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AN ABSTRACT OF THE THESIS OF Peter Bruce Valentine Jacky for the
Master of Science in Biology presented May 19, 1976.

Title: The Chromosome Constitutions of a Hybrid Salmonid and Its
Parental Types: Salmo Gairdneri and Salmo Clarki.

APPROVED BY MEMBERS OF THE THESIS COMMITTEE:

Lester J. Newman, Chairman

Richard Forbes

Trygve P. Steen

Herman E. Wyandt

Arthur F. Scott

A study was undertaken to investigate the application of tissue culture and histochemical banding techniques normally used in mammalian studies to fish cells and to examine the mechanisms of chromosome evolution and speciation in salmonids by comparing the chromosomes

of the hybrid to those of the parental types. The application of banding techniques was generally unsuccessful in revealing bands, though quinacrine staining did reveal the presence of two fluorescent spots in the vicinity of the centromere. These were thought to be heterochromatic blocks, possibly related to the high incidence of centric fusion or fission in salmonids. Results of chromosome analysis showed a higher incidence of acrocentric chromosomes in the hybrid than in either parental type which together with evidence for tetraploidization in salmonids is consistent with some of the requirements for species diversification. Conditions of divergence in chromosome evolution and changes which would have to occur in the hybrid for divergence from the parental types to take place are discussed. The results of a preliminary electrophoretic examination of LDH and esterase activity tended to confirm hybridization.

THE CHROMOSOME CONSTITUTIONS OF A HYBRID SALMONID AND

ITS PARENTAL TYPES: SALMO GAIRDNERI

AND SALMO CLARKI

by

PETER BRUCE VALENTINE JACKY

A thesis submitted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE
in
BIOLOGY

Portland State University
1976

TO THE OFFICE OF GRADUATE STUDIES AND RESEARCH:

The members of the Committee approve the thesis of
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I am disgruntled with Lucky Strikes for all the breath they've taken. An finally, I am very grateful to Lee for her loving patience both on and off this track, her clearer than my own language, and the neck rubs that ultimately kept this whole thing rolling.

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CHAPTER I

INTRODUCTION

Tissue culture and histochemical chromosome banding techniques have been routinely applied in the cytogenetic analysis of mammals. The application of these techniques to lower vertebrates, and in particular to fishes, has been relatively unstudied. The study of lower vertebrate chromosomes should offer significant evidence about speciation tendencies in vertebrates. The application of banding techniques to salmonids has been limited to C-banding (Thorgaard, 1976, and Zenzes and Vioculiescu, 1975). It does not appear that comparative chromosome studies, employing chromosome banding techniques, of a salmonid hybrid and its parental types have been published to date.

Salmonid fishes have recently received attention in chromosome studies because of a high incidence of Robertsonian-type translocations. The function of a Robertsonian translocation, centric fusion, is to reduce the number of chromosomes while maintaining the number of chromosome arms. In other words the process is one of joining two acrocentric chromosomes, each with one arm, to form one metacentric chromosome with two arms (Ohno, 1970, and Gold and Gall, 1975). Ohno has postulated that salmonids have undergone a very recent tetraploidization and are evolutionarily in the process of diploidization using the Robertsonian type translocation mechanisms (Ohno, et al., 1968, 1969, and Ohno and Atkins, 1966).

By comparing the karyotypes of S. gairdneri and S. clarki, Simon (1963) hypothesized that S. gairdneri is evolutionarily a recent derivative of the S. clarki species and that a general tendency of speciation in these particular species is toward a reduction in chromosome number. S. gairdneri has a diploid number of 60 with 44 metacentrics and 16 acrocentrics. S. clarki on the other hand has a generally accepted diploid number of 64 with 42 metacentrics and 22 acrocentrics. The chromosome arm number (N.F.) of S. gairdneri is 104 and of S. clarki, 106. Simon postulates that the transition from S. clarki to S. gairdneri must have taken place by two alterations of the haploid chromosome set: 1) a reduction in chromosome number by centric fusion of two acrocentrics and 2) a reduction in arm number by a single centromere shift, transposing a metacentric into a long arm acrocentric, that is, by a pericentric inversion. The evolutionary tendency seems to be to reduce chromosome number while functionally maintaining the same amount of genetic material.

