[Salmonidae research]

UNIVERSITY OF WASHINGTON

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We have carefully read the thesis entitled Cytogenetics, Relationships and Evolution in Salmonidae.

submitted by Raymond C. Simon in partial fulfillment of the requirements of the degree of Doctor of Philosophy and recommend its acceptance. In support of this recommendation we present the following joint statement of evaluation to be filed with the thesis.

The question of speciation of the family Salmonidae has provoked a series of re-appraisals by taxonomists through the years. The present taxonomy is based upon a wide variety of morphological features. The candidate has seized upon a new approach for a more complete description of the speciation problem by using chromosome morphology for comparison. Through a series of studies on selected materials he has provided a sound basis for the differentiation of the species based upon chromosome numbers and arms. He has derived a concept relating total genetic transfer and described the probable development of the various Salmonidae. Based upon these studies he has concluded that the modern designation Oncorhynchus should be included in Salmo. The theories of Arm transfer and translocation mechanisms have provided a genetic basis for the inclusion of Oncorhynchus in the genus Salmo. The work constitutes a valuable contribution not only to the taxonomy of confusing genera but provides a guide for comprehension of their evolution.

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INTRODUCTION

Studies of the family Salmonidae have resulted in many statements concerning the morphological plasticity and the consequent systematic difficulty of the several component species. These facets of complexity are inextricably related in understanding the evolution of this widely distributed group of teleosts. The elusive nature of systematic problems is evident even at the family level and extends through subsequent taxa down to the subspecific level.

For example, the Pacific salmons, the trouts, and chars have been placed in the family Salmonidae while the whitefishes and grayling have been placed in separate families (Coregonidae and Thymallidae respectively) according to the recommendations of Schultz (1936). More recently Berg (1940) has placed the whitefishes with the family Salmonidae but has retained the distinction of Thymallidae. The current trend appears to favor the inclusion of all three groups in the family Salmonidae to include the subfamilies Salmoninae, Coregoninae and Thymallinae (Bailey, et al., 1960; Norden, 1961). Within this framework the Salmoninae are represented by five reasonably consistent genera: Brachymystax, Hucho, Oncorhynchus, Salmo, and Salvelinus. Two additional genera, Cristovomer and Salmothymus, have been variably included or rejected.

This scheme has been generally, although not universally, adopted. Very frequently in the Scandinavian literature no distinction is made nominally between Salvelinus and Salmo with both included in the latter. Regan (1914) in a study of cranial characters and anal fin rays in Salmo and Oncorhynchus arrived at two alternative conclusions related to generic

distinction between the two: (a) since counts of anal fin rays overlapped extensively in comparing S. clarkii and O. masou, he believed that generic status of Oncorhynchus could not be maintained, or (b) if differences in cranial characters (size and shape of the mesethmoid) were deemed to warrant retention of generic distinction, then Oncorhynchus would perforce include the Pacific trouts with S. trutta and S. salar alone constituting the genus Salmo. Tchernavin (1937) rejected these conclusions by recognizing five species of Oncorhynchus and by tacitly ignoring the existence of O. masou as a sixth species upon which the weight of Regan's argument hinged. The recommendations of Tchernavin have been followed up to the present despite the fact that O. masou is widely recognized to be a "good" species.

While a relatively enormous number of papers has appeared in the past eighty years which were directly concerned with taxonomic problems and evolution of these fishes, there has been no attempt to review the systematic status of Salmoninae from the viewpoint of more recent concepts of species. Most taxonomic studies have been conducted largely or exclusively within the framework of the "type species" concept employing morphological criteria.

There seems to be unity in the view that systematic arrangements are constructed to represent evolutionary trends in the best possible manner. It is evident that knowledge of evolution should ideally precede the formulation of a classification; however, the beginnings of a study are rarely made under such desirable circumstances to which the classification of fishes is no exception. The alternative is to collect new information which may bear upon the understanding of evolutionary processes.

Systematics, interrelationships, and evolution are dependent

segments of a single, complex problem. Petrunkevitch (1952) has succinctly stated this interdependency:

> Yet the essence of systematics is not the creation of handy classifications, not the preparation of keys useful for the identification of species, not the production of check-lists for zoogeographical studies. All this is done because it has to be done when one is confronted with the problems of evolution. For systematics is that branch of biological science which is engaged in unraveling the mystery of evolution by the only method by which this can be done, namely by a comprehensive study of relationships between living beings, based on all available data.

Taxa are constructed on the basis of similarities and dissimilarities. Those animals which form "natural" groups do so because taxonomists have placed them there on the basis of characters in common. Simpson (1961) commented that ". . . the concept of homology grew out of the observation of characters in common. Homology does always involve characters in common, but it has also been sufficiently shown that the mere existence of characters in common or the possibility of abstracting an archetype or its modern synonym, a morphotype, is not a sufficient criterion of homology." Simpson (1961, p. 81) elaborates further that "it was Darwin (1859) who produced the explanation and a definite criterion for homologues; they are, as defined above, organs or, more inclusively, any similarities inherited from a common ancestry." The problem of recognizing those characters in common which have been inherited from a common ancestry seems to be a formidable one since it not only involves discrimination between truly homologous characters and those which are the

consequence of parallel adaptations, but it also demands that the direction toward a primitive condition be correctly assessed.

It would appear that the chromosomes of salmonids can provide helpful clues indicating generally a decrease in chromosome number with increased specialization, at least within Oncorhynchus (Simon, 1963). In Salmoninae the genera Brachymystax and Hucho are considered to be most primitive on the basis of morphological characters while increasing specialization is thought to progress through Salvelinus, Salmo, and Oncorhynchus (i.e., Norden, 1961). Additional support with respect to the order of the latter three is gained through consideration of degree of anadromy (Rounsefell, 1958), behavioral aspects (Hoar, 1958), and geographical distribution (Neave, 1958). Salvelinus is also suggested to be more primitive than Salmo and Oncorhynchus on a chromosomal basis by observing that all chars studied thus far possess 80-84 chromosomes in the diploid condition (Svardson, 1945; Wahl, 1960) representing the upper extreme in Salmoninae. The lower extreme in diploid number (2n = 52) is represented by the highly specialized pink salmon, O. gorbuscha (Simon, 1963). New extremes may be established when more populations of Salmoninae are subjected to chromosome study. The very high numbers proposed by Nogusa (1960) for several salmonids will be considered in the discussion.

Among Salmo the brown trout (S. trutta) is conspicuous in possessing a diploid number of 80 and in this respect is similar to the species of Salvelinus. This similarity prompted Wright (1955) to comment, "Rainbow trout (2n = 60) and brown trout (2n = 80) have been classified as belonging to the genus Salmo, whereas the brook trout (2n = 84) has been placed in Salvelinus. It is apparent that the chromosome numbers and morphology

differ more between the two species of the same genus than between the members of different genera. It has been known for some time, moreover, that hybridization between brown trout and brook trout is feasible, whereas hybridization of rainbow trout with brown trout or brook trout is extremely difficult. On the basis, therefore, of both chromosome-number relationships and ability to hybridize, the species belonging to different genera are more closely related than are the two that have been placed in the same genus." Alm (1955) stated in diametric opposition (based on F_1 hybrid mortality) that even though the brown trout chromosome number is nearer that of the chars, the chromosomes of the brown trout were more nearly homologous with those of Salmo salar. Alm's extensive data indicate that survival in some lots of S. trutta x S. fontinalis is better than in some control lots of S. trutta x S. trutta; although in general the latter is more favorable.

Success of F_1 hybrids would seem to be a somewhat questionable measure of relationships as indicated by the comments of Hubbs (1955) and White (1945). The importance of interbreeding is notwithstanding of paramount utility in the species concepts of the "new systematics" (Huxley, 1940; Mayr, et al., 1953; Simpson, 1961; Mayr, 1963) by virtue of the fact that populations cannot become distinct species if they share a common gene pool. The apparent conflict in the preceding statements is resolved by recognizing that artificial hybridization may not even remotely reflect interbreeding potential under natural conditions. For example, the results of artificial crosses (successful) of Lota marmorata x Salmo trutta (Fraas, 1854) and Gadus macrocephalus x Oncorhynchus keta (Terao, 1935) do not demand that the families Gadidae and Salmonidae be placed in the

same order. In the evolutionary sense interbreeding is important only when the hybrids are fertile. Attempts to deduce relationships must therefore consider degree of hybrid sterility as well as potential for interbreeding under natural conditions.

Questions on relationships in Salmoninae appear to be amenable to clarification by obtaining new information on chromosome numbers and morphology. The wide range in diploid chromosome number (52-84) in this subfamily would at first appear to indicate a wide diversity in hereditary material of the species studied thus far. This diversity is minimized by observing that most species possess approximately 100 chromosome arms and that numerical differences in diploid number can be explained by considering the proportion of metacentric (two-armed) and acrocentric (single-armed) chromosomes (Simon, 1963). Chromosome study would also appear to offer a means of evaluating hybridization since chromosome segregation to gametes in maturing F_1 hybrids is of the essence in determining the success of the F_2 generation (i.e., F_1 fertility).

The value of chromosome data would appear to be enhanced by:

(a) explanation of species such as S. salar with atypical arm numbers (72 rather than ca. 100); as reported by Svardson (1945) and Boothroyd (1959);

(b) study of meiotic chromosomes in maturing testis to clarify or confirm the highly different chromosome numbers reported by Nogusa (1960) for several salmonids;

(c) determining chromosome numbers in F_2 hybrids of species with widely different chromosome numbers as a means of assessing segregation behavior of meiotic chromosomes

to the F_2 zygote;

(d) study of meiotic divisions in testis of F_1 hybrids to reflect upon pairing homology and gamete viability;

(e) considering published information on interbreeding and chromosome morphology together with new information on these subjects to obtain a current synthesis on species evolution in Salmoninae together with any systematic implications which may follow.

