayr, B., Kalat, M., Rab, P., and Lambrou, M. 1987. Band karyotypes and specific types of heterochromatin in several species of European percid fishes (Percida, Pisces). Genetica (The Hague), **75**: 199-205.

McElreavey, K., Vilain, E., Cotinot, C., Payen, E., and Fellous, M. 1993. Control of sex determination in animals. Eur. J. Biochem., **218**: 769-783.

McKeown, M. 1994. Sex determination and differentiation. Dev. Genet., **15**: 201-204.

McKeown, M., and Madigan, S.J. 1992. Sex determination and differentiation in invertebrates: *Drosophila* and *Caenorhabditis elegans*. Current Opinion in Cell Biology, **4**: 948-954.

McLaren, A. 1991. Sex determination in mammals. Oxford Reviews of Reproductive Biology, **13**: 1-33.

Medrano, J.F., Aasen, E., and Sharrow, L. 1990. DNA extraction from nucleated red blood cells. BioTechniques, **8**: 43.

Mestriner, C.A., Bertollo, L.A.C., and Galletti, P.M., Jr. 1995. Chromosome banding and synaptonemal complexes in *Leporinus lacustris* (Pisces, Anostomidae): analysis of a sex system. Chromosome Res., **3**: 440-443.

Michele, J.L., and Takahashi, C.S. 1977. Comparative cytology of *Tilapia rendalli* and *Geophagus brasilliensis* (Cichlidae, Pisces). Cytologia, **42**: 535-537.

Michele, J.L., Takahashi, C.S., and Ferrari, I. 1977. Karyotypic study of some species of the family Loricariidae (Pisces). Cytologia, **42**: 539-546.

Michelmore, R.W., Paran, I., and Kesseli, R.V. 1991. Identification of markers linked to disease-resistance genes by bulked segregant analysis. A rapid method to detect markers in specific genomic regions by using segregating populations. Proc. Nat. Acad. Sci., USA, **88**: 9828-9832.

Moreira-Filho, O., Bertollo, L.A.C., and Galetti, P.M., Jr. 1993. Distribution of sex chromosome mechanisms in neotropical fish and description of a ZZ/ZW system in *Parodon hilarii* (Parodontidae). Caryologia, **46**: 115-125.

Morescalchi, A. 1991. Chromosomes, sex determination and environment in teleosteans. *In* International symposium on origin and evolution of sex. Siena, Italy. *Edited by* R. Dallai. Mucchi, Modena. pp. 137-149.

Morescalchi, A., Hureau, J.C., Olmo, E., Ozouf-Costaz, C., Pisano, E., and Stanyon, R. 1992. A multiple sex-chromosome system in Antarctic ice-fishes. Polar Biol., **11**: 655-661.

Moses, M.J. 1977. Synaptonemal complex karyotyping in spermatocytes of the Chinese hamster (*Cricetulus griseus*). II. Morphology of the XY pair in spread preparations. Chromosoma, **60**: 127-137.

Moses, M.J. 1980. New cytogenetic studies on mammalian meiosis. *In* Animal models in human reproduction. *Edited by* M. Serio and L. Martini. Academic Press, New York. pp. 169-190.

Moses, M.J., and Poorman, P.A. 1981. Synaptonemal complex analysis of mouse chromosomal rearrangements. II. Synaptic adjustment in a tandem duplication. Chromosoma, **81**: 519-535.

Mulcahy, D.L., Weeden, N.F., Kesseli, R., and Carroll, S.B. 1992. DNA probes for the Y-chromosome of *Silene latifolia*, a dioecious angiosperm. Sex. Plant Reprod., **5**: 86-88.

Muller, H.J. 1925. Why polyploidy is rarer in animals than in plants. Am. Nat., **59**: 346-353.

Murofushi, M. 1980. Sexual dimorphism in chromosomes of fishes. (In Japanese). Fish Genet. Breed. Sci. (Suisan Ikushu), **5**: 27-30.

Nace, G.W., Richards, C.M., and Asher, J.H., Jr. 1970. Parthenogenesis and genetic variability. I. Linkage and inbreeding estimations in the frog, *Rana pipiens*. Genetics, **66**: 349-368.

Nakayama, I., Foresti, F., Tewari, R., Schartl, M., and Chourrout, D. 1994. Sex chromosome polymorphism and heterogametic males revealed by two cloned DNA probes in the ZW/ZZ fish *Leporinus elongatus*. Chromosoma, **103**: 31-39.

Nanda, I., Feichtinger, W., Schmid, M., Schröder, J. H., Zischler, H., and Epplen, J. T. 1990. Simple repetitive sequences are associated with differentiation of the sex chromosomes in the guppy fish. J. Mol. Evol., **30**: 456-462.

Nanda, I., Schartl, M., Feichtinger, W., Epplen, J. T., and Schmid, M. 1992. Early stages of sex chromosome differentiation in fish as analyzed by simple repetitive DNA sequences. Chromosoma, **101**: 301-310.

Navin, A., Prekeris, R., Lisitsyn, N.A., Sonti, M.M., Grieco, D.A., Narayanswami, S., Lander, E.S., and Simpson, E.M. 1996. Mouse Y-specific repeats isolated by whole chromosome representational difference analysis. Genomics, **36**: 349-353.

Nikonorov, S.I., Ryabova, G.D., Kutergina, I.G., and Ofitserov, M.V. 1985. Electrophoretic analysis of genetic variability of the starred sturgeon *Acipenser stellatus*. Dokl. AN SSSR, **284**: 209-211.

Nishikawa, S., and Sakamoto, K. 1978. Comparative studies on the chromosomes in Japanese fishes - IV. Somatic chromosomes of two lizardfishes. (In Japanese with English summary). J. Shimonoseki Univ. Fish., **27**: 113-117.

Nogusa, S. 1957. Chromosome studies in Pisces. VI. The X-Y chromosomes found in *Cottus pollux* Günter (Cottidae). J. Fac. Sci. Hokkaido Univ. Ser. VI Zool., **13**: 289-292.

Ohno, S. 1967. Sex chromosomes and sex-linked genes. *Edited by* A. Labhart, T. Mann, and L. T. Samuels. Monographs on endocrinology. Vol. I. Springer-Verlag, Berlin.

Ohno, S. 1970. Evolution by gene duplication. Springer-Verlag, New York.

Ohno, S., Muramoto, J., Stenius, C., Christian, L., Kittrell, W.A., and Atkin, N.B. 1969. Microchromosomes of the Holocephalian, Chondrostean and Holostean Fishes. Chromosoma, **26**: 35-40.

Ojima, Y. 1983. Fish cytogenetics. *In* Chromosomes in evolution of eukaryotic groups. Vol. I. *Edited by* A. K. Sharma and A. Sharma. CRC Press, Boca Raton, Fl. pp. 111-146.

Oliveira, C., Foresti, F., Rigolino, M.G., and Tabata, Y.A. 1995a. Synaptonemal complex analysis in spermatocytes and oocytes of rainbow trout, *Oncorhynchus mykiss* (Pisces, Salmonidae): the process of autosome and sex chromosome synapsis. Chromosome Res., **3**: 182-190.

Oliveira, C., Foresti, F., Rigolino, M.G., and Tabata, Y.A. 1995b. Synaptonemal complex formation in spermatocytes of the autotriploid rainbow trout, *Oncorhynchus mykiss* (Pisces, Salmonidae). Hereditas, **123**: 215-220.

Paran, I., and Michelmore, R.W. 1993. Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. Theor. Appl. Genetics, **85**: 985-993.

Park, E.H., and King, Y.S. 1979. Karyological confirmation of conspicuous ZW sex chromosomes in two species of Pacific anguilloid fishes (Anguilliformes: Teleostomi). Cytogenet. Cell Genet., **23**: 33-38.

Penman, D.J., Shah, M.S., Beadmore, J.A., and Skibinski, D.O.F. 1986. Sex ratios of gynogenetic and triploid tilapia. *In* Proc. world symposium on selection, hybridization, and genetic engineering in aquaculture, Bordeaux, France, 27-30 May, 1986. Vol. II. *Edited by* K. Tiews. Heenenmann, Berlin. pp. 267-274.

Peterson, D.G., Stack, S.M., Healy, J.L., Donohoe, B.S., and Anderson, L.K. 1994. The relationship between synaptonemal complex length and genome size in four vertebrate classes (Osteicthyes, Reptilia, Aves, Mammalia). Chromosome Res., **2**: 153-162.

Peykani, G.R. 1993. Economic feasibility analysis of sturgeon aquaculture in the United States: an application of dynamic linear programming. Ph.D. Dissertation, University of Florida, Gainesville.