A study was undertaken involving two species of salmonids, Salmo gairdneri (rainbow trout) and Salmo clarki (cutthroat trout), and their hybrid for two purposes: 1) to investigate the degree to which tissue culture and histochemical banding techniques normally used in mammalian studies could successfully be applied to fish cells, and 2) to study the mechanisms of chromosome evolution and speciation in salmonids by comparing the chromosomes of the hybrid to those of the parental types. The investigation proceeded out of the following question: in view of the occurrence of heterosis, is hybridization a normal intermediate in speciation or is it a dead

end in terms of chromosomal evolution ?

CHAPTER II

MATERIALS AND METHODS

Collection Techniques. Twenty-nine fish were collected by hook and line from 5 different locations in the Bull Run watershed and data were recorded on morphological type (S. gairdneri, S. clarki, or hybrid) with particular notation of sex, coloration, and presence or absence of basibranchial teeth.¹ Individuals were photographed and aseptically rinsed in 70% EtOH in preparation for removal of tissue samples in the field. Ovary or testes, spleen, liver, and muscle tissues were taken from each individual and transported to the laboratory in growth medium. The growth medium consisted of Eagle's Minimal Essential Media which was supplemented with 15-20% fetal calf serum (Flow Laboratories), 1% L-glutamine, Gentamicin at a concentration of 100 mcg/ml, 2% non-essential amino acids, and 5% whole egg ultra-filtrate (Gibco). The mixture was pH balanced with 7.5% sodium bicarbonate. Primary tissue culture lines were initiated from gonad, spleen, and in some cases liver using modifications of the techniques of Denton (1973) and Wolfe and Quimby (1969).

Culture Techniques. Three modifications of the suggested poikilothermic culture methodology were found to be advantageous in

1. Morphological indices for S. gairdneri are silvery pastel coloring on the sides, small spotting or none at all, and small scales. S. clarki has larger spots and scales, basibranchial (hyoid) teeth, red pectoral fins, red marks on the branchiostegal membrane, and yellow to olive coloration on the sides (deep red in spawners).

establishing cell lines. Tryptic digestion of the tissue explants in preparation for seeding the primary flask was generally too severe and was routinely replaced by thorough mincing. Growth medium was supplemented with 15 to 25% fetal calf serum with an apparent optimum at 19%. Also, the volume of whole egg ultra-filtrate used was 5% rather than 2½% (Wolfe and Quimby, 1969).

Gram negative bacterial contaminants were prevented with Gentamicin at 50-100 mg/ml. Mycostatin (at 100 units/ml), Anti-PPIO (6000 mg/ml), and Fungizone (25 mg/ml) were therapeutically applied against yeast and mold contaminants. The cultures were gassed with 5% CO₂ in air before incubating to achieve a pH range of 6.9 to 7.5. A temperature optimum of 19° C (suitable range: 17-25° C) was maintained by placing the incubator in a walk-in refrigerator and checking daily.

Cultures usually entered into an exponential growth phase within 5 to 9 days and were periodically split upon flask confluence. Lines not maintained for immediate chromosome analysis were frozen in liquid nitrogen after two or three passages.

Chromosome Techniques. Cultures grown for 5 to 9 weeks were harvested for chromosome analysis using conventional mammalian cell techniques (Yunis, 1974) with the following modifications: Colcemid at a final concentration of 1 mcg/ml was introduced 10-15 hours prior to harvest to increase the metaphase index; hypotonic treatment with 0.075 M KCl was extended to 25 minutes at room temperature followed by 10 minutes of centrifugation; a single prolonged treatment in KCl was found preferable to two short treatments; cells were fixed in

methanol: acetic acid (3:1) at 5⁰ C for 10 minutes. Slides were made by routine air dry techniques using wet slides and by slide flaming to improve metaphase spreading. The slides were then subjected to histochemical banding procedures.