These desiderata constitute the objectives of the present study.

MATERIALS AND METHODS

Sources and preparation of embryonic, juvenile and adult materials

Samples of ten populations of "pure strain" origin and one of hybrid origin were obtained as embryonic material at the blastula stage. Ova of two or more females were fertilized, incubated, and preserved in separate groups. One to four ounces of fertilized ova were preserved from each female after incubating for a predetermined period dependent upon temperature and incubation rate of the species (Simon, 1963). Data pertaining to these samples are contained in Table 1.

Chromosomes of embryonic tissue were stained in 2% orcein (G. T. Gurr) prepared in acetic acid according to the instructions of Ritter (1958). Temporary preparations were prepared by the squash method described by Simon (1964) which is schematized in Figure 1. The reference to 32 temperature units in the upper left of Figure 1 applies to the rainbow and cutthroat trouts and has been adjusted to equal about 1/24 the total incubation period in other species.

Criteria for selecting embryonic cells for chromosome counts

Several criteria were adopted and followed in selecting cells of blastulae for chromosome counts. This was deemed to be essential in reducing errors arising from counting chromosomes in fragmented cells with reduced chromosome numbers (Hungerford, 1955; Hsu and Klatt, 1958; Simon, 1963). Two general aims of these critera are: (a) to exclude ruptured cells to minimize the possibility of counting chromosome sets which have lost (or gained) one or more chromosomes as a consequence of mechanical

Table 1

Species, sample size, collection area and date of sampling for embryonic chromosome materials (blastulae)

species	date	females sampled	collection locality
Salmo g. gairdneri	April, 1962	5	Soos Creek, Pierce Co., Wn.
	Feb., 1963	2	Minter Creek, Pierce Co., Wn.
Salmo c. clarki	Jan., 1963	10	Washougal River, Clark Co., Wn.
	Feb., 1963	2	Minter Creek, Pierce Co., Wn.
Salmo c. henshawi	May, 1963	10	Summit Lake, Summit Lake Indian Res., Nev.
Salmo c. lewisi	April, 1963	10	Leavenworth National Fish Hatchery, Wn.
	June, 1963	5	Lauri Lake, Flathead Co., Mont.
	June, 1963	10	Jackson National Fish Hatchery, Wyo.
Salmo aguabonita	June, 1963	5	Alpine Lake, Wind River Indian Res., Wyo.
Oncorhynchus masou	Sept., 1963	5	Vicinity of Sapporo, Hokkaido, Japan
O. keta x *O. gorbuscha* (F_2)	Sept., 1963	7	Hoodsport Salmon Hatchery, Mason Co., Washington

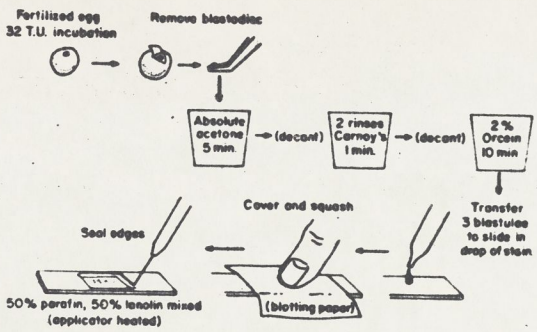


Figure 1

Method employed for temporary preparation of embryonic chromosomes

displacement from squashing pressure, and (b) to avoid the confusion of counting chromosome sets which are grossly atypical or insufficiently spread. The criteria adopted are:

1. chromosomes not extensively distorted as evidenced by circular (rather than ovoid) arrangement on the metaphase plate;
2. all chromosomes in sharp focus in a single plane at a magnification of 400 x (high-dry objective);
3. extensive or confusing overlapping of chromosomes not present;
4. cell of rounded shape without interruption of cytoplasmic outline;
5. mitotic stage of mid to late metaphase, or earliest anaphase without extensive separation of chromatids;
6. obviously hypo-, or hyperdiploid cells excluded;

7. entire embryos excluded which display abnormal components such as anaphase chromatin bridges;
8. blastulae rejected which display lobed, or otherwise non-spherical outline upon macroscopic inspection; these blastulae are typically mosaics of two or more cell populations with different chromosome number.

These criteria are illustrated in Plates I, II and III (Figs. 2-11) with further explanatory comment, with exception of #8 which is obvious upon inspection. Strict adherence to the criteria was considered to be necessary in order to obtain reproducible results.

Preparation of testis sections

Pieces of testis ca. 2 mm. in maximum dimension were excised from maturing males (two each) of Salmo gairdneri irideus, Oncorhynchus gorbuscha and Oncorhynchus gorbuscha x O. keta (F_1). This material was obtained from the University of Washington fish hatchery, the Stillaguamish River (Snohomish Co., Wn.) and the Hoodsport Salmon Hatchery (Mason Co., Wn.) respectively. Fixation in Flemming's mixture (water 250, osmium tetraoxide 1.0, chromic acid 1.8, acetic acid 12.5) for 24-36 hours was followed by paraffin embedding. About 30 cc. of fixative were used for each gram of tissue. Sections were cut at 5 microns and stained in Heidenhain's iron-hematoxylin for 24 hours following a one-hour mordanting in 2.5% ferric ammonium sulfate (aqueous solution). Destaining was accomplished with the latter solution. No counterstain was employed. Duplicate samples were also fixed in Carnoy's fluid and prepared by the squash method as for embryonic tissue.

Plate I

Illustration of Counting Criteria

Figure 2

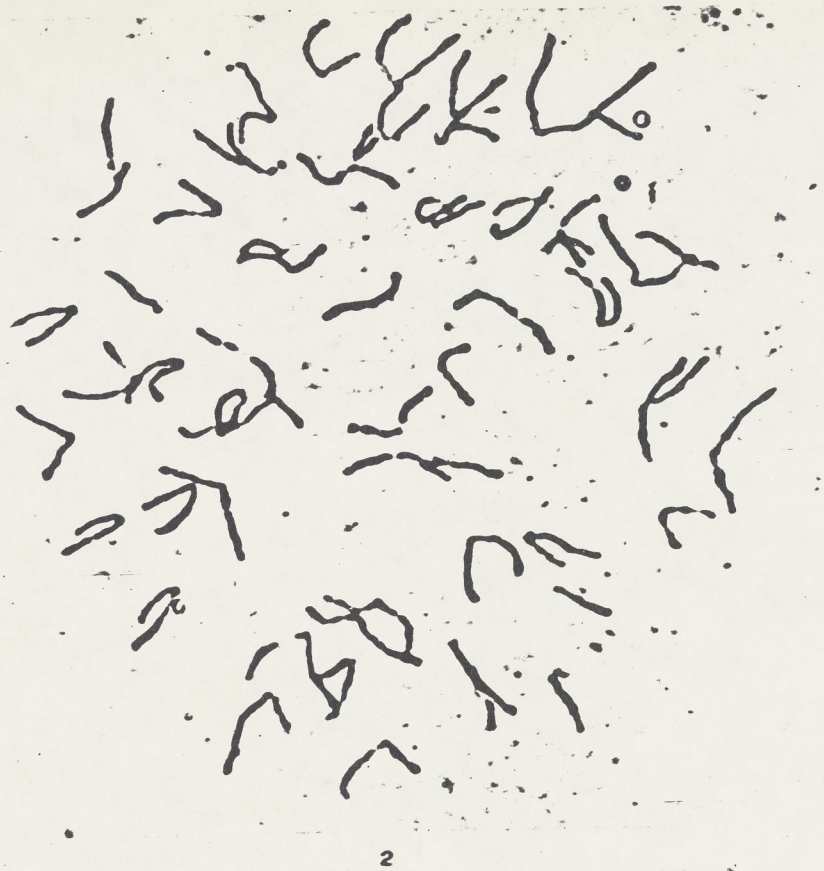
Chromosomes in focus on a single optical plane. Phase-contrast with orcein staining. S. gairdneri blastula, late metaphase, magnification 3000 x.

Figure 3

Ovoid aggregation of chromosomes indicates disruption by squashing pressure despite clarity in single focal plane. Phase-contrast, orcein staining, Oncorbynchus keta x O. gorbuscha F_2 hybrid, magnification 1145 x.

Figure 4

Confusing overlap of chromosomes at lower center. Phase-contrast with orcein staining. O. keta x O. gorbuscha F_2 hybrid, magnification 2500 x.