Pezold, F. 1984. Evidence for multiple sex chromosomes in the freshwater goby, *Gobionellius shufeldti* (Pisces: Gobiidae). Copeia, **1984**: 235-238.

Phillips, R.B., and Hartley, S.E. 1988. Fluorescent banding patterns of the chromosomes of the genus *Salmo*. Genome, **30**: 193-197.

Phillips, R.B., and Ihssen, P.E. 1985. Identification of sex chromosomes in lake trout (*Salvelinus namaycush*). Cytogenet. Cell Genet., **39**: 14-18.

Ponce de León, F.A., S. Ambady, G. A. Hawkins, S. M Kappes, M. D. Bishop, J. M. Robl, C. W. Beattie. 1996. Development of a bovine X chromosome linkage group and painting probes to assess cattle, sheep, and goat X chromosome segment homologies. Proc. Natl. Acad. Sci. USA, **93**: 3450-3454.

Pongthana, N., Penman, D.J., Karnasuta, J., and McAndrew, B.J. 1995. Induced gynogenesis in the silver barb (*Puntius gonionotus* Bleeker) and evidence for female homogamety. Aquaculture, **135**: 267-276.

Postlethwait, J.H., Johnson, S.L., Midson, C.N., Talbot, W.S., Gates, M., Ballinger, E.W., Africa, D., Andrews, R., Carl, T., Eisen, J.S., Horne, S., Kimmel, C.B., Hutchinson, M., Johnson, M., and Rodriguez, A. 1994. A genetic linkage map for the zebrafish. Science, **264**: 699-703.

Purdom, C.E. 1983. Genetic engineering by the manipulation of chromosomes. Aquaculture, **33**: 287-300.

Purdom, C.E., and Lincoln, R.F. 1973. Chromosomal manipulation in fish. *In* Genetics and mutagenesis of fish. *Edited by* J. H. Schröder. Springer-Verlag, New York. pp. 83-89.

Rab, P. 1986. A note on the karyotype of the sterlet, *Acipenser ruthenus* (Pisces, Acipenseridae). Folia Zool., **35**: 73-78.

Rab, P., Reed, K.M., Ponce de León, F.A., and Phillips, R.B. 1996. A new method for detecting nucleolus organizer regions in fish chromosomes using denaturation and propidium iodide staining. Biotechnic and Histochemistry, **71**: 157-162.

Rab, P., Arefjev, V.A., and Rabova, M. 1996. C-banded karyotype of the sterlet, *Acipenser rethenus*, from the Danube river. Sturgeon Quart., **4**: 10-12.

Rasmussen, S.W., and Holm, P.B. 1979. Chromosome pairing in autotetraploid *Bombyx* females. Mechanism for exclusive bivalent formation. Carlsberg Res. Commun., **44**: 101-125.

Reed, K.M., Sudman, P.D., Sites, J.W., Jr., and Greenbaum, I.F. 1990. Synaptonemal complex analysis of sex chromosomes in two species of *Sceloporus*. Copeia, **1990**: 1122-1129.

Refstie, T., Stoss, J., and Donaldson, E.M. 1982. Production of all female coho salmon (*Oncorhynchus kisutch*) by diploid gynogenesis using irradiated sperm and cold shock. Aquaculture, **29**: 67-82.

Rishi, K.K. 1973. Somatic karyotypes of three teleosts. Genen. Phaenen., **16**: 101-107.

Rishi, K.K. 1975. Somatic and meiotic chromosomes of *Trichogaster fasciatus* (Bl. & Sch.) (Teleostei, Perciformes: Osphronemidae). Genen. Phaenen., **18**: 49-53.

Rishi, K.K. 1976a. Karyotypic studies on four species of fish. Nucleus, **19**: 95-98.

Rishi, K.K. 1976b. Mitotic and meiotic chromosomes of a teleost, *Callichtomis bimaculatus* (Boch) with indications of male heterogamety. Ciencia Cult., **28**: 1171-1173.

Rishi, K.K. 1979. Somatic G-banded chromosomes of *Colisa fasciatus* (Perciformes: Belontidae) and confirmation of female heterogamety. Copeia, **1979**: 146-149.

Rishi, K.K., and Gaur, P. 1976. Cytological female heterogamety in jet-black molly, *Mollienesia sphenops*. Curr. Sci., **45**: 669-670.

Rodionova, M.I., Nikitin, S.V., and Borodin, P.M. 1996. Synaptonemal complex analysis of interspecific hybrids of *Poecilia* (Teleostei, Poecilidae). Brazilian J. Genet., **19**: 231-235.

Romashov, D.D., Golovinskaia, K.A., Beliayeva, V.N., Bakuline, E.D., Pokrovskia, G.L., and Cherfas, N.B. 1960. Diploid radiation gynogenesis in fish. Biophysics, **5**: 524-532.

Romashov, D.D., Nikolyukin, N.I., Belyaeva, V.N., and Timofeeva, N.A. 1963. Possibilities of producing diploid radiation-induced gynogenesis in sturgeons. Radiobiology, **3**: 145-154.

Ryabova, G.D., and Kutergina, I.G. 1990. Allozyme variability of the sturgeon *Acipenser stellatus* (Pallus) from the North Caspian. Genetika, **26**: 902-911.

Sadler, J.R., and Novick, A. 1965. The properties of repressor and the kinetics of its action. J. Mol. Biol., **12**: 305-327.

Saitoh, K. 1989. Multiple sex-chromosome system in a loach fish. Cytogenet. Cell Genet., **52**: 62-64.

Saitoh, Y., and Laemmli, U.K. 1994. Metaphase chromosome structure: bands arise from a differential folding path of the highly AT-rich scaffold. Cell, **76**: 609-622.

Salvador, L.B., and Moreira-Filho, O. 1992. B chromosomes in *Astyanax scabripinnis* (Pisces, Characidae). Heredity, **69**: 50-56.

Sánchez, L., Martínez, P., Viñas, A., and Bouza, C. 1990. Analysis of the structure and variability of nucleolar organizing regions of *Salmo trutta* by C-, Ag-, and restriction endonuclease banding. Cytogenet. Cell Genet., **54**: 6-9.

Santos, J.L., Del Cerro, A.L., and Diéz, M. 1993. Spreading synaptonemal complexes from the grasshopper *Chorthippus jacobsi*: pachytene and zygotene observations. Hereditas, **118**: 235-241.

Schmid, M., and Guttenbach, M. 1988. Evolutionary diversity of reverse (R) fluorescent chromosome bands in vertebrates. Chromosoma, **97**: 101-114.

Schmid, M., and Haaf, T. 1987. Origin and evolution of sex chromosomes in amphibia: The cytogenetic data. *In* Evolutionary mechanisms in sex determination. *Edited by* S. S. Wachtel. CRC Press, Boca Raton, Fl. pp. 37-56.

Scott, M.P., Haymes, K.M., and Williams, S.M. 1992. Parentage analysis using RAPD PCR. Nucleic Acids Res., **20**: 5493.

Serebryakova, E.V. 1969. Some data on the chromosome complexes in Acipenseridae. *In* Genetics, selection and hybridization of fish. *Edited by* Y. Sobel. Keter Press, Jerusalem. pp. 98-106.

Serebryakova, E.V., Arefjev, V.A., Vasiliev, V.P., and Sokolov, L.I. 1983. The study of the karyotype of giant sturgeon, *Huso huso* (L.) (Acipenseridae, Chondrostei) with reference to their systemic position. *In* Genetics in commercial fishes and aquaculture objects, Moscow. pp. 63-69.

Shelton, W.L., Mims, S.D., and Clark, J.A. 1995. Induced gynogenesis in paddlefish, *Polyodon spathula*. *In* Aquaculture '95, San Diego, Ca. World Aquaculture Society. pp. Abstract 411.

Slyn'ko, V.I. 1976. Multiple molecular forms of malate dehydrogenase and lactate dehydrogenase in Russian sturgeon (*Acipenser güldenstädti*) and great sturgeon (*Huso huso* L.). Dokl. AN SSSR, **228**: 470-472.

Sola, L., Cataudella, S., and Capanna, E. 1981. New developments in vertebrate cytotaxonomy III. Karyology of bony fishes: A review. Genetica (The Hague), **54**: 285-328.

Sola, L., Cordisco, C., Bressanello, S., and Cataudella, S. 1994. Cytogenetic characterization of the North American white sturgeon *Acipenser transmontanus* (Pisces, Acipenseridae). *In* Proceedings VIII congress societas Europaea ichthyologorum, Oviedo, Spain, 26 September - 2 October, 1994. pp. 64-65.