Chromosome Banding Techniques. Cell lines from five individuals were chromosomally analyzed and karyotyped. The application of histochemical chromosome banding techniques was initiated to facilitate the distinguishing of chromosome pairs as well as to assess the applicability of conventional mammalian chromosome banding techniques to lower vertebrate cell lines. Slides were sequentially stained in the following order with the technique modifications noted:

1. Giemsa (MCB, Gurr's buffer at pH 6.9).
2. Q-banding (Casperson et al., 1970, 1972), Quinacrine Mustard Dihydrochloride (Gibco Control No. R253156).
3. R-banding, reverse banding, with Acridine Orange (Dutrillaux and Lejeune, 1971) in A.O. phosphate buffer at pH 6.5 Incubation in 85⁰ C buffer varied from 19-25 minutes as a function of slide age. Staining time in AO/cold buffer varied from 5 to 9 minutes.
4. The banding of constitutive heterochromatin, C-banding (Arrighi and Hsu, 1971, 1974), and with Giemsa at pH 11 (Wyandt et al., in press) were also attempted. The procedures were followed without modification.

Destaining, when required, was done sequentially with ethanol hydration, HCl in 70% ethanol, and 95% ethanol.

Photographs of metaphases were taken on a Zeiss Photoscope 3 with a 3RS illuminator. Appropriate enhancement and barrier filters were used for photomicrography with Kodak Pan-X Film (ASA 32). Film was developed with Diafine developer. Karyotypes were analyzed for chromosome number, N.F. and significant chromosome rearrangements.

Biochemical Technique. A preliminary biochemical examination of enzyme activity in tissues from 15 individuals, chosen on the basis of morphology to include specimens from both parental types and the hybrid, was undertaken to look for confirmatory evidence of hybridization. Electrophoresis of samples was carried out under the supervision of Fred Utter at the University of Washington, Seattle. Electro-starch gels were prepared as described by Utter et al., (1976), and the samples run against two buffer systems for best resolution. The supernatant fraction of a combined slurry of liver, spleen, and muscle tissue fragments which had been frozen after collection in Hanks balanced salt solution was used as the protein source. In the processes of freezing and thawing, sufficient quantities of the enzymes to be assayed had leached out of their particular tissues. The supernatant fraction could then be generally assessed for all enzyme markers. Creatine kinase (CK), aspartate aminotransferase (AAT), sorbitol dehydrogenase (SDH), phosphohexoisomerase (PHI), and lactate dehydrogenase (LDH) activities were assayed in a Ridgeway buffer system and malate dehydrogenase (MDH) and esterase (Est) activity were assayed in an acidic amino citrate gel. After slicing the gels, enzyme activities were assayed using standard NAD-dependent tetrazolium salt reactions (Allendorf, 1975). Photographs of both the slides and the gels were then compiled and analyzed.

CHAPTER III

RESULTS

Primary ovary and spleen explants were consistently the most successful in culture. Liver tissue required from 3 to 5 weeks before even moderate numbers of fibroblast type cells could be seen growing from seeded tissue pieces and was thus discontinued as a potential cell line source.

Minced pieces of ovary showed good fibroblastic growth in 3 to 5 days (Figure 1A and B). Spleen, while more responsive initially, required 5 to 7 days before substantial fibroblastic cell growth was detectable. A piece of spleen underwent three notable changes in these first 5 days of culture: the first stage was a general sloughing-off of tissue-bound red blood cells in the first two days followed by a very rapid proliferation of rhomboid-epitheloid cell types from the tissue explant. These were densely packed (Figure 1C) and unsuitable as a source for chromosome analysis because of a contact inhibited slow turn-over rate (Denton, 1973).

On the fifth day, spleen fibroblasts were seen growing out from beneath the initial peripheral rhomboid layer. The fibroblasts continued in rapid turnover to flask confluence. Fibroblasts cause the epithelial cells to lift off of the flask bottom which seemed to indicate cell death. Trypan Blue was used to check free-floating cells for viability. Both ovary and spleen reached flask confluence (25 cm²) in 2 to 5 weeks and could be split into larger flasks.

Figure 1. Typical cell morphology from cultured ovarian and spleen tissues (phase;150x): A and B, ovary at 5 weeks, typical fibroblast morphology, mitosing cells (mit) are rounded up from cytoplasmic accumulation in the cell main body. Note binucleate cell in A. C, 5 day old spleen culture with a preponderance of epitheloid type cells and indications of secondary fibroblast cell types. D, 9 week old spleen fibroblast line with perinuclear chromatophores.

General fibroblast morphology, with particular attention to increases in vacuolation and lifting-off was checked as culture progressed. It was usually possible to return unhealthy cultures to normal growth by supplementing them with fetal calf serum and L-glutamine and reducing therapeutic applications of Fungizone by 90%.