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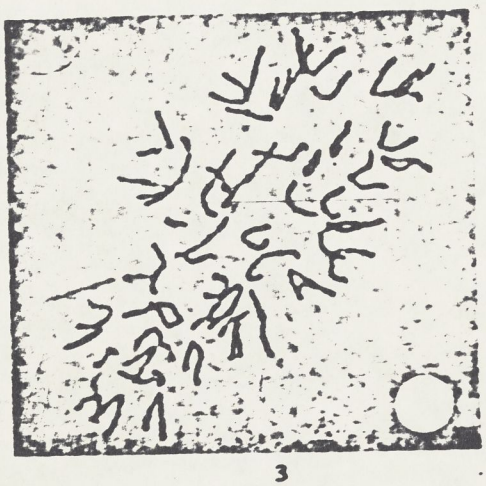

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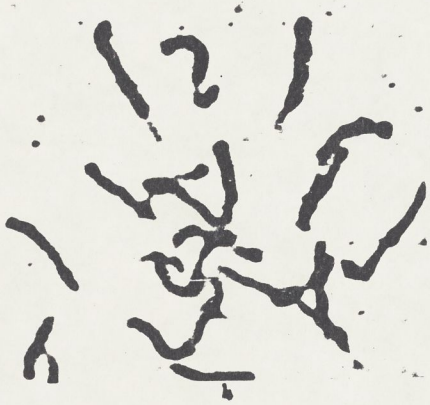

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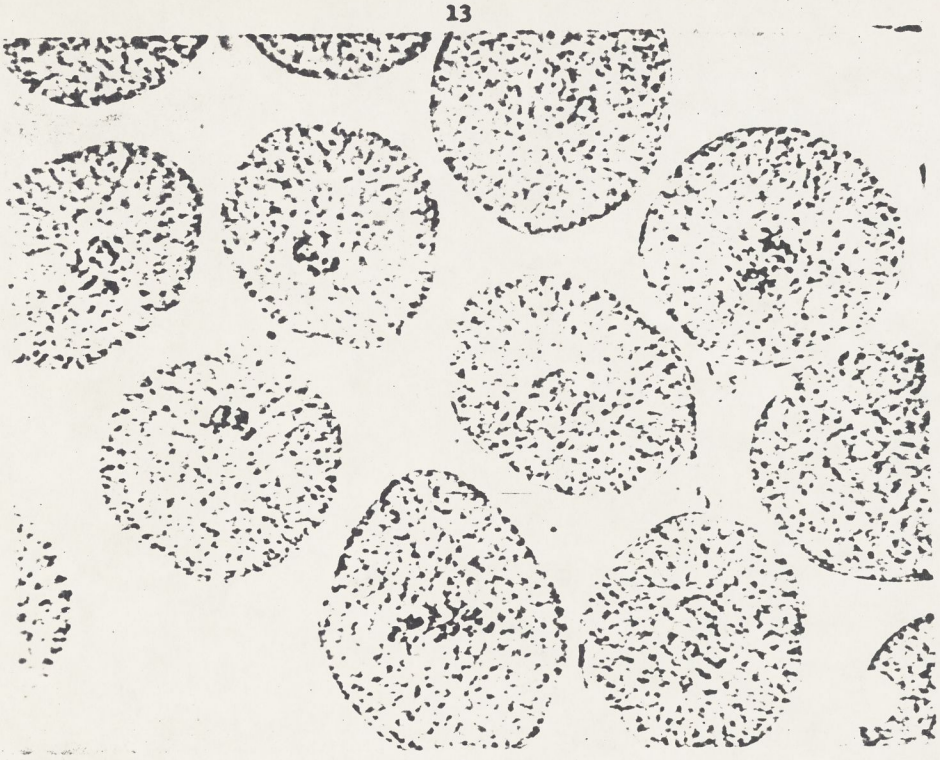

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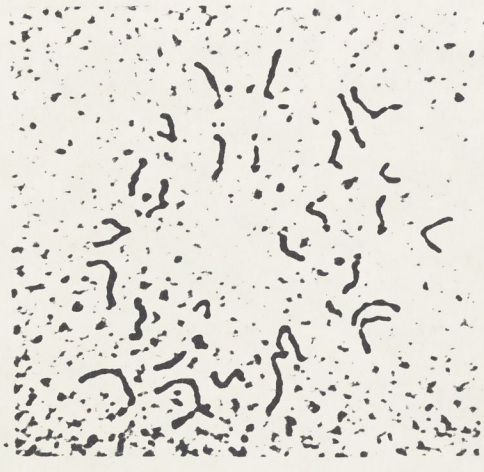

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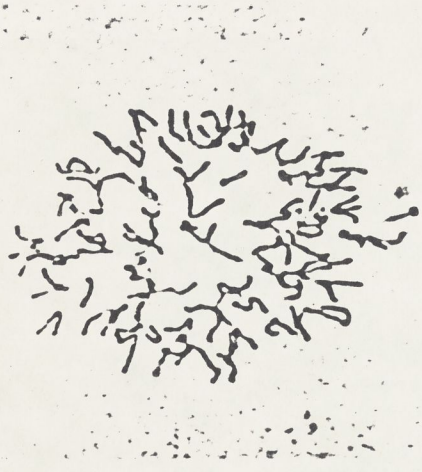

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Plate III

Illustration of Counting Criteria (continued)

Figure 8

Mitotic defect in metaphase of S. gairdneri. Note tenuous connection of two peripheral chromosomes at 2 o'clock. Other cells from the same embryo often display atypical chromosome numbers. The number of cells affected is a function of the time at which the defect was acquired. Brightfield illumination, orcein staining. Magnification 600 x.

Figure 9

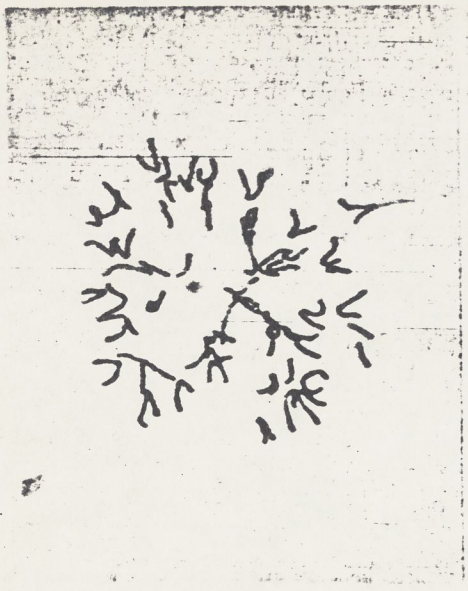
Mitotic defect in anaphase of S. gairdneri. Distribution of chromosomes to the daughter cells will be determined by breakage or non-breakage of the chromatin bridge and further by the extent of chromatin movement prior to cytokinesis. Brightfield illumination, orcein staining. Magnification 600 x.

Figure 10

Chromatin clumping suggestive of telophase but without chromatid separation or cell division. The same blastula of O. keta displayed numerous aneuploid cells. Phase-contrast with orcein staining. Magnification 350 x.

Figure 11

Telophase-like clumping of chromatin in the absence of cell division in O. keta. Embryos which contained any of the defects illustrated in Figures 8-11 were discarded even though many normal cells appeared to be present in many cases. Phase-contrast with orcein staining. Magnification 350 x.



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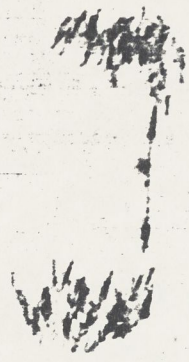

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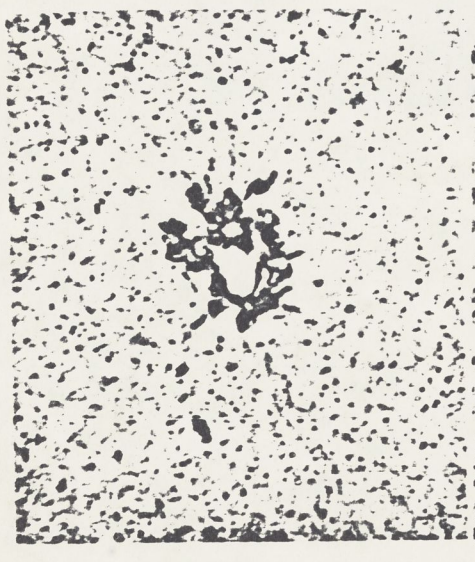

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Chromosome preparations from corneal tissue

Juvenile corneal tissue was prepared by the squash method described for embryonic tissue for purposes of verifying numbers obtained from embryos and testis. Modification was necessary only in the preparation of tissue prior to fixation in Carnoy's fluid. The modification consisted of a 45-60-minute treatment of freshly excised tissue in a 45% dilution of modified Hiu-Twitty solution (Hungerford and DiBerardino, 1958). This solution is compounded as in Table 2 and is diluted with distilled water to 45% of full strength prior to covering the fresh, finely minced cornea.

Table 2

Composition of modified Hiu-Twitty solution used in prefixation treatment of corneal tissue

Solution A, 500 ml.		Solution B, 250 ml.		Solution C, 250 ml.	
NaCl	2943 mg.	Na_2HPO_4	1300 mg.	$NaHCO_3$	200 mg.
KCl	50 mg.	KH_2PO_4	116 mg.		
Sterilize by boiling separately, mix when cooled.					

Tissue thus prepared was used to determine chromosome number in F_1 hybrids of Oncorhynchus keta x O. gorbuscha and in the rainbow trout (S. gairdneri).

Preparation and comparison of karyograms

Camera lucida drawings were made of metaphase chromosome complements

which were considered to represent each of the previously unstudied populations considered in the present study. These drawings were made on tracing paper which was subsequently placed in a photographic enlarger and projected at a final magnification of approximately 5400 x. Chromosome outlines were measured to enable arrangement by pairs in a series beginning with the longest metacentrics and terminating with the shortest acrocentric "pair." These pairs are not strictly intended to represent homologous mates and surely do not in many cases. The chrocosome complements arranged in this manner do, however, enable systematic comparisons of the several species being considered (i.e., White, 1945).

Measurements of individual chromosomes have not been used *per se* in comparing species complements since the chromosomes diminish in length with each cell division during early stages of embryonic development. This diminution is particularly rapid in the blastular cleavages which have provided much of the material upon which the present study is based. Proportionate lengths of chromosomes (expressed as percent of total complement length) would appear to offer a basis for comparison which minimizes the differences in length attributable to differences in cleavage stage. A comparison of proportionate lengths was undertaken in three species in an attempt to reflect upon the aberrant nature of *S. salar* (72 chromosome arms) and *O. kisutch* (110 chromosome arms) as extremes in departure from a seemingly typical arm number of approximately 100. *Salmo gairdneri* (104 chromosome arms) was chosen as the third species for purposes of this comparison. All three species are characterized by a diploid number of 60. Measurements of *S. salar* chromosomes were estimated from Svardson's (1945) Figure 12 (p. 45) while those of *O. kisutch* were obtained from

Simon (1963). Measurements of S. gairdneri chromosomes were made from a camera lucida drawing of material obtained at the American Falls Hatchery, American Falls, Idaho.