Solari, A.J. 1974. The relationship between chromosomes and axes in the chiasmatic XY pair of the Armenian hamster (*Cricetulus migratorius*). Chromosoma, **48**: 89-106.

Solari, A.J. 1977. Ultrastructure of the synaptic autosomes and the ZW bivalent in chicken oocytes. Chromosoma, **64**: 155-165.

Solari, A.J. 1994. Sex chromosomes and sex determination in vertebrates. CRC Press, Boca Raton, Fl.

Stanley, J.G. 1976. Female heterogamety in grass carp (*Ctenopharyngodon idella*) determined by gynogenesis. J. Fish. Res. Board Canada, **33**: 1372-1374.

Stebbins, G.L., Jr. 1950. Variation and evolution in plants. Columbia University Press, New York.

Stebbins, G.L., Jr. 1976. Chromosome, DNA and plant evolution. *In* Evolutionary biology. Vol. 9. *Edited by* M. K. Hecht, W. C. Steere, and B. Wallace. Plenum Press, New York. pp. 1-34.

Sumner, A.T. 1972. A simple technique for demonstrating centromeric heterochromatin. Exp. Cell Res., **75**: 304-306.

Swarup, H. 1959. Production of triploidy in *Gasterosteus aculeatus* (L.). J. Genet., **56**: 129-142.

Świtoński, M., Gustavsson, I., Höjer, K., and Plöen, L. 1987. Synaptonemal complex analysis of the B-chromosomes in spermatocytes of the silver fox (*Vulpes fulvus* Desm.). Cytogenet. Cell Genet., **45**: 84-92.

Thorgaard, G.H. 1977. Heteromorphic sex chromosomes in male rainbow trout. Science, **196**: 900-902.

Thorgaard, G.H. 1978. Sex chromosomes in the sockeye salmon: a Y-autosome fusion. Can. J. Genet. Cytol., **20**: 349-354.

Thorgaard, G.H. 1983. Chromosome set manipulation and sex control in fish. *In* Fish physiology. Vol. IX, Part B. *Edited by* W. S. Hoar, D. J. Randall, and E. M. Donaldson. Academic Press, New York. pp. 405-434.

Thorgaard, G.H. 1986. Ploidy manipulation and performance. Aquaculture, **57**: 57-74.

Thorgaard, G.H., Rabinovitch, P.S., Shen, M.W., Gall, G.A.E., Propp, J., and Utter, F.M. 1982. Triploid rainbow trout identified by flow cytometry. Aquaculture, **29**: 305-309.

Thorgaard, G.H., Scheerer, P.D., and Parsons, J.E. 1985. Residual paternal inheritance in gynogenetic rainbow trout: implications for gene transfer. Theor. Appl. Genet., **71**: 119-121.

Tiersch, T.R., Chandler, R.W., Wachtel, S.S., and Elias, S. 1989. Reference standards for flow cytometry and application in comparative studies of nuclear DNA content. Cytometry, **10**: 706-710.

Tiersch, T.R., Simco, B.A., Davis, K.B., and Wachtel, S.S. 1992. Molecular genetics of sex determination in channel catfish: Studies on SRY, ZFY, Bkm, and human telomeric repeats. Biol. Reprod., **47**: 185-192.

Utter, F.M., Johnson, O.W., Thorgaard, G.H., and Rabinovitch, P.S. 1983. Measurement and potential applications of induced triploidy in Pacific salmon. Aquaculture, **35**: 125-135.

Uyeno, T., and Miller, R.R. 1971. Multiple sex chromosomes in a Mexican cyprinodontid fish. Nature (London), **231**: 452-453.

Uyeno, T., and Miller, R.R. 1972. Second discovery of multiple sex chromosomes among fishes. Experientia, **28**: 223-225.

Van Eenennaam, A.L., Van Eenennaam, J.P., Medrano, J.F., and Doroshov, S.I. 1996. Rapid verification of meiotic gynogenesis and polyploidy in white sturgeon (*Acipenser transmontanus* Richardson). Aquaculture, **147**: 177-189.

Vasiliev, V.P., Sokolov, L.I., and Serebryakova, Y.V. 1980. Karyotypes of the Siberian sturgeon, *Acipenser baeri*, of the Lena River and some aspects of karyotype evolution in Acipenseriformes. J. Ichthyol., **20**: 37-45.

Vassetzky, S.G. 1966. The thermosensitivity of sturgeon eggs at the stages of maturation and early cleavage. (In Russian). Zhurnal Onshchei Biologii, **27**: 583-595.

Vassetzky, S.G. 1967. Changes in the ploidy of sturgeon larvae induced by heat treatment of eggs at different stages of development. Dokl. AN SSSR, **172**: 1234-1237.

Villagómez, D.A.F. 1993. Zygotene-pachytene substaging and synaptonemal complex karyotyping of boar spermatocytes. Hereditas, **118**: 87-99.

Vitturi, R., Catalano, E., and Lafargue, F. 1991. Evidence for heteromorphic sex chromosomes in *Zeus faber* (Pisces, Zeiformes): nucleolus organizer regions and C-banding pattern. Cytobios, **68**: 37-43.

Volckaert, F.A.M., Galbusera, P.H.A., Hellemans, B.A.S., Van den Haute, C., Vanstaen, D., and Ollevier, F. 1994. Gynogenesis in the African catfish (*Clarias gariepinus*). I. Induction of meiogynogenesis with thermal and pressure shocks. Aquaculture, **128**: 221-233.

Wardell, B.B., Sudweeks, J.D., Meeker, N.D., Estes, S.E., Woodward, S.R., and Teuscher, C. 1993. The identification of Y chromosome-linked markers with random sequence oligonucleotide primers. Mamm. Genome, **4**: 109-112.

Warmke, H.E., and Blakeslee, A.F. 1940. The establishment of a 4N dioecious race in Melandrium. Am. J. Bot., **27**: 751-762.

Wattendorf, R.J. 1986. Rapid identification of triploid grass carp with a Coulter counter and channelyzer. The Progressive Fish Culturist, **48**: 125-132.

Welsh, J., and McClelland., M. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic. Acids. Res., **18**: 7213-7218.

Wieland, I., Bolger, G., Asouline, G., and Wigler, M. 1990. A method for difference cloning - gene amplification following subtractive hybridization. Proc. Natl. Acad. Sci. USA, **87**: 2720-2724.

Wigger, G., Fries, R., and Stranzinger, G. 1996. Isolation of bovine Y-specific probes applying genetically directed representational analysis. (In German with English summary). Schweiz. Arch. Tierheilk., **138**: 167-171.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., and Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res., **18**: 6531-6535.

Winge, O. 1922. One-sided masculine and sex-linked inheritance in *Lebistes reticulatus*. J. Genet., **12**: 145-162.

Wise, D.A., and Nail., B. 1987. A technique for analyzing fish meiotic chromosomes with light and electron microscopes. Copeia, **1987**: 499-503.

Yajuan, L., and Qixing, Y. 1991. Electron microscopic observation of synaptonemal complexes in spermatocytes of six species of fish. Chinese J. Genet., **18**: 273-282.

Yamamoto, T. 1953. Artificially induced sex reversal in genotypic males of the medaka (*Oryzias latipes*). J. Exp. Zoology, **123**: 571-594.

Yamamoto, T., and Kajishima, T. 1969. Sex-hormone induction of reversal of sex differentiation in the goldfish and evidence for its male heterogamety. J. Exp. Zool., **168**: 215-222.

Yamazaki, F. 1983. Sex control and manipulation in fish. Aquaculture, **33**: 329-354.

Yosida, T.H., and Hayashi, M. 1970. Preliminary note on karyotype of guppy and topminnow. Ann. Rep. Nat. Inst. Genet. (Japan), **21**: 52-53.

Yu, X., Zhou, T., Li, K., Li, Y., and Zhou, M. 1987. On the karyosystematics of cyprinid fishes and a summary of fish chromosome studies in China. Genetica (The Hague), **72**: 225-236.

Zubova, S.E. 1971. The ontogeny of gonadal differentiation and male:female ratio in juvenile sterlet (*Acipenser ruthenus*) of Volga river. J. Ichthyol., **11**: 524-526.