Peri-nuclear chromatophores were more abundant in older cell lines (Figure 1D). Occasional large fibroblasts were observed, suggesting that polyploid cells had arisen by endoreduplication. This observation was confirmed with Giemsa stained slides showing cells with 2,4,6, and 8 nuclei, with a preponderance of cells having 4 to 6 nuclei in some cultures (Figure 1A). It should be emphasized however that this was not a normal circumstance.

Quinacrine fluoresced brightly at the centromere, or in a region adjacent to it, and on some chromosomes at the telomeric ends, apparently distinguishing regions of constitutive heterochromatin (Thorgaard, 1976). Two distinct centromeric spots were clearly visible on most acrocentric chromosomes and could occasionally be discerned on each arm of larger metacentric chromosomes. It is of interest that the fluorescence pattern was observed to be distinctly divided into two spots. The significance of these seemingly chromatid associated, heterochromatic blocks will be discussed later.

The banding of constitutive heterochromatin, C-banding, required reducing the length of denaturation in .07N NaOH to less than a minute (15-45 seconds). Constitutive heterochromatin is located at the centromeres and to a lesser extent at the telomeres of the larger metacentric chromosomes. Data from Q-banding were consistent with

this result.

Sequential staining of chromosomes with Giemsa and quinacrine provided a clearer delineation of homolog pairs and better resolution of particular chromosome rearrangements. On the basis of banding information and centromere position, it was possible to group the chromosomes as follows (Figures 2,3,4,5, and 6):

Group A: 9-10 pairs of metacentric chromosomes in descending size order.

Group B: 9-10 submetacentric pairs in descending size order.

Group C: chromosomes having clearly defined secondary constrictions.

Group D: chromosomes involved in rearrangements.

Group E: acrocentric and telocentric chromosomes.

In a number of the karyotypes, two acrocentrics were placed end-to-end to make one homolog of a metacentric pair. Q-banding facilitated making such homologies since it was obvious that particular metacentric chromosomes occasionally had no metacentric homolog (Figure 4). Whether metacentric breakage was the result of persistent cell culturing, as is the occasional occurrence of polyploid cells, or whether it is a tendency in the hybrid to meiotically break metacentrics in second generation pairing could not be determined. Frequency of both broken metacentrics and submetacentrics in the first 3 to 4 pairs of groups A and B bears some significance.

Of the two specimens of S. gairdneri chromosomally analyzed, the total arm number varied from 101 to 105. A single specimen of S. clarki showed a N.F. of 105-106. The two hybrids showed a variation of 90-107 in N.F. (Table 1). The number of chromosome

Figure 2. Giemsa stained karyotype from an ovary cell line of S. gairdneri (No. 21). Two acentric fragments have been appropriately placed to account for discrepant lengths in particular homologs. A submetacentric telomere / acrocentric short arm association (arrow) may be an alternative to the Robertsonian type translocation mechanism in salmonids for functionally reducing the no. of linkage groups while maintaining the same amount of genetic material.

21 Sp5A 10/19 Fig.3 *S. gairdneri*

Figure 3. Quinacrine karyotype of *S. gairdneri* from a spleen cell line. Quinacrine fluorescence shows bright regions at the telomeric ends and at or proximal to the centromere. Quenching of the stain is clearly evident at the 2^o constriction region of the C-group chromosomes. The first pair of submetacentric chromosomes (B-group) is either heteromorphic for centromere position or the discrepancy can be explained by chromatid overlap of the short arm.

Figure 4. Quinacrine stained karyotype: ovary line, hybrid 13.

Figure 5. Quinacrine staining of a spleen cell line from hybrid no. 13. Note particularly that the inverted D-group chromosome shows bright fluorescence in two distinct areas, suggesting a dicentric chromosome. "Dicentricity" may be a structural intermediate in the fusion of two acrocentrics with short arms.