F_2 Hybrids of Oncorhynchus (O. keta x O. gorbuscha)

In the Fall of 1961 the Washington State Department of Fisheries produced 150,000 first-generation hybrids by artificial fertilization of O. gorbuscha ova with sperm of O. keta. These fish were released as fry with yolk absorbed in early 1962 into Hood Canal at the site of the rearing station (Hoodsport Salmon Hatchery, Mason Co., Wn.). More than 4% of these hybrids returned to the hatchery as mature adults in the Fall of 1963.

Mr. Charles Ellis, Mr. Richard Noble, and Mr. Rudy Schwab of the Washington State Department of Fisheries were responsible for performing second-generation crosses and for providing space and care for the samples used in the present study.

Ova of seven female hybrids were fertilized and incubated for purposes of chromosome study in embryos of these second-generation hybrids. These ova were mixed and incubated in a single wire-mesh basket contained in a standard hatching trough. Mortality data were accumulated on this group from fertilization to the commencement of feeding.

Microscopy and photomicrography

A Zeiss model WL research microscope was employed for purposes of routine examination of squashed preparations and tissue sections, in preparation of camera lucida drawings and in preparation of all photomicrographs. Preparations were scanned at a magnification of 200 x

and studied in detail at 1250 x. Camera lucida drawings were made at an initial magnification of 1250 x with further enlargment to 3000 x resulting from the characteristics of the camera lucida arrangement. Optics consisted of 12.5 x oculars and 16 x or 100 x fluorite objectives. Positive phase-contrast was used routinely except for examination of spermatocytes which were better suited to ordinary brightfield illumination with transmitted light. Kodak Panatomic-X film was suitable for general use. Satisfactory results were also obtained where appropriate by use of Kodak Kodalith Ortho II or Ansco Versapan. A Zeiss microreflex camera was used exclusively with 6.5 x 9.0 cm. film (120 U. S. designation). Developers and developing times were in accordance with manufacturers' specifications.

RESULTS

Meiotic chromosome numbers in primary spermatocytes

Haploid chromosome numbers were determined in six salmonid species by Nogusa (1960). Nogusa's findings have cast serious doubt on the validity of chromosome counts obtained from embryonic tissue by several authors. The observations of Nogusa are contained in Table 3.

Table 3

Haploid chromosome numbers in several salmonid fishes reported by Nogusa, 1960

Species	Haploid Number (observed)	Diploid Number (inferred)
Oncorhynchus		
nerka	54	108
rhodurus	50	100
masou	50	100
keta	50	100
Salmo irideus	52	104*
Salvelinus fontinalis	50	100

*verified by spermatogonial counts

These proposed numbers are astonishingly high when compared to the results of other authors based on studies of chromosomes from blastulae (O. nerka, 2n = 56; O. keta, 2n = 74; S. irideus, 2n = 60; S. fontinalis,

2n = 84). These conflicting results have been reviewed by Simon (1963) who concluded that Nogusa had interpreted each of the two arms of metacentrics as constituting individual bivalents. This opinion was based on the close numerical agreement in arm number proposed by other authors as compared to the diploid numbers proposed by Nogusa. This assumption was, however, without experimental verification.

The findings of the present study are in agreement with this supposition and are illustrated (Figs. 12-15) by considerations of meiosis in Oncorhynchus gorbuscha and Salmo gairdneri (= irideus).

It has been found in the former species (2n = 52, all chromosomes of the diploid complement are metacentric) that 52 "dots" are evident in diakinesis of primary spermatocytes viewed in polar aspect. A possible (although incorrect) interpretation would be that the haploid number in this species is 52, especially in the absence of any information on the morphology and number of chromosomes in the diploid complement. Closer scrutiny of the chromatin "dots", however, reveals that all are associated in two-by-two fashion (Figs. 12 and 13). This association is seen particularly well in chromosomes at the periphery of the chromosome

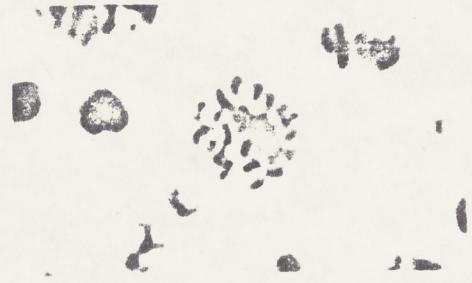


Figure 12
Polar view of Meiosis I in Oncorhynchus gorbuscha; note associations of peripheral chromosomes.
4000 x

Figure 13
Of the total of 52 chromatin dots present, all seem to be associated in two-by-two fashion. Meiosis I in Oncorhynchus gorbuscha.
4000 x

aggregation. If the meiotic stage is recognized to be diakinesis it is evident that these "paired" elements are not bivalents, but are the consequence of viewing metacentric chromosomes with terminalized chiasmata in the form of diakinesis rings (Fig. 14) typical of chromosomes with median, or submedian, centromeres. As a consequence of their positioning on the metaphase plate, the chromosomal elements are most clearly resolved to be separate on that optical plane which is between centromere levels, since at higher or lower levels of focus the paired elements (arms of metacentrics) will blend together at the centromeric apex. Non-polar views of such diakinesis rings (Fig. 14) readily illustrate the manner in which such a false interpretation could be made. This immediate observation is supported by viewing diakinesis in polar aspect of species which possess acrocentric chromosomes, such as the rainbow trout (Fig. 15). In this case some peripheral elements can be seen to be isolated without "pairing" associations.

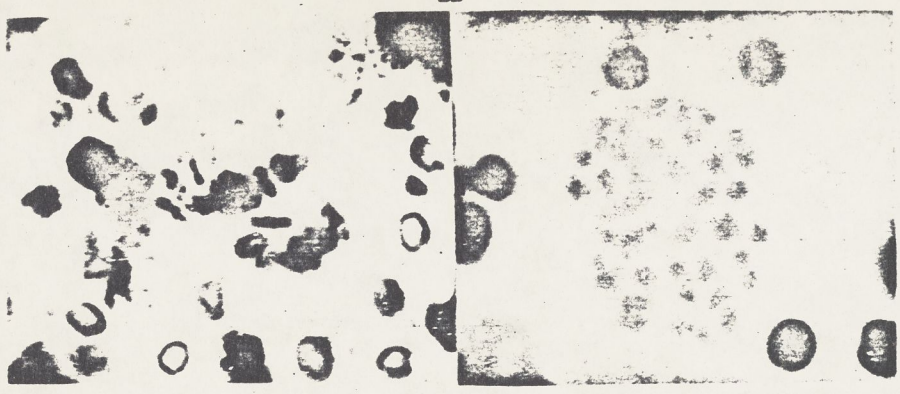


Figure 14
Axial view of diakinesis rings in O. gorbuscha; chiasmata have terminalized. Iron hematoxylin, paraffin section, 4000 x.

Figure 15
Polar view of diakinesis in S. gairdneri; note that some chromatin dots are not associated in two-by-two fashion, i.e., at 10 o'clock. Orcein-squash preparation, 4000 x.

As a consequence of these findings the determinations of chromosome numbers in salmonids made by Nogusa (1960) are considered to be invalid. The findings of Nogusa are nonetheless of considerable value in documenting the fact that a general equivalence in chromosome arm number exists in salmonid fishes despite considerable differences in diploid number of some species. This equivalence is deemed to be of paramount significance in understanding the evolutionary pathway of species divergence and will be elaborated upon further in subsequent sections.

Blastular chromosome numbers and chromosome morphology in previously unstudied populations of Salmoninae

Results of chromosome number determinations in blastula cells of several populations of salmonids are summarized in Table 3. The diploid chromosome number of O. masou could not be ascertained with assurance. The five samples which were procured from this species were all at an advanced

pre-gastrula stage characterized by many small cells which were resistant to attempts to disperse their chromosomes in a manner to allow chromosome counts with a high degree of certainty. The principal confusion resulted from a failure in separation of individual chromosomes so that an aggregate of four "arms," for example, could be interpreted as two metacentrics; as one metacentric and two acrocentrics; or as four acrocentrics. Counts of ten metaphase complements yielded estimated diploid numbers ranging from 63 to 70. Although the exact diploid number was not determined, it is apparent that the arm number in this species is in very close accord with other Oncorhynchus species. The arm number of 104 has been obtained repeatedly despite an inability to be even modestly certain of the exact diploid number. A particular point of interest in the morphology of acrocentric chromosomes of O. masou is the fact that many (perhaps all) possess a short second arm. This occurrence is closely comparable with the chromosome complement of Salmo c. clarki which is characterized by short second arms on all acrocentrics. Such a close correspondence in types of acrocentric chromosomes is emphasized by the fact that a predominance of acrocentrics with short second arms is not otherwise known in Salmo or Oncorhynchus, nor for that matter in Salvelinus. This observation seems to be of potential importance in understanding the intermediate nature of O. masou.

The inability to obtain regularly repeatable counts in O. masou has been considered to be a consequence of preserving the embryonic material at a time removed from the most suitable cleavage period. It has been estimated that embryos of this species had entered the 16th or 17th cleavage. This slight departure from cleavage 10-13 seems to have

been sufficient to obviate successful chromosome counts.

Better success was enjoyed in determinations of chromosome number in the remaining unstudied populations. It has become evident, however, that even in good cytological preparations the chromosome number is not rigidly invariable. Haploid (or near-haploid) cells account for most of this variability, but have been excluded by adhering to counting criteria. Other deviations from the usual diploid number have been observed which cannot be attributed to counting error in the following cases:

(1) Two samples of fertilized ova of S. c. clarki of a total of 10 samples obtained from the Washougal River yielded counts of 68 or 69 in contrast to regular counts of 70 observed in the remaining lots from this locality.