APPENDIX 1. PROTOCOLS

1A. DNA EXTRACTION FROM STURGEON SPLEEN

(Modified from a protocol for DNA extraction from mouse spleen tissue obtained from Ben Taylor, Jackson Laboratories, Bar Harbor, ME)

Work on ice

1. Homogenize 0.02g of sturgeon spleen with 600 µl LST[1]. Transfer into 2 ml graduated eppendorf tubes.
2. Add 200 µl TNLB[2] and gently shake.
3. Microfuge 3000 rpm, 5 min. There should be a whitish pellet visible and a red supernatant. Do not let the tubes sit for too long after centrifugation or the pellet will start to diffuse. Remove the red supernatant being careful not to disrupt the pellet.
4. Add 800 µl ACE[3] and resuspend by pipetting up and down with 1 ml blue pipette tips.
5. Add 80 µl 10% SDS.

Proceed at room temperature

6. Rock on the nutator for 5-10 min.
7. Add 800 µl phenol.
8. Rock on the nutator for 5 min.
9. Microfuge 6000 rpm, 10 min.
10. Transfer the aqueous (upper) phase to another 2 ml graduated eppendorf tube using a 1 ml transfer pipette. The upper phase at this time is often milky white and viscous.
11. Add 400 µl phenol and 400 µl chloroform to the tube.
12. Rock on the nutator for 5 min.
13. Microfuge 6000 rpm, 10 min.
14. Transfer the aqueous phase to another 2 ml graduated eppendorf tube. The upper phase should be clearer now. Try to avoid disturbing the white layer between the aqueous phase and the organic phase when you remove the supernatant - this can be achieved by drawing the aqueous phase into the transfer pipette very slowly.
15. Repeat steps 11-13. The aqueous phase should be very clear at this time.
16. Transfer the aqueous phase to another 2 ml graduated eppendorf tube.
17. Add 800 µl chloroform to the tube.
18. Rock on the nutator for 5 min.
19. Microfuge 6000 rpm, 10 min.
20. Transfer the aqueous phase to a 5 ml culture tube.
21. Add 2-3 ml ice cold 100% ethanol and invert the tube several time until the DNA precipitates out of solution.
22. Wind the DNA around a hooked Pasteur pipette, and wash with 70% ethanol.
23. Air dry for 10 minutes until the DNA is clear.
24. Resuspend the DNA in 500 µl TE in a 1.5 ml eppendorf tube and rock overnight on the nutator at 37°C.
25. Yields are typically 400 ng/µl (total 200 µg), The A1/A2 ($\lambda_{260}/\lambda_{280}$) ratio should be ≈ 1.8.

[1]LST	[2]TNLB	[3]ACE
20 mM Tris-HCl (pH 7.4)	5% sucrose (wt/vol)	50 mM sodium acetate
10 mM sodium chloride	4% Nonidet P-40 (vol/vol)	10 mM sodium EDTA
3 mM magnesium chloride	in LST	pH = 5.9

1B. SUBTRACTIVE HYBRIDIZATION

1. Preparation of genomic DNA

Equal amounts (10 μg) of genomic DNA from 10 white sturgeon full-sib females were combined and randomly sheared by sonication (10 sec) to a mean size of 500-2000 bp, as determined by comparison to a 123 bp ladder and *Hind*III-digested λ DNA on a 1% agarose gel. Sheared female DNA (50 μg) was labeled with photobiotin acetate using the photobiotin labeling system (Gibco BRL, Gaithersburg, MD). Equal amounts (2 μg) of genomic DNA from 10 full-sib males (2 μg) were combined and partially digested with *Sau*3AI to a mean size of 500-1000 bp.

2. Subtractive hybridization and paramagnetic particle capture

Digested male DNA (2 μg) and photobiotinylated female DNA (30 μg) were mixed, ethanol precipitated, washed with 70% ethanol, dried and resuspended in 10 μl of 0.12M sodium phosphate buffer, pH 6.8 containing 1mM EDTA, 0.1% SDS, and 50% deionized formamide in a siliconized microfuge tube. The mixture was overlaid with 35 μl mineral oil, denatured at 100°C for 5 min, and incubated at 42°C for 5 days. After incubation, the hybridization mixture was diluted to 500 μl with 75 mM sodium chloride and 7.5 mM sodium citrate, pH 7.0, and added to 250 μg streptavidin-coated paramagnetic particles (SAV-PMPs) (Promega, Madison, WI) that had been washed three times with 0.5X SSC immediately before use and incubated at room temperature for 30 min. The SAV-PMPs were captured magnetically and this process was repeated with two fresh aliquots of SAV-PMPs. The DNA remaining in solution was precipitated by the addition of magnesium chloride and sodium acetate to final concentrations of 1 mM and 300 mM, respectively, and 2.5 vol ethanol. The paramagnetic particle capture of biotin-labeled hybrids was repeated with the precipitated "male-enriched" DNA and an additional 8 μg of biotin-labeled female DNA. The "male-enriched" DNA was then resuspended in 20 μl TE.

Cloning procedure

Two micrograms of the vector Bluescript S/K- (Stratagene, La Jolla, CA) was digested with 20 U *Bam*HI at 37°C for 2 h, dephosphorylated with 1U calf intestinal alkaline phosphatase at 37°C for 1 h, extracted with phenol/chloroform, precipitated with ethanol, and resuspended in 10 μl TE. The male-enriched DNA (0, 2, 4 μl) was ligated with 1 μl vector, 1 μl 10x T_4 DNA ligase buffer, 1U of T_4 DNA ligase in a final volume of 10 μl. Each ligation was allowed to proceed at room temperature for 1 hr and then transferred to 4°C overnight.

Ligation mixes (2 μl) were added to 80 μl supercompetent XL1-Blue MRF' cells (Stratagene) following the manufacturer's protocols, and plated (20 and 200 μl) on LB-ampicillin (50 μg/ml) plates containing Xgal (40 μg/ml) and isopropylthiogalactosidase (0.5 mM). Inserts were amplified by PCR using the M13 reverse and -20 universal sequencing primers (Promega).

Analysis of isolated clones

Plasmid DNA from overnight cultures of 40 putative recombinant plasmids (white colonies) was prepared using the QIAquick Spin Plasmid Miniprep Kit (Qiagen, Chatsworth, CA). Double restriction digests (*Xba*I, *Xho*I) revealed that all of the plasmids contained small inserts (100-400 bp). Whole genomic DNA (1 μg total from 10 full-sib males, 1 μg total from 10 full-sib females) was separately labeled with 5'-(α-^{32}P) dATP by nick translation and hybridized (.25 M sodium phosphate buffer, pH 7.0, 7% SDS at 65°C) to Southern blots of miniprep plasmid DNA, and PCR amplified inserts run on a 1.5% agarose gel and transferred onto Zeta Probe GT membrane (Bio-Rad, Hercules, CA) following the manufacturer's protocols. Inserts that had a greater hybridization affinity for the male genomic DNA were themselves labeled by PCR incorporation of 5'-(α-^{32}P) dATP and hybridized to Southern blots of male and female full-sib genomic DNA digested with either *Bam*HI, *Bgl*II, or *Sau* 3AI restriction enzymes and run on a 0.7% agarose gel to examine for sex-specific hybridization patterns.

Reference

Fletcher, S., Darragh, D., Fan, Y., Grounds, M.D., Fisher, C.J., and Beilharz, M.W. 1993. Specific cloning of DNA fragments unique to the dog Y chromosome. GATA, **10**: 77-83.

1C. REPRESENTATIONAL DIFFERENCE ANALYSIS

(Modified from a protocol obtained from Nikolai Lisitsyn and Michael Wigler, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY)

To avoid cross-contamination from the beginning of the RDA procedure, all pipetting should be done using filtered pipette tips. It is important to always treat the driver and the tester DNA the same way except where indicated. DNA should be sufficiently pure to achieve complete digestion since partial digestion can cause multiple problems. Positive controls can be included by adding appropriate target DNA into the tester DNA (e.g. 16 pg λ phage DNA per microgram of human DNA) for *Bgl*II and *Hind*III representations prior to preparing the amplicons.

Oligonucleotides

Purified oligonucleotides (adaptors and primers for PCR) designed for RDA using *Bgl*II, *Bam*HI, or *Hind*III restriction digests (shown below) can be purchased from Operon (Cat. No. RDA Bgl, RDA Bam and RDA Hind). Resuspend the primers at 62 pmol/ml (12 OD_{260}/ml for 24-mers and 6 OD_{260}/ml for 12-mers).