TABLE I

CHROMOSOME COUNTS AND MORPHOLOGICAL CRITERIA FOR
FIVE FISHES CHROMOSOMALLY ANALYZED

Species	Fish No.	Sex	BASIBRANCHIAL 'HYOID' TEETH PRESENCE	EXTERNAL MORPHOLOGY	TISSUE CULTURED OVARY or SPLEEN CELL LINE No.	No. of CELLS COUNTED	DIPLOID NUMBER	KARYOTYPE ANALYSIS			FIGURE No.
								NUMBER of METACENTRICS	NUMBER of ACROCENTRICS	NUMBER of ARMS, N.F.	
<u>S. gairdneri</u> (rainbow)	15	♂	-	rainbow character- istics	Ov 3A	9 1 2	59 69 68	45 37 36	14 31 32	104 105 104	
<u>S. gairdneri</u> <u>irideus</u>	21	♀	-	steelhead rainbow trout	Ov 5A	5 3	59 57	45 43	14 14	104 100	Fig. 2
					Sp 5A	3 3	57 29	44 25	13 4	101 54	Fig. 3
<u>S. clarki</u> (cutthroat)	14	♂	+	cutthroat	Sp 2A	5 2	64 63	42 42	22 21	106 105	
<u>S. clarki</u> x <u>S. gairdneri</u> (hybrid)	13	♀	+	rainbow w/ throat marks, red tip pector- als, <u>clarki</u> spotting	Ov 3A	5 2 1 1	63 66 69 119	34 34 37 (endoreduplication)	29 33 32	97 101 106	Fig. 4
					Sp 5A	3 1 1	66 47 53	33 43 52	33 4 1	99 90 105	Fig. 5
<u>S. clarki</u> x <u>S. gairdneri</u>	19	♀	+	hybrid intermedi- ate	Sp 4A	1 2	66 59	39 43	29 16	107 102	Fig. 6

arms in S. gairdneri and S. clarki have been shown to be 104 and 106 respectively (Ohno, 1966; Fukuoka, 1972; Simon and Dollar, 1963; and Thorgaard, 1976). While the data show a relative consistency in the number of chromosome arms, there are obvious discrepancies in total chromosome number within a particular fish. Fluctuations in chromosome number have generally been attributed to Robertsonian translocations (Roberts, 1970; Simon, 1963; Nygren, 1975; and Ohno, 1974).

From the data in Table 1, it appears that fishes 14, 15, and 21 are consistent in N.F. and chromosome number with data already available on S. clarki and S. gairdneri, and can be considered representative of the parental types.

Fishes 13 and 19 morphologically appeared to be hybrids. That is, they had the pastel coloring of S. gairdneri while also having S. clarki throat marks, basibranchial teeth, and spotting. Chromosomally, these two fish showed wide discrepancies in N.F. and diploid number. There was a notable increase in the number of acrocentric chromosomes with a concomitant reduction in the number of metacentrics, suggesting considerable centric fission not evidenced in non-hybrid individuals.

Heteromorphic chromosome pairs were found in both hybrid fish. Group C chromosomes indicated a contribution from both parental types: the secondary constriction was more distally located on the long arm of the chromosome contributed by the S. gairdneri parent while the secondary constriction was more proximal to the centromere in the chromosome contributed by the S. clarki parent. Heteromorphic chromo-

some pairs were not evident in fishes 14, 15, and 21. There were no sexual dimorphisms.

An unpaired and presumably inverted chromosome which was not evident in either parental type was found in each hybrid fish. The parental types did occasionally show rearrangements in chromosome morphology. The long arm association of a submetacentric chromosome with an acrocentric chromosome (Figure 2) indicates an alternative to the Robertsonian type translocation mechanism for functionally reducing N.F. and chromosome number. Polyploid cells, thought to be the results of extended Colcemid treatment, were occasionally found (Figure 8).

A triradial association was noted in hybrid 19 (Figure 6). This apparently involves an acrocentric and two metacentric arms which seem to be in the process of fission. Satellite-like blocks of chromatin were observed in this association which were thought to be synonymous with the heterochromatic blocks evident in quinacrine fluorescence. No other such associations were found in fishes 13, 14, 15, or 21.

In conclusion, sequential staining did not clearly show an appreciable number of chromosome bands. That is, with the possible exceptions of quinacrine mustard and C-banding procedures (Figure 7), the histochemical banding techniques including reverse banding with acridine orange and Giemsa at pH 11 were unsuccessful.