(2) One of two samples of the same subspecies from Minter Creek was also found to be typified by a diploid number of 70, while the remaining sample contained embryos with the following chromosome numbers in 10 embryos: 62, 63, 63, 63, 64, 65, 65, 66, 66, 67.

(3) Both of two samples of S. g. gairdneri also from Minter Creek deviated from the consistent diploid number of 60 obtained from other samples of the same subspecies collected from Soos Creek. Of 20 counts made on the Minter Creek samples, only one was found to be 60 while the remainder ranged up to 66 with intermediate counts of 61, 62, 63, 64, and 65 represented.

(4) One of five samples of S. clarki lewisi from Lauri Lake, Montana, yielded counts of 66 while a second was

variably counted as 65 or 66. The remaining samples were uniformaly determined as 64 which is the usual diploid number in other populations of this subspecies (Simon and Dollar, 1963).

These counts are not considered to be the result of poor technique or the result of counting errors. While deviations of similar or greater magnitude are not uncommon in cells which do not conform to the counting criteria adopted, they are striking deviants from the usual findings where counting criteria are satisfied.

Departure of one or two counts in twenty-five has been the usual case observed in the samples not commented upon further. These deviations may be real or may be the result of counting errors. Chromosome counts of several previously unstudied populations are contained in Table 4.

Several observations are of interest in connection with these results:

(1) The chromosome complement of the coastal cutthroat, S. c. clarki (Figure 16) is distinctive in comparison with its inland relatives both with respect to abundance of subtelocentric chromosomes and with respect to diploid number (Table 4).

(2) The diploid number is apparently the same in several populations of inland cutthroats, one of which (S. c. benshawi) has been assigned subspecific status as distinctive from the Yellowstone and Montana black-spot cutthroats (S. c. lewisi). Differences have been noted

in morphology of particular chromosomes in different populations of inland cutthroats. The small sample sizes in the present study (even if they adequately represent respective populations) would appear to preclude any presumption of significance in this observation. Detailed analysis of chromosome differences at the substructural level is beyond the scope of the present study.

(3) Chromosome morphology and number in the anadromous rainbow trout (S. g. giardneri) appears to be indistinguishable from the complements of nonmigratory populations (Figures 18 and 19).

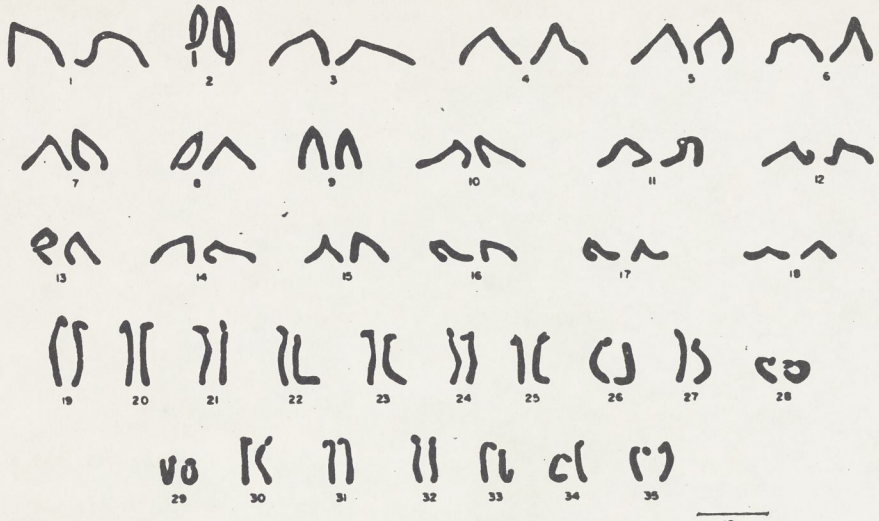
(4) The chromosome number of the golden trout (S. aguabonita) is the smallest of any Salmo species studied thus far. The arm number in this species is the same as that found in the cutthroat trout (viz., 106). A comparison of Figures 17 and 18 suggests that this difference in diploid chromosome number can be accounted for by centromeric "fusion" of acrocentric chromosomes (with consequent reduction in their number) which results in an increased number of metacentric chromosomes accompanied by a decrease in diploid number. The fact that arm numbers are equal in these species lends support to such an interpretation since it is known that fracture of metacentrics to produce acrocentric elements results in loss of one or both of the newly formed acrocentrics (White, 1945). This loss is a consequence of impaired function of broken centromeres (McClintock, 1934) and due to the lack of anaphase movement of acentric fragments.

Figure 16

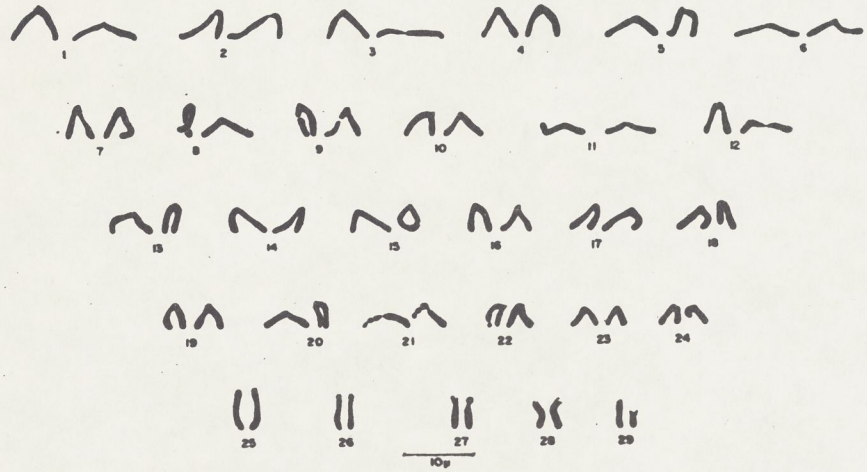
Karyogram of the diploid complement of the coastal cutthroat, Salmo c. clarkii. The most frequent number depicted here (2n = 70) has not been invariably obtained in some samples (see text). Note the short second arm on each acrocentric.

Figure 17

Karyogram of the diploid complement of the golden trout, Salmo aguabonita (2n = 58). Note the very short acrocentric included in "pair" 29. It is suggested that this chromosome may be associated with sex determination.



Somatic Chromosomes, Salmo clarki clarki
Washougal River, Washington



Somatic Chromosomes, Salmo equidensis
Alpine Lake, Wyoming

Table 4

Chromosome numbers and morphology from blastular cells of several salmonid populations

Species, locality	2n	acrocentrics	metacentrics	arms	total counts
O. masou Hokkaido	63-70?	?	?	ca. 104	10
S. c. clarki* Washington	70	34	36	106	60
S. c. lewisi* Montana	64	22	42	106	25
S. c. lewisi Washington	64	22	42	106	50
S. c. lewisi Wyoming	64	22	42	106	25
S. g. henshawi Nevada	64	22	42	106	25
S. g. gairdneri* Washington	60	16	44	104	45
S. aguabonita Nevada	58	10	48	106	25

*inconstant counts as noted above

Heteromorphic acrocentric chromosomes

Throughout the course of this study it has been observed that a small acrocentric chromosome is variably present or absent in some species. This has been observed in the golden trout (Fig. 17), in the rainbow trout and in inland cutthroat trouts. When karyograms were compared in the latter two species it was noted that a mate of similar length to the largest acrocentric chromosome was present only when the small acrocentric

was absent (the diploid number remaining the same in either case). A comparison of 20 karyograms of the rainbow trout indicates an approximate occurrence of 50% presence or absence of a mate of similar size as the longest acrocentric (8 present, 12 absent). This distribution is suggestive of a sex difference since that character occurs in roughly equal proportion. It is not known whether this circumstance is common in other species.

The longest acrocentric in the rainbow and cutthroat trouts seems to be one of a heteromorphic pair which appears in two alternate conditions with approximately equal frequency. In the golden trout, the smallest acrocentric seems to be variably with, or without, a mate (Fig. 17). The findings of Svardson (1945) are somewhat akin to this situation in that he reported the variable presence of a small acrocentric "fragment" in Coregonus lavaretus. Svardson decided that this small chromosome was a sex chromosome without a mate and thus resulted in fluctuation of the chromosome number in that species according to presence or absence of the fragment. Such a mechanism is of course quite different from the one postulated in the present study.

It seems to be likely that the small acrocentric observed in the present study may in fact be one of a pair involved in sex determination as indicated by its frequency of occurrence. Figures 18 and 19 have been prepared with this tentative conclusion in mind in order to illustrate the observed differences (compare pair 23 in each figure).

Even if these acrocentrics are indeed pairs, and if they are involved with sex determination, it is not possible to speculate as to which sex is heterogametic. Questions on sex determination in fishes have

provided the substance of elaborate confusion and debate (see Svardson's review, p. 112-118). Spermatocyte metaphases do not appear to offer clarification of this situation.