Primer set	Name	Sequence
1.	R Bgl 24	5'-AGCACTCTCCAGCCTCTCACCGCA-3'
	R Bgl 12	5'-GATCTGCGGTGA-3'
2.	J Bgl 24	5'-ACCGACGTCGACTATCCATGAACA-3'
	J Bgl 12	5'-GATCTGTTCATG-3'
3.	N Bgl 24	5'-AGGCAACTGTGCTATCCGAGGGAA-3'
	N Bgl 12	5'-GATCTTCCCTCG-3'
1.	R Bam 24	5'-AGCACTCTCCAGCCTCTCACCGAG-3'
	R Bam 12	5'-GATCCTCGGTGA-3'
2.	J Bam 24	5'-ACCGACGTCGACTATCCATGAACG-3'
	J Bam 12	5'-GATCCGTTCATG-3'
3.	N Bam 24	5'-AGGCAACTGTGCTATCCGAGGGAG-3'
	N Bam 12	5'-GATCCTCCCTCG-3'
1.	R Hind 24	5'-AGCACTCTCCAGCCTCTCACCGCA-3'
	R Hind 12	5'-AGCTTGCGGTGA-3'
2.	J Hind 24	5'-ACCGACGTCGACTATCCATGAACA-3'
	J Hind 12	5'-AGCTTGTTCATG-3'
3.	N Hind 24	5'-AGGCAGCTGTGGTATCGAGGGAGA-3'
	N Hind 12	5'-AGCTTCTCCCTC-3'

1. Restriction of the DNA

a. Digest separately 1-2 µg driver and tester DNA in a volume of 200 µl with the restriction enzyme (New England Biolabs, Beverly, MA) chosen for the representation. Use 10U restriction enzyme per µg DNA. Check the digestion for completion by running it on a 1% agarose gel.

b. Add 10 µg of tRNA (Sigma, type X-SA, Cat. No. R 8759) after digestion and extract the DNA with equal volumes of phenol and then phenol/chloroform (1:1).

c. Add 1/10 vol of 3 M sodium acetate, mix by pipetting and add 3 vol ice cold 100% ethanol. Mix again by inverting the tube several times, incubate at -70°C for 10 min, microfuge 13000 g for 10 min, wash the pellet with 1 ml of 70% ethanol, vacuum dry, and resuspend at a concentration of 0.1 mg/ml.

2. Ligation of the adaptors

a. Mix 10 µl (1 µg) of digested driver or tester DNA, 7.5 µl each of "R" 12-mer and 24-mer (primer set 1), 2 µl water and 3 µl of 10x ligase buffer.

b. To anneal the oligonucleotides, place the tube in a heating block (holes filled with glycerol) at 50-55°C and then place the block at 4°C for approximately 1h, until the temperature drops to 10-15°C.

c. Place the tubes on ice for 3 min, add 1 µl of T4 DNA ligase (400U/µl, New England Biolabs), mix by pipetting and incubate overnight at 12-16°C.

3. PCR amplification

a. Prepare 2 tubes for the preparation of tester amplicon and 12 tubes for the preparation of driver amplicon, each containing 352 µl of standard PCR mixture composed of: 80 µl of 5x PCR buffer (335 mM Tris-HCl, pH 8.9 at 25°C, 20 mM $MgCl_2$, 80 mM $(NH_4)_2SO_4$, 50 mM β-mercaptoethanol, 0.5 mg/ml bovine serum albumin); 32 µl dNTP solution (dATP, dGTP, dCTP, dTTP, 4 mM each), and 240 µl water.

b. Dilute each ligate from Section 2c with 970 µl TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)

c. Add 40 µl of the dilution of the ligate (40 ng DNA) and 8 µl of the "R" 24-mer (primer set 1) to each tube prepared above (Section 3a) and place the tubes in a thermocycler prewarmed to 72°C. At this temperature the small oligonucleotide dissociates.

d. To fill-in the 3'-recessed ends of the ligated fragments, add 3 µl (15U) of AmpliTaq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) to each tube, mix by pipetting, overlay with 110 µl of mineral oil and incubate at 72°C for 5 min.

e. Amplify for 20 cycles (95°C for 1 min, 72°C for 3 min) with the last cycle followed by an extension at 72°C for 10 min.

f. To check the quantity and quality of the amplicons run 10 µl of the PCR product on a 2% agarose gel.

4. Cleaving the adaptors from the amplicons

a. Remove mineral oil with a pipette, combine the contents of two PCR tubes in a 2 ml eppendorf tube for the tester amplicon, and twelve into six for the driver amplicon. Extract with 600 µl of phenol and phenol/chloroform (1:1).

b. Add 1/10 vol of 3 M sodium acetate and 800 µl isopropanol, incubate for 15 min in a wet ice bath and recover the DNA by centrifugation. Resuspend driver and tester amplicons in TE buffer at concentration of 0.2-0.4 mg/ml, and check DNA by gel electrophoresis. Save 20 µg of driver and tester amplicons for amplicon southern analysis.

c. To cleave the adaptors digest the driver DNA (150 µg) and the tester DNA (10 µg) with the initially chosen restriction endonuclease (10U/µg) in the volumes 800 µl and 200 µl correspondingly. Add 10 µg of tRNA to tester amplicon digest, phenol and phenol/chloroform extract and recover both DNA's by isopropanol precipitation.

d. Resuspend driver and tester amplicon DNA digests in 125 µl of TE buffer. Use at least two vortex shakings, 30 sec each, to dissolve the driver DNA pellet. The final concentration of DNA should be 1 mg/ml in the driver tube and .1 mg/ml in the tester tube. To check the concentrations, dilute a 2 µl aliquot of the driver amplicon in 18 µl of water. Load approximately 0.2, 0.4, and 0.6 µg of driver and tester amplicon DNA digests on a 2% agarose gel. Load undigested amplicon to check digestion and appropriate concentration standards.

e. Estimate the concentration of amplicon DNA digests by comparison of the intensities of the lanes. Adjust driver DNA concentration to 0.5 µg/µl and tester DNA concentration to 50 ng/µl with TE buffer. Check digestion of the amplicons by comparison of the mobilities of intensive bands of repeated sequences in the amplicon before and after digestion.

5. Removal of the adaptors from the amplicons

a. Load 100 µl (5 µg) of digested tester amplicon and 80 µl (40 µg) of digested driver amplicon on separate 2% NuSieve low melting point agarose (FMC Bioproducts, Rockland, ME) gels and make a short run such that you can separate the 24 bp adaptor from 150-1500 bp fragments.

b. Cut agarose slices (< 0.5 g) containing 150-1500 bp fragments and place them in 2 ml eppendorf tubes.

c. Extract the DNA from the gel slices using the QIAEX II gel extraction kit (Qiagen) following the manufacturer's protocols.

d. Adjust the concentration of extracted tester DNA to 0.1 mg/ml and driver DNA to .5 mg/ml. You will need at least 240 µl (120 µg) of driver DNA (320 µl for *Hind*III representations).

6. Ligation of adaptors to tester amplicon

a. Ligate 1 µg of tester DNA amplicon digest to the "J" primers (primer set 2) as in Section 2.

b. Adjust the DNA concentration of the tester amplicon ligate to 10 µg/ml (25 µg/ml for *Hind*III representations) by adding TE buffer containing tRNA (20 µg/ml).

7. First round of hybridization

a. Mix 80 µl (40 µg) of driver amplicon DNA digest and 40 µl of tester amplicon ligate in a 0.5 ml eppendorf tube, extract once with an equal volume of phenol/chloroform.

b. Add 30 µl of 10 M ammonium acetate, mix by pipetting, add 380 µl of ice cold ethanol, chill at -70°C for 10 min, incubate at 37°C for 2 min, and recover the DNA by centrifugation. Wash twice with 70% ethanol and vacuum dry.

c. Resuspend the pellet in 4 µl of EE x 3 buffer (30 mM EPPS buffer (Sigma), pH 8.0 at 20°C, 3 mM EDTA) by vortexing for 2 min, collect sample on bottom of tube by brief centrifugation and overlay with 35 µl mineral oil. If the DNA is too viscous and does not form a meniscus, add one additional microliter of EE x 3 buffer and a total of 1.5 µl of 5M sodium chloride (see next step).

d. Denature DNA for 3-4 min at 98°C in a heating block, carefully add 1 µl of 5M sodium chloride to the DNA drop and incubate at 67°C for 20 h.