Although the banding techniques were generally unsuccessful in revealing bands, two large fluorescent heterochromatic blocks could be discriminated at, or proximal to, the centromere as a result of

Figure 7. C-banding of a hypermodal cell. Major constitutive heterochromatic blocks are at or proximal to the centromere (C) with detectable banding at the telomeric ends (T) on some chromosomes. Note the pronounced heterochromatic regions on the acrocentrics (h) thought to be involved in Robertsonian rearrangements while the more stable metacentrics (m) band only minimally.

Figure 8. An endoreduplicated cell from an ovarian cell line of hybrid No. 13. Interphase cells having undergone endomitosis showed multiple nuclei (Figure 1A).

quinacrine staining and C-banding. These were consistently found on acrocentrics and occasionally on metacentrics.

The results of electrophoretic examination of 15 fish for 3 biochemical markers are presented in Figure 9. Although good band resolution was obtained, evidence for hybridization would require further study. It is noteworthy that fishes 19 and 33, both morphological hybrids, are apparently heterozygous for the LDH-4 locus. Heterozygosity for fishes 19 and 33 was confirmed by esterase activity though this is not generally accepted as a reliable indicator of specific hybrid gene activity (May, et al., 1975, and Utter, personal communication).

Figure 9. Electrophoresis of 3 enzyme markers: LDH, PHI, and Esterase activity. The 3 gels were sliced from the same starch slab and differentially stained for specific enzyme activity. In control standards, Ct=*S. clarki* and St = *S. gairdneri* for particular tissues electrophoresed. Hybrids No. 19 and No. 33 show heterozygosity at the LDH-4 locus. No. 19 is also heterozygous for Esterase activity. The significance of hybrid heterozygosity to adaptive plasticity in speciation is discussed.

CHAPTER IV

DISCUSSION

Although the application of mammalian chromosome banding techniques to lower vertebrate fishes met with limited success in the experiments presented in this thesis, it is the opinion of the author that further experimentation would show them to be as successful with lower vertebrate as with mammalian cell lines. A noteworthy outcome of quinacrine staining was the appearance of fluorescent spots near the centromere. It is possible that these represent blocks of heterochromatin that may functionally protect structural gene material of acrocentric arms that are involved in centric fusion. It is also possible that these heterochromatic blocks are either the result of, or necessary for, the frequent occurrence of centric fusion and fission in salmonids. Is the presence of heterochromatin proximal to the centromere essential to a high incidence of Robertsonian translocations?

Susumu Ohno, known for his work on evolution at the chromosome level, has presented several characteristics of speciation and divergence. First, speciation and diversification of an organism require that it be less specifically adapted (less fixed) and geographically isolated. If the organism is not geographically isolated, it must be chromosomally isolated in order to avoid breeding back to the ancestral species. If these requirements are met, then through normal mutation, divergence occurs.

A second condition of speciation and diversification is that the organism must have genetic redundancy. Duplicated gene material, for which the organism has no immediate use, provides the potential for acquiring a new genetic function. Genetic redundancy can be achieved by polyploidization or tandem gene duplication, e.g. unequal sister chromatid exchange. Finally, Robertsonian translocations and pericentric inversions are the mechanisms which facilitate chromosomal isolation and which are responsible for associating particular genes into specific linkage groups.

In this study Robertsonian translocations involved almost exclusively the large metacentric and submetacentric chromosomes of Groups A and B, indicating that Robertsonian translocation is probably not a completely random mechanism. This hypothesis is supported by the findings of Davisson (1972, 1973) where pseudo-linkage and segregation of the LDH A and LDH B alleles were demonstrated in a trisomic male of Salvelinus fontinalis. The aneuploidy involved a single new metacentric, each arm of which was linked to an LDH locus. A non-random association of two acrocentrics to form one metacentric may put functionally related genes proximal to one another for more efficient regulation and guarantees their segregation as a metacentric unit.

Chromosome studies of the hybrid fishes showed a number of marked differences from the parental types which may be helpful in understanding the role of hybridization in evolution at the chromosome level. Perhaps most significant is the increased number of acrocentric chromosomes in the hybrid. It is thought to be the tendency

in chromosome evolutions to reduce the total number of chromosomes by centric fusion (Comings, 1972).

The hybrid appears to have reverted to a more elementary karyotype. The increased number of acrocentric chromosomes will increase the number of bivalents at metaphase-I of meiosis and thereby increase the genetic variability within the gametes through independent assortment. Thus there is increased variability on which natural selection may act.