Somatic chromosomes from cornea

Limited results (six counts) with cornea squash preparations from rainbow trout have indicated:

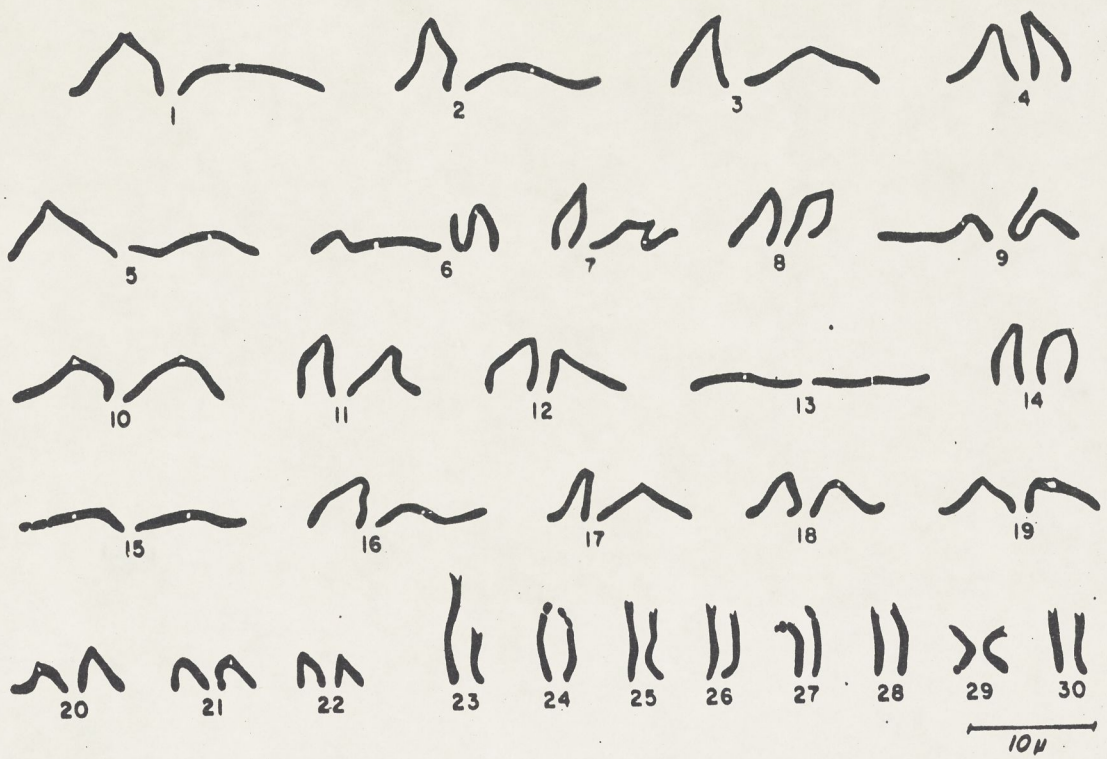
(a) study of chromosomes in this tissue is feasible,

(b) total chromosome number and morphology are entirely in agreement with results from blastula cells.

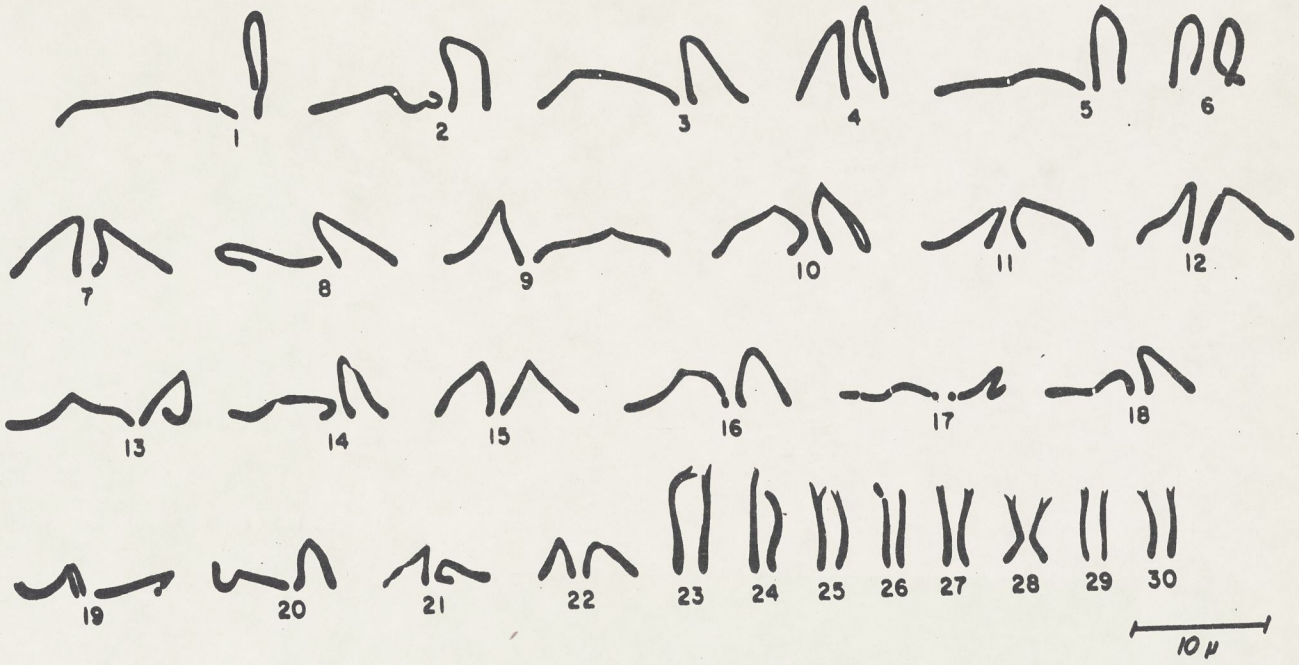
The sex of fish employed as cornea donors was clearly discernible but no further information was gained on possible sex-chromosome mechanisms since sharp decrements in chromosome length are not apparent in this material (Figure 20). However, these findings have been of value in supporting the validity of chromosome counts derived from embryonic material contrary to the objections of Nogusa (1960) who considered embryonic tissue to be poorly suited to chromosome study.

Although mitoses are not abundant in corneal tissue, good results are sporadically obtained (Figure 21) suggesting a possible utility of this tissue for future chromosome studies.

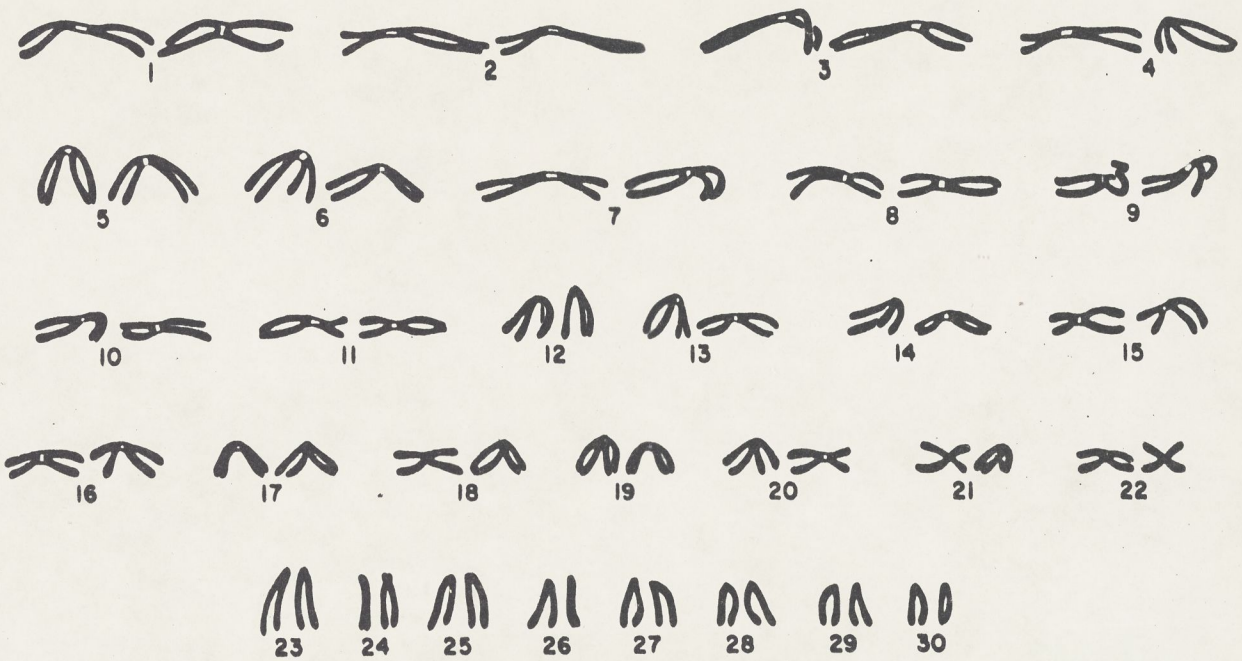
Cornea preparations of five F_1 hybrids of *O. keta* x *O. gorbuscha* have confirmed the expectation of 63 somatic chromosomes in all five samples from newly-hatched fry.