8. Selective amplification

a. Remove oil, add 8 µl of tRNA solution (5 mg/ml) to reannealed DNA, mix thoroughly by pipetting, add 390 µl TE buffer and mix again.

b. To fill in the adaptor ends make 2 tubes with 352 µl of standard PCR mixture (Section 3a). Add 40 µl of DNA dilution to each tube, place in the thermocycler at 72°C, add 3 µl (15U) of AmpliTaq DNA polymerase, mix by pipetting and incubate for 5 min.

c. To amplify self-reannealed tester amplicon molecules add 10 µl of "J" 24-mer primer, mix, overlay with mineral oil and perform 10 cycles of PCR as in Section 3e. For J Bgl 24 primer (*Bgl*II or *Hind*III representations), use slightly different PCR conditions (95°C for 1 min, 70°C for 3 min). This yields the first round difference product.

d. Remove oil by pipetting, combine the contents of the two PCR tubes in one 2 ml eppendorf tube, add 10 µg of tRNA. Phenol and phenol/chloroform extract and recover the DNA as in Section 4a,b. Dissolve the pellet in 40 µl of TE buffer.

e. Take 20 µl of the first round difference product, add 20 µl of 2x mung bean nuclease buffer, mix by pipetting, add 2 µl of mung bean nuclease (10U/µl, New England Biolabs), mix again and incubate at 30°C for 30 min.

f. Add 160 µl of 50 mM Tris-HCl, pH 8.9, and inactivate the mung bean nuclease by 5 min incubation at 98°C. Prepare 2 tubes with 352 µl of standard PCR mixture (Section 3a), add 10 µl of "J" 24-mer, add 40 µl of nuclease-treated difference product to each tube and PCR for 20 more cycles as in Section 3e. For J Bgl 24 primer (*Bgl*II or *Hind*III representations), use again use the 95°C for 1 min, 70°C for 3 min PCR amplification profile.

g. Remove mineral oil and run 10 µl of the PCR product on a 2% agarose gel, estimate the quantity of DNA (usually 0.1-0.3 µg) and, if necessary to improve the yield, make 1-3 supplementary cycles adding 3 µl of fresh AmpliTaq DNA polymerase.

9. Change of adaptors for subsequent hybridization/amplifications

a. Remove oil by pipetting, combine the contents of the two PCR tubes in one 2 ml eppendorf tube, add 10 µg of tRNA. Phenol and phenol/chloroform extract and recover the DNA as in Section 4a,b. Dissolve the pellet in 100 µl of TE buffer, check the DNA by 2% agarose gel electrophoresis and adjust the DNA concentration to 0.1 mg/ml.

b. To cleave the adaptors digest 5 μg tester DNA with the initially chosen restriction endonuclease (10U/μg) in 100 μl. After digestion add 10 μg of tRNA to the digest mixture, phenol and phenol/chloroform extract and recover DNA by ethanol precipitation as in Section 1c. Dissolve the pellet in 100 μl of TE buffer and adjust the concentration to 20 ng/μl.

c. Take 5 μl (100 ng) of the digested difference product and directly ligate it to the "N" primer set (primer set 3) in a volume of 30 μl as described in Section 2. Add 50 μl of TE buffer (10 μl for *Hind*III representations) containing tRNA (20 μg/ml) to the difference product DNA ligate.

10. Subsequent hybridization/amplification steps

a. For the second hybridization, mix 40 μl of the first round difference product ligated to the "N" primer set (50 ng for most restriction endonucleases, 100 ng for *Hind*III representations) and 40 μg in 80 μl of driver amplicon DNA digest (adaptors cleaved off). Proceed through hybridization and selective amplification steps as above (Sections 7 and 8). Change the adaptors on the second round difference product with the "J" primer set as described in Section 9. Adjust the concentrations of the DNA ligate to 2.5 pg/μl (10 pg/μl for *Hind*III representations) by making two consecutive dilutions with TE buffer containing tRNA (20 μg/ml).

b. For the third hybridization mix 40 μl of the second round difference product ligated to the "J" primer set (100 pg from most restriction endonucleases, 400 pg for *Hind*III representations) with 40 μg in 80 μl of driver amplicon DNA digest. Proceed through hybridization and selective amplification steps as above (Sections 7 and 8).

c. For *Hind*III representations a fourth hybridization is needed. Change the adaptor on the third round difference product with the "N" primer set and adjust the concentration of the DNA ligate to 0.125 pg/μl by making three consecutive dilutions with TE buffer containing tRNA (20 μg/ml). Mix 5 pg in 40 μl third round difference product ligated to the "N" primer set with 40 μg in 80 μl of driver amplicon DNA digest. Proceed through hybridization and selective amplification steps as above (Sections 7 and 8) with the final amplification after mung bean nuclease treatment for an additional 27 cycles (Section 8f).

Reference

Lisitsyn, N., Lisitsyn, N., and Wigler, M. 1993. Cloning the differences between two complex genomes. Science, **259**: 946-951.

1D. LYMPHOCYTE CULTURE FROM STURGEON BLOOD

(Modified from a protocol obtained from F. Fontana, Università di Ferrara, Ferrara, Italy)

Materials required

- 5 ml Sodium Heparin vacutainers (green top), and 21 gauge sterile needles
- 25 ml tissue culture flasks
- 25°C cell culture incubator @ 8% CO_2
- PBS (Phosphate Buffered Saline), pH 7.2 Gibco # 20012-027
- PSN Antibiotic Mixture (5 mg penicillin, 5 mg streptomycin, 10 mg neomycin/ml) Gibco # 15640-055
- DMEM (Dulbecco's modified Eagle, 1X, low glucose) media Gibco # 11885-084
- Fetal bovine serum, qualified, heat-inactivated Gibco # 10438-018
- Phytohemagglutin, M-form, lyophilized (PHA-M) Gibco #10576-015
- Pokeweed mitogen Gibco # 15360-019
- "Karyomax" colcemid, 10 µg/ml in PBS Gibco # 15212-012
- Methotrexate Sigma # M 8407
- Thymidine Sigma # T 1895
- Acetic Acid
- Methanol
- Hypotonic solution (0.075 M Potassium chloride (KCl))
- Giemsa stain R66-GURR and Gurr's buffer, pH 6.8 (Bio/medical Specialities Inc.)

PROCEDURE

1. Collect blood from the caudal blood vessel using a 5 ml vacutainer (green top) and needle. Keep the tube at ambient temperature, do not put on ice.

2. Spin the tube at 500 rpm (Sorval benchtop) for 10 min at room temperature.

3. Remove the green top (do not discard) and remove 1 ml of the plasma supernatant. This will be very cloudy if the fish is undergoing vitellogenesis. Add 1 ml of PBS to the tube, replace the top, and gently rock the tube backwards and forwards several times.

4. Spin the tube at 500 rpm (Sorval benchtop) for 10 min at room temperature.

5. Using a transfer pipette remove the buffy coat (located between the clear aqueous supernatant and the lower red erythrocyte layer) and add it (approximately 8-10 drops) to a 25 ml tissue culture flask containing 1.5 ml fetal bovine serum, 200 µl PSN Antibiotic Mixture, 200 µl PHA-M, 100 µl Pokeweed, and 8 ml DMEM.

6. Gently mix the blood in with the media, loosen the lid to allow gas exchange, and place the flask in an incubator at 25°C, 8% CO_2 for 5 days.

7. At 5 pm on the 4th day add 100 µl of 10 µM methotrexate (10^{-7} M final) to block DNA replication. Mix gently. Incubate 16 to 18 hr (overnight).

8. On the following day (e.g. at 8:30 am) add 100 µl of 1 µM thymidine (10^{-5} M final) to release the methotrexate block. Mix gently. Incubate 4 hr (e.g. until 12:30 pm).

9. Initiate harvest by adding 100 µl of colcemid. Mix gently. Incubate for 4 hours.

10. Pour the cells and media into a 15 ml tube. Spin the tube at 500 rpm (Sorval benchtop) for 10 min at room temperature. Discard the supernatant.

11. Add 10 ml of hypotonic solution to the tube and gently resuspend the cells. Let stand at room temperature for 30 min, gently inverting the tube every 10 min. Remove any large viscous red pellets with the tip of a Pasteur pipette.

12. Spin the tube at 500 rpm (Sorval benchtop) for 10 min at room temperature. Discard the supernatant.

13. Repeat step 11.

14. Add 10-15 drops of freshly prepared fixative (3 parts methanol: 1 part acetic acid). Invert the tube until the color of the mixture goes from red to brown (brown being the color that phenol red turns in an acid environment).

15. Spin the tube at 500 rpm (Sorval benchtop) for 10 min at room temperature. Remove all but 0.5 ml of the supernatant carefully. Resuspend the remaining supernatant by gently flicking the bottom of the tube.

16. Add 1 ml of fixative (3 parts methanol: 1 part acetic acid) and immediately mix the contents of the tube gently so that an even suspension is formed. Add an additional 8 ml fixative to approximately 10 ml. At this stage the tube can be left in the freezer overnight if a stop point is required.