In investigating hybridization from the standpoint of evolution, we must ask: what necessary changes must take place to lead from hybridization to species diversification? We have noted that the increased number of acrocentrics would provide greater flexibility in meiotic pairing and therefore greater potential variation of offspring. However, the existence of a heteromorphic chromosome pair in the hybrid, one chromosome from each parental type, indicates that, if the hybrid population were inbred, 50% of the offspring (Mendelian inheritance) would revert to the parental chromosomal type². In order for a hybrid to stabilize into a new species, it would be necessary for some change to occur in the heteromorphic pair which would make it a homologous pair different from either parental contri-

2. Although hybrid fertility was not proved or disproved by this study, it was assumed on the basis of anatomy that hybrid specimens, fish 13, 19, 25, and 33, were fertile. Fishes 13 and 19 contained healthy appearing egg-laden ovaries; fish 25 had a streaked ovary which may have been due to physical immaturity since the fish was apparently juvenile; and fish 33, a *S. clarki* and salmon hybrid, was heavily laden with fully developed egg skeins. Studies by Nygren (1975) confirmed hybrid fertility in a *S. salar*, *S. trutta* cross, and fertile hybrids were found by Simon and Nobel (1968) in the genus *Oncorhynchus* despite widely disparate diploid numbers.

bution.

The question, whether the increased number of acrocentric chromosomes in hybrids 13 and 19 is a direct consequence of meiotic pairing of the haploid contributions from both parental types in the F_1 hybrid, or if the increased incidence of acrocentrics is a function of long term tissue culture, deserves some attention. The consistency of increased acrocentric number in both hybrids and a notable increase in acrocentrics in two different cell lines from the same hybrid individual (ovary and spleen) as compared to no increase in parental acrocentric number, is evidence that long term cell culture is not responsible for the acrocentric increase. In human chromosome breakage studies, short-term lymphocyte cultures are used because of a tendency for chromosome breaks to either stabilize or to be overcome by normal cell proliferation (Yunis, 1974).

There is an apparently inverted C-group chromosome found in both tissue lines of hybrid 13 that can not be paired with a homolog in karyotyping. It is postulated that this inverted chromosome is dicentric and could have arisen as a result of a meiotic pairing irregularity involving a metacentric and a large acrocentric in an F_1 hybrid. Simon (1963) pointed out that a single centromere shift in a metacentric giving rise to along arm acrocentric was partially responsible for the divergence of the S. gairdneri species from the S. clarki ancestral line.

Electrophoretic examination of F_1 hybrid individuals generally showed patterns of enzyme activity associated with both parental types. This has been interpreted as an initially codominant relationship

between contributing parental genes (Nyman, 1970). F_2 individuals however show patterns of a single parent. Apparently in the F_2 , preferential selection of a single genotype is dominant and therefore suppresses the expression of one contributing parent.

Goldberg (1966) in an examination of the hybrid splake trout (speckled x lake) found F_1 individuals to be heterozygous for the LDH-5 locus. Heterozygosity for the LDH-4 locus has been observed in hybrids 19 and 33 in this study.

On the basis of esterase activity, hybrid 19 showed electrophoretically detectable heterozygosity. The 2-band pattern strongly suggests contributions from both S. clarki and S. gairdneri for the esterase locus (Figure 9). Although esterase activity is not accepted as a reliable indicator of specific gene activity in salmonid fishes (Allendorf, 1975), recent studies with Salvelinus fontinalis (brook trout), S. clarki, and S. gairdneri have shown 3 distinct bands for a monomeric liver esterase (Utter, personal communication). The similarity of results in this study with those using liver esterase activity tends to confirm fish 19 as a hybrid.

In examining hybrids for adaptive plasticity, the question arises: is there a selective advantage in the maintenance of heterozygosity at a particular enzyme locus? Koehn (1969), studying serum esterases, found a clinal distribution of esterase heterogeneity. Three phenotypes were distinguished in a 2-allele system. Each enzyme exhibited a markedly different temperature optimum. Koehn pointed out that it is reasonable to postulate, similar functional restrictions on other "heterotically maintained two allele polymorphisms". Select-

ive variation in temperature optimums for particular enzyme systems would certainly be of significance in poikilothermic organisms, possibly allowing for adaptation to and isolation in a particular geographic area.

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