Somatic Chromosomes, Salmo gairdneri gairdneri
Soos Creek, Washington



Somatic Chromosomes, Salmo gairdneri
American Falls Hatchery, American Falls "Strain"



Somatic Chromosomes from Cornea of Salmo gairdneri
University of Washington Hatchery

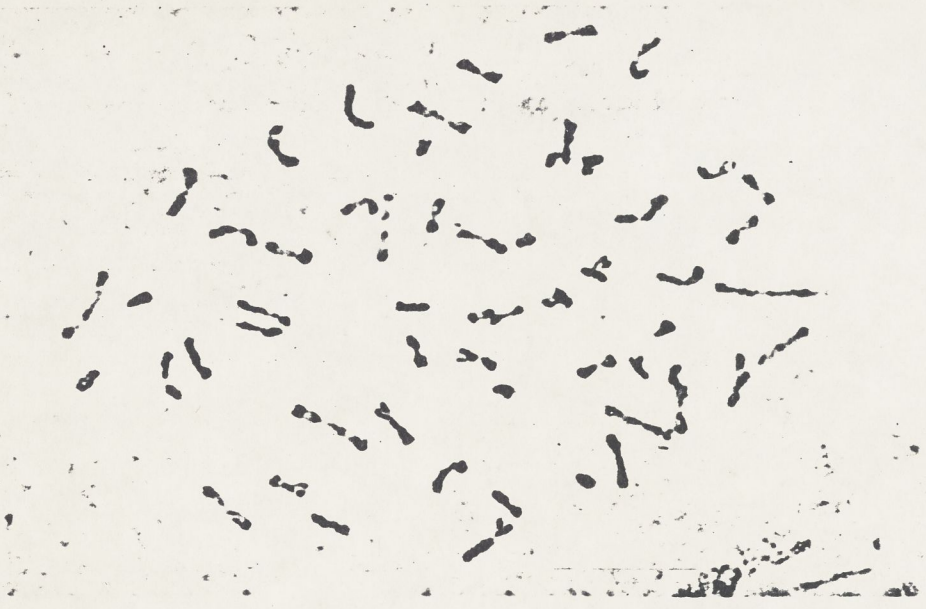


Figure 21
Somatic chromosomes from cornea of the rainbow trout, S. gairdneri. Chromatids have separated except in the region of centromere thus assisting in easy location of centromere. This separation is a consequence of treatment with hypotonic solution prior to fixation. Orcein-squash preparation with phase-contrast, magnification 1780 x.

Second generation hybrids of O. keta x O. gorbuscha

Temporary preparations of 300 F_2 blastulae have formed the basis of the results reported below. Initial viability of this hybrid (O. gorbuscha ♀ x O. keta ♂ exclusively) was observed to be near 95% as evidenced microscopically by active cleavage. Subsequent mortality through completed hatching and yolk-sac absorption is summarized in Table 5.

Table 5

Survival data on F_2 hybrids of *O. keta* x *O. gorbuscha*

	September, 1963 originally fertilized	Survival after "rough handling" prior to hatching	January, 1964 survival at fry stage
number	8975	6575	3832
percent	100.0	73.2	42.7

A total mortality of 57.3% was thus observed up to the time when the hybrid offspring had begun active feeding. A very rough estimate of mortality up to this period in the original parent species might range from 12-15% on the average. Much higher losses than those observed in the hybrids are observed on occasion in "pure strain" crosses of parent species, however.

Samples of yolk-sac fry were observed shortly after hatching had begun. At this time abnormalities were noted in eye diameter and spine formation. The eye is the most heavily pigmented structure at this time and is hence easily noticeable. The eye varied from essentially absent without melanin to completely formed and well pigmented (Figure 22). About 70% of those hybrids which had hatched successfully were superficially normal.

Figure 22
Living, yolk-sac fry of second generation hybrids. Note variable eye diameter and defects in spinal column.

Chromosome morphology and numbers in F_2 hybrids

In general, cleavages appeared to be entirely normal in hybrid blastulae. Atypical chromosome numbers were largely represented by near-tetraploid cells (often typical of entire embryos) which accounted for 18 of the 300 embryos surveyed. Three haploid embryos were found. Two embryos displayed chromosome numbers which could not be considered to be either haploid or tetraploid. Counts associated with these atypical findings were: near-tetraploid, 106-124; near-haploid, 28, 31 and 32; otherwise atypical, 45 and 75. These atypical cells were subjected to chromosome counts, but were otherwise ignored for purposes of analysis.

Typical chromosome numbers ranged from 56 to 69 in a sample of 50 embryos in which chromosome spread was sufficient to enable accurate determination of metacentric and acrocentric frequency. The metacentric-acrocentric distribution in this sample of 50 observations is depicted

Figure 23

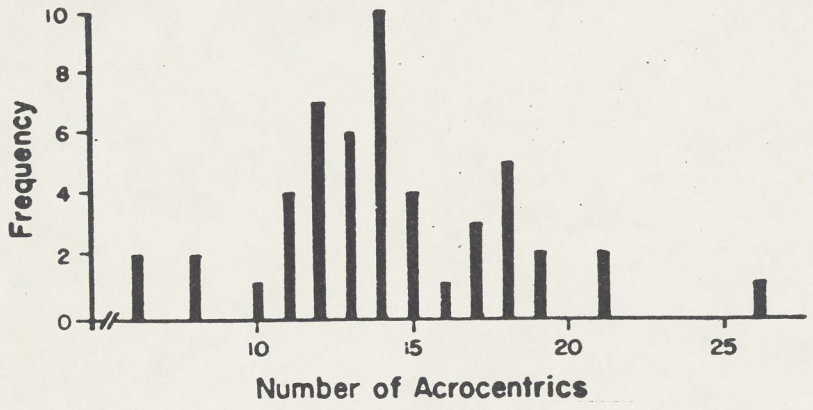
Regression of metacentric chromosome number on acrocentric chromosome number in 50 F_2 embryos. The broken line is the expected if diploid number tends to remain constant, the solid line was fitted from the observed data. Line slope of minus 1.25 suggests diploid number tends to remain more constant than does the arm number (arm constant slope = minus 2.0). One observation was omitted from polygon (2n = 69) but included in chi-square test.

The inset frequency polygon depicts the approximation to a normal distribution.

The frequency distributions of metacentric and acrocentric chromosomes in F_2 blastulae are presented in Figure 24. The most obvious feature of chromosome numbers in F_2 embryos is the greater-than-expected abundance of metacentric chromosomes (all but two embryos have more metacentrics than the F_1) accompanied by a less-than-expected number of acrocentrics (only one of 50 embryos with 25 acrocentrics and all others less than 23 as in the F_1). The acrocentric-metacentric ratio of the F_1 (23A : 40M = 1 : 1.74) is not faithfully reproduced in the F_2 (mean 14A : 45M = 1 : 3.21). The mean chromosome number of 59 in F_2 embryos (Figure 25) cannot be explained either on the basis of chance distribution (univalent segregation resulting from complete pairing failure) or on the basis of ordered randomness resulting from pairing of two acrocentrics contributed by O. keta with each of the metacentrics contributed by O. gorbuscha, since either would be expected to result in reconstitution of the F_1 number of 63 as a mean occurrence. Before proceeding to analytical considerations which may explain chromosome distribution to the F_2 it is desirable to review observations on gametogenesis in maturing testis of F_1 males. These observations are intended to constitute a cytological basis for later treatment of meiotic chromosome pairing.

Meiosis and spermatogenesis in F_1 hybrids

Pairing, or absence of pairing, in meiotic chromosomes is the only information which will enable decisions on the degree of species relatedness. In addition, pairing information will reflect directly upon explanation of the variable chromosome number observed in second-generation hybrids. Observation on pairing of meiotic chromosomes is



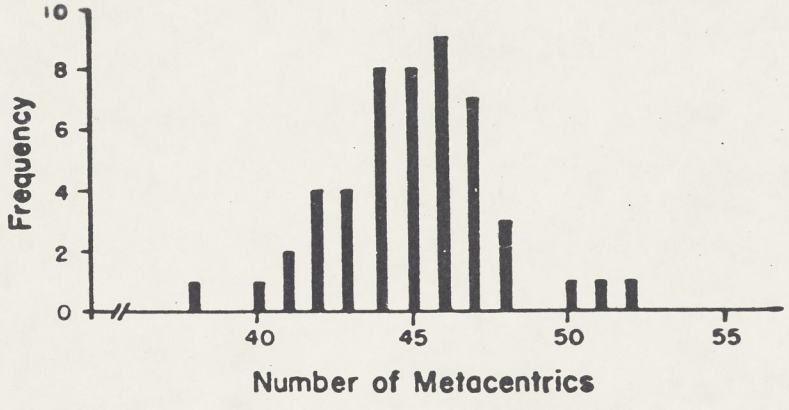
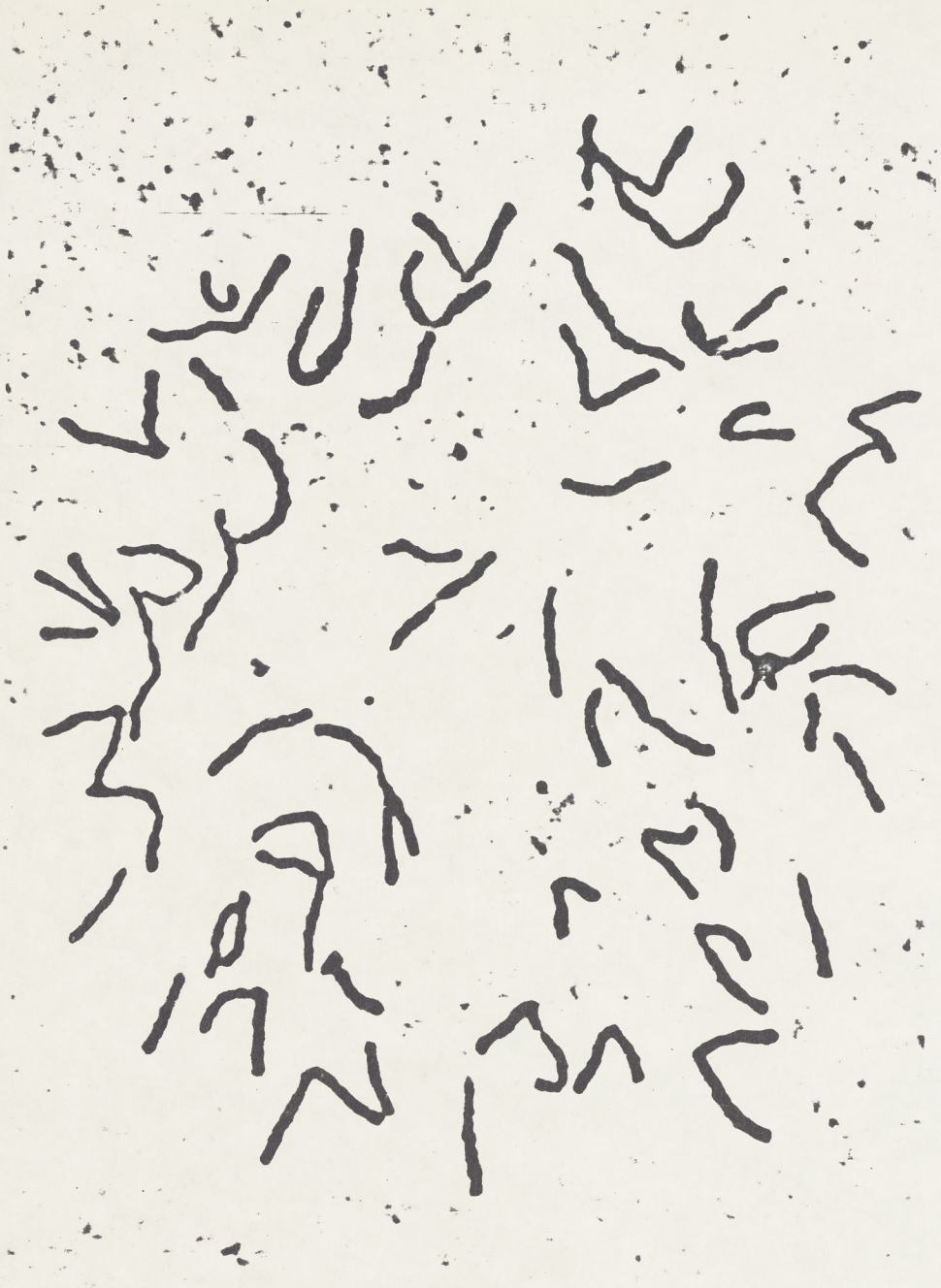