17. Spin the tube at 500 rpm (Sorval benchtop) for 10 min at room temperature. Remove all but 0.5 ml of the supernatant carefully. Resuspend the remaining supernatant by gently flicking the bottom of the tube. Add 8 ml fixative.

18. Spin the tube at 500 rpm (Sorval benchtop) for 10 min at room temperature. Remove the supernatant and resuspend the pellet in a volume of fixative sufficient to produce a light milky suspension. Allow to stand 30 min at room temperature or store overnight at 4°C. Drop onto methanol cleaned microscope slides using a Pasteur pipette and stain the spreads for 20-30 min using 4% Giemsa in pH 6.8 Gurr buffer.

APPENDIX 2. WHITE STURGEON DNA SEQUENCES

1. Sequence of the A12 clone insert isolated by subtractive hybridization.

```
1     GATCTTACCC AGCATTCAGG AAACAGTACA ACAGCAAAAC CACCTGGGTT
51    TGGAAAAATC CTTCCAACAA ACAATTCCCC AATGGAAAGG CAGCTCAGAG
101   AAAAGATC
```

Forward Primer: 3' ATCTTACCCA GCATTCAGG 5'
Reverse Primer: 3' TTCTCTGAGC TGCCTTTCC 5'

2. Partial female DNA template sequence of the OP C7 RAPD primer polymorphic band.

Foward

```
1     GTCCCGACGA GATGCAATGT TCATGTCTAG ATTTGGTATT AACAAACAAA
51    GAAGCAAGGA TACCAACTTA CAGGTAATAG AACCACAAGG AATACGTGAC
101   CATAATATGA TGAGGTTTGT GGCAAATAAA CCAAGGGAAG TACATAAATC
151   AAAGACAATG GTTTACAATT CCAGGACAGC AGACTACAAG GAATGCAACA
201   GGAGCTTCTA AACACAGACT GGGATAGAAT CTAATAGATA .....
```

Reverse

```
1     GTCCCGACGA ACACAGAGCC ATATAATGAG AGGGATCAGA ATTGCCCTGT
51    GTCCCGACGA .....
```

Scar OPC7 forward primer: 3' GTCCCGACGA GATGCAATGT TC 5'
Scar OPC7 reverse primer: 3' GTCCCGACGA ACACAGAGCC 5'

3. Partial male DNA template sequence of the UBC 661 RAPD primer polymorphic band.

Foward

```
1     CCTGCTTACG AAATAATGCA CAAAAAAATG CGAACCTCCAT CTTTTTTTT
51    TGTTGTTTGT TTATTGAAAG TAAGGTGTGT TAAAGTTGGG ACACATCAAA
101   ACAGCTACAT CAATAAGAGA TTTGGGCAAA CGCTGATTCA TTACAAGTCT
151   AAAATAGCAG GAATGGTTAA AATGAGGCAA AACCCAACTG AGATTGTTCT
201   GAAGTTGAAA TTAATTAATC AGTATATGGA CAACACATAT AATTACTAAT
251   ATTAATGGAA AGAAGACCTA TGATACATCG AGAT .....
```

Reverse

```
1     CCTGCTTACG TGGCAAATCT GGAGAAGAAT ACGTCTTCAC CAGAATCTAA
51    GGTTTTAGAA ATTTGTTCCA AGCTTTAGGG CTTTTTTGTG GTCTTTAACT
101   TCTAAAAACA GCGTGACACA CAGTTAGTGT GCGATTTACA ACCGAGCCAC
151   CACCACCAT G .....
```

Scar UBC 661 forward primer: 3' CCTGCTTACG AAATAATGCA C 5'
Scar UBC661 reverse primer: 3' CTGCTTACGT GGCAAATCTG 5'

APPENDIX 3. FLUORESCENCE *IN SITU* HYBRIDIZATION

Introduction

Genomic DNA *in situ* hybridization has proven to be a useful tool for analyzing the genomes of cell hybrids (Bouffler 1994). For example, the equine chromosome complement of mouse-horse hybrids can be analyzed by hybridization of genomic equine DNA to the hybrids (Lear et al. 1992). In this experiment we hybridized labeled male and female sturgeon genomic DNA to metaphase chromosome spreads of each sex to determine if we could identify a sex-specific chromosome that hybridized only to genomic DNA of the same sex.

Materials and Methods

The methods used were based on those of Lichter et al. (1990) and were performed according to Ponce de León et al. (1996). A detailed protocol from the laboratory of Dr. F. A. Ponce de León is outlined at the end of this appendix. Male and female sturgeon genomic DNA was fluorescently labeled with biotin-16-dUTP and passed through a sephadex G-50 column to remove unincorporated nucleotides. Each of six hybridization mixtures contained different ratios of labeled genomic DNA and competitor DNA in a 1 μg/μl hybridization solution (50% formamide, 10% dextran sulphate, 1X SSC). The mixture was denatured at 75°C for 5 min, reannealed at 37°C for 10 to 15 min, and deposited on denatured (70% formamide, 2X SSC at 70°C for 2 min) male and female sturgeon metaphase chromosome preparations, sealed with rubber cement, and incubated overnight in a 37°C humidified incubator. The slides were washed in 50% formamide/2X SSC at 42°C for 15 min and 0.1X SSC at 60°C for 15 min. Blocking was done using 2% blocking reagent (Boehringer

Mannheim) and the signals were detected using avidin-fluorescin isothiocyanate (5 μg/ml, Vector labs) in 1% blocking solution. Slides were washed in 4X SSC/0.1% Tween-20 for 15 min at 42°C, stained for 10 min in propidium iodide (400 ng/ml in 2X SSC) and rinsed for 2 min in 2X SSC/0.01% Tween-20. Finally, the slides were mounted in antifade and observed under a microscope (Zeiss Axioskop) equipped for epifluorescence microscopy. Propidium iodide and fluorescin isothiocyanate (FITC) were observed with 546/590 and 450/520 excitation/barrier filter combinations, respectively.

Results and Discussion

A total of six experiments were performed. In the first two experiments we used a hybridization mix DNA ratio of 1 labeled sturgeon DNA probe: 50 unlabeled competitor sturgeon DNA of the opposite sex: 9 bovine competitor DNA. Hybridization between the probe and the unlabeled competitor DNA was allowed to proceed for a limited time to allow repetitive sequences common to both sexes in the probe to complex with the competitor DNA while leaving labeled sex-specific sequences in a single-stranded form. We found that this high proportion of sturgeon competitor DNA removed too much of the labeled probe such that the green FITC signal was not seen on most of the macrochromosomes. Green signal was localized to the microchromosomes and centromeric regions. We therefore reduced the amount of sturgeon competitor DNA in the subsequent experiments to 1 labeled sturgeon DNA probe: 12.5 unlabeled competitor sturgeon DNA of the opposite sex: 46.5 bovine competitor DNA (Experiments 3 and 4), and 1 labeled sturgeon DNA probe: 59 bovine competitor DNA (Experiments 5 and 6). The best results were achieved when no sturgeon

competitor DNA was included in the hybridization mix (Experiment 5 and 6). An even green signal was seen over the length of all of the chromosomes. There was no evidence of a chromosome that was hybridizing to the genomic probes in a sex-specific fashion in metaphase spreads from either sex.

Conclusions

Fluorescence *in situ* hybridization of labeled male and female sturgeon genomic DNA to metaphase chromosome spreads of each sex did not reveal a chromosome that was seen to be specifically hybridizing only to the genomic DNA of the same sex.

Acknowledgments

We would like to thank F. A. Ponce de León and S. Ambady for considerable assistance with the fluorescence *in situ* hybridization experimental design and execution.

FLUORESCENCE *IN SITU* HYBRIDIZATION PROTOCOL

(Protocol from the laboratory of Dr. F. A. Ponce de León, University of Massachusetts)

1. Preparation of competitor DNA

Reagents required

1. 10X nick translation buffer (0.5M Tris-HCl, pH 7.8-8.0; 50 mM $MgCl_2$, 0.5mg/ml BSA)
2. 0.1 M β-mercaptoethanol
3. 1 µg/ml DNAse I (1 mg/ml in 50% glycerol) at -20°C. Dilute 1 µl in 1 ml of icecold water immediately before use. Discard afterwards.

Resuspend 1000 µg of genomic DNA in a total of 5 ml of 1X nick translation containing 0.01 M β-mercaptoethanol (final). Pass the mixture through a 30G needle several times to mechanically shear the DNA. Add 25-30 µl of DNAse I (working dilution), mix very well and incubate at 15°C with intermittent mixing. Monitor the size of digested DNA periodically (beginning 3 hours after the addition of the DNAse I) by adding a 200 ng DNA aliquot to a tube containing a final concentration of 15mM EDTA, boiling the mixture for 10 min, and running it on a 2% agarose gel alongside a 1kb ladder DNA marker. Store the reaction tube on ice while the gel is running. If the DNA has not digested to 500 bp or below, add 25 µl of **freshly diluted DNAse** to the reaction and repeat the above steps until the DNA is digested to the correct size (< 500 bp).

Stop the reaction by adding EDTA and SDS to a final concentration of 15 mM and 0.1%, respectively. Perform a phenol-chloroform extraction and precipitate the DNA. Resuspend the DNA in sterile water and check the size of the DNA again by running it on a gel. If DNA streaks above 500 bp are seen, repeat the digestion in a smaller volume. Continue the digestion until the correct size range is achieved. Optimally the bulk of the DNA should be in the 200 to 300 bp range. Remove the DNA below 125 bp by passing the DNA through a microcon-100 (Amicon, Inc). Resuspend the DNA (0.5 - 1 µg/µl) in 1X TE.

2. Preparation of the probe

Reagents required

Nick-Translation Stock Solutions:

[A] Filter sterilized 10X nick translation buffer (0.5M Tris-HCl, pH 7.8-8.0; 50 mM $MgCl_2$, 0.5mg/ml BSA)

[B] Nucleotide Stock 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dCTP, 0.5 mM biotin-11-dUTP (Sigma No. B-7645) in 50 mM TRIS, pH 8.0

[C] Filter sterilized 0.1 M β-mercaptoethanol

[D] 1 µg/ml DNAse I (1 mg/ml in 50% glycerol) at -20°C. Dilute 1 µl in 1 ml of icecold water immediately before use. Discard afterwards.

The following mix is for a 100 µl reaction mix:

Add the reagents in the following order, all on ice:

Probe DNA		2 µg
Solution [A]		10 µl
Solution [B]		10 µl
Solution [C]		10 µl
dd H2O	Up to	100 µl
Solution [D]		1-12 µl
E. coli DNA polymerase (10U/µl), need 5 U/µg DNA		1 µl

Do not vortex DNA polymerase. Immediately place tube onto 15°C waterbath for 2 h. Terminate the reaction by adding EDTA and SDS to a final concentration of 15 mM and 0.1%, respectively. Incubate at 65-68°C for 15 min. Use a Sephadex G-50 spin-column to separate the unincorporated nucleotides. Equilibrate Sephadex G-50 with 50mM Tris, 10 mM EDTA, 0.1% SDS and wash the column with the same solution prior to use. Run an aliquot of the labeled probe, after heat denaturation (5 min in a boiling water bath, 2 min on ice) on a 2% agarose gel with a 1 kb ladder marker. Probe size should be less than 500 bp, best size is 200 bp. Keep the reaction on ice or freeze it overnight. Use a dark colored eppendorf tube to store the probe to protect it from light.

3. Preparation of the slides

Slides should be precleaned in methanol prior to use. For chromosome preparations use the air drying technique. Fresh slides can be used within a month, frozen slides in a sealed box can be used for years. Prepare 50 ml of denaturing solution (70% deionized formamide, 2X SSC) at EXACTLY 70°C in a Coplin jar, and 4 other Coplin jars (2 x 70%, 1 x 90%, 1 x 100% ethanol) on ice. Incubate each slide individually for EXACTLY 2 min in the denaturing solution, and then dehydrate the slide by placing it for approximately 3 min in the first 70% EtOH jar, and 5 minutes in each of the remaining 70%, 90% and 100% Coplin jars and then air dry.

4. Preparation of the hybridization solution

Reagents required

1. Formamide (Aldrich or Boehringer) deionized by ion-exchange column (BioRad AG.501.X8). Freeze in 35 ml aliquots after deionization,. For washing use the formamide as supplied.
2. Autoclaved dextran sulphate (Pharmacia) 50% stock in water, must be autoclaved to reduce background.
3. Autoclaved 20X SSC

Hybridization solution: need 10 µl/slide for a 18 x 18 mm coverslip, and 30 µl/slide for a 24 x 50 mm coverslip. Prepare the hybridization buffer (20% dextran sulphate solution in 2X SSC) mix well and place at 42°C. The total DNA concentration should be 10 µg/10 µl hybridization solution. The exact ratios will depend upon the experiment. In our experiments with labeled genomic sturgeon probe we used the following ratios:

DNA type	Experiment Number					
	1	2	3	4	5	6
Male probe	1		1		1	
Female probe		1		1		1
Unlabeled male competitor		50		12.5		
Unlabeled female competitor	50		12.5			
Bovine competitor	9	9	46.5	46.5	59	59

Mix DNAs for 20 minutes in a vortex, and then do an ethanol precipitation. Leave the tube at -70°C for at least 30 min or at -20°C overnight, spin, wash in 70% ethanol, spin and dry the tube in a speed-vac for 5-15 min. For DNA volumes less than 10 µl, speed-vac only. Resuspend the DNA in ½ final volume 100% deionized formamide. Vortex for 20 min. Add ½ final volume prewarmed 20% dextran sulphate in 2X SSC (mark pipette tip after test with water to assure pipetting the exact amount). Vortex 3-5 minutes. Denature the DNA at 75°C for 5 min, and then incubate the tube at 37°C for 5-15 min to allow the probe to reanneal with the competitor DNA.

5. Hybridization

During the entire in situ *hybridization procedure, slides should never be allowed to dry.*

Prewarm the slides and coverslips to 42°C and pipette the hybridization solution onto the center of the slide or premarked area. Carefully lower the coverslip to avoid air bubbles. Seal with rubber cement from a 10 ml plastic syringe. Incubate at 37°C in moist chamber overnight (16-20 hr).

6. Washes

Remove the rubber cement from the slide being careful not to disturb the coverslip. Wash the slides for 5 min in three separate Coplin jars containing 50% formamide, 2X SSC and prewarmed to 42°C in a shaking water bath. Then perform a high stringency wash by washing the slides in three separate Coplin jars containing 0.1X SSC prewarmed to 60°C in a shaking water bath. Slides can sit in the last wash, don't let them dry out.

7. Blocking

Add 200 µl 2% blocking reagent (Boehringer Mannheim, Cat # 1096 176) in maleic acid solution (1M maleic acid, .15 M NaCl, pH 7.5). Add an extra large coverslip and incubate at 37°C in a moist chamber for 1 h. After the incubation drain off the blocking solution, there is no need to wash it off.

8. Detection (in the dark)

Add 2.5 µl Avidin-FITC (2 mg/ml; Vector labs) to 1 ml of blocking solution in maleic acid solution. Incubate this solution at 37°C for 30 min. Add 200 µl/slide, cover with an oversized coverslip and incubate at 37°C for 30 min in a moist chamber covered with aluminum foil. Wash the slides for 5 min in three separate Coplin jars containing 4X SSC, 0.1% Tween 20 and prewarmed to 42°C in a shaking water bath. The coverslip will come off during the first wash. Immediately after the last wash counterstain the slides by placing them in a Coplin jar containing 200 ng/ml propidium iodide in 2X SSC at room temperature for 10 min. Wash the slides for 2 min in 2X SSC, 0.05% Tween 20 at room temperature. Add 30 µl (2 drops) antifade*, and cover with a large coverslip. Store the slides in the dark at 4°C.

* 10 ml stock DAPCO antifade
0.233g DAPCO (Sigma # D-2522)
800 µl dd H_2O
200 µl 1M Tris-HCl, pH 8.0
9 ml glycerol
Keep at 4°C in the dark

References

Bouffler, S.D. 1994. Whole-chromosome hybridization. Int. Rev. Cyt., **153**: 171-232.

Lear, T.L., Trembicki, K.A., and Ennis, R.B. 1992. Identification of equine chromosomes in horse x mouse somatic cell hybrids. Cytogenet. Cell Genet., **61**: 58-60.

Lichter, P., C.-J. C. Tang, K. Call, G. Hermanson, G. A. Evans, D. Housman, D. C. Ward. 1990. High-resolution mapping of human chromosome 11 by *in situ* hybridization with cosmid clones. Science, **247**: 64-69.

Ponce de León, F.A., S. Ambady, G. A. Hawkins, S. M Kappes, M. D. Bishop, J. M. Robl, C. W. Beattie. 1996. Development of a bovine X chromosome linkage group and painting probes to assess cattle, sheep, and goat X chromosome segment homologies. Proc. Natl. Acad. Sci. USA, **93**: 3450-3454.