Fig. 24 Distribution of metacentric and acrocentric frequency in 50 F_2 blastula metaphases. Each observation is from a different embryo.

Figure 25

Blastula metaphase in F_2 hybrid of *O. keta* x *O. gorbuscha*. The somatic chromosome number of 59 in this cell is typical of the mean number in a sample of fifty counts. Note that distinction between metacentrics and acrocentrics is relatively simple. Orcein staining with phase-contrast. Magnification 3500 x.



very difficult because of their small size and high number in the species thus far observed cytologically. A complete pairing analysis does not seem possible with the methods employed because lateral views of diakinesis present dense chromatin masses within which the course of single chromosomes is highly obscure. Limited results have been obtained, however, by selecting fragments of the meiotic complement which have been separated as a consequence of sectioning.

The following observations suggest that pairing is very nearly complete, thus indicating that the original parent species have not diverged sufficiently to obscure chromosome homology:

(a) although a single univalent has been frequently observed, (Figure 26) meiotic figures otherwise appear to be constituted of closely-approximated elements which separate cleanly at anaphase;

(b) fragmentary portions of the meiotic complement contain elements in zig-zag array like the teeth of a saw (Figure 27); these arrays appear to be the consequence of acrocentric (A) and metacentric (M) pairing in chains up to six chromosomes in length in the fashion, A-M-M-M-M-A;

(c) despite the fact that many hundreds of diakinesis figures have been observed in lateral view, not one diakinesis pairing of only two metacentrics has been noted, but rather many have been seen to consist of three or more elements.

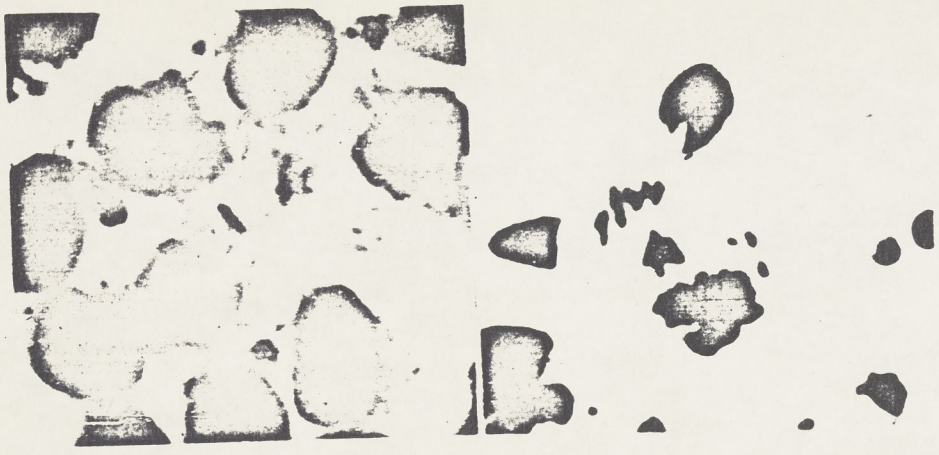


Figure 26
Meiotic metaphase in F_1 hybrid. Note univalent near spindle apex. Iron-hematoxylin, magnification 4000 x.

Figure 27
Diakinesis in F_1 hybrid. Note chain-like pairing. Only small portion of total complement is present. Iron-hematoxylin, magnification 4000 x.

Some spermatocytes at the periphery of testis tubules are clumped together in dense masses which persist even in fully mature gonads otherwise filled with mature spermatozoa. These cells appear to have aborted at an early meiotic stage and appear to constitute from 5-10% of the total chromatin present. This finding has been interpreted as an indication of gamete elimination, since the clumped chromatin is within tubule lumina.

Considerations of pairing and segregation

Although cytological data on meiotic pairing are severely limited, it would appear that extensive pairing does take place. This implies that the observed variability in arm number, metacentric number, etc. is not the consequence of chance assortment and should thus be explicable

in terms of pairing and segregation.

Possible modes of pairing are now examined with the fact in mind that the contribution of one parent species is comprised solely of metacentrics, while that of the other parent consists of a majority of acrocentrics, as below:

gamete contribution, O. keta = 23A, 14M (51 arms)
" " O. gorbuscha = 0A, 26M (52 arms)

diploid constitution, F_1 = 23A, 40M (103 arms)

Pairing of homologous elements must be on an arm basis during meiosis in the F_1 since chromosome numbers contributed by the parent species are greatly different. Table 7 contains the basic pairing possibilities applicable to the present situation.

Table 7

Pairing configurations and segregation results in meiosis I of first-generation hybrids

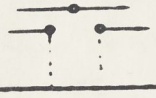
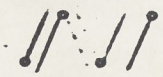
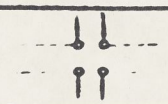
pairing configuration	segregation results
	not possible since all acrocentrics are from haploid set of O. keta, none can be homologous to other acrocentrics.
	haploid number same in each gamete, as are numbers of arms and numbers of metacentrics
	haploid number $\pm$ one, arm numbers equal, acrocentrics $\pm$ two; metacentrics $\pm$ one
	haploid number $\pm$ one; arm number $\pm$ two; metacentrics $\pm$ one

Table 7 (continued)

Pairing configurations and segregation results in meiosis I of first-generation hybrids

pairing configuration	segregation results
	haploid, arm, acrocentric and metacentric numbers equal in each gamete
	haploid, arm and metacentric numbers equal in each gamete

If this scheme adequately represents actual pairing conditions, then several statements are possible concerning pairing:

(a) the only configurations which can result in unequal distributions to gametes are those which involve odd numbers of centromeres - of these, only the rings composed solely of metacentrics can influence arm number;

(b) rings or chains with odd numbers of centromeres will cause the same fluctuation in the segregation of pairing elements irrespective of the number of chromosomes involved, so long as that number is not even;

(c) chains of pairing elements will be terminated at each end by an acrocentric (except one, due to odd number of acrocentrics);

(d) since 23 acrocentrics are involved, 12 chains can be expected to form, each containing at least one metacentric;

(e) at least 5 rings consisting of odd numbers of metacentrics are assumed to have formed and resulted in the observed variation in arm number (mean 104 ± 9);

(f) the 5 rings considered in (e) would also account for a variability of 10 in the range observed in metacentric distribution to the F_2 (mean 45 ± 7) thus of the 12 chains formed, 3 would be expected to contain odd numbers of centromeres to account for the remainder of the observed metacentric variability;

(g) the 3 chains with odd numbers of centromeres could be expected to contribute 12 to the range in variation observed in acrocentrics (mean 14 ± 12) thus an additional 3 chains must be specified to contain unequal numbers of centromeres.

This pairing model is sufficient to account for the pairing relationships of all but 5 metacentrics in the F_1 complement as follows:

1. 6 chains with odd number of centromeres (minimum 6 M)
2. 6 chains with even number of centromeres (minimum 12M)
3. 1 chain with even number of centromeres (observed to contain 4M) must be one within #2, since <u>all</u> acrocentrics are accounted for (additional 2M)
4. 5 rings with odd number of centromeres, all associated with metacentrics (minimum 15M)

(F_1 contained 23A, 40M) minimum total 23A, 35M

It should be stressed that this model is postulated on the basis that complete pairing has taken place. This is supported by the observation of only one univalent per complement in meiosis I (if indeed any are visible) and by the compact association of pairing elements. Further verification of pairing has been gained in observation of diakinesis chains, and in numerous two-stranded associations at pachytene. None of these cytological observations provide much detail concerning meiotic pairing but they do indicate that extensive pairing has taken place.

The theoretical considerations of pairing and segregation indicate the distinct possibility of numerical fluctuation of pairing elements even in the presence of precise homology. The five metacentrics which cannot be accounted for could easily be dispersed in several ways throughout chains or rings without affecting segregation variability, since that variability is dependent upon whether the association contains an odd or even number of centromeres and is hence unaffected by simple weight of chromosome numbers (Table 7).

The immediate question is concerned with how well the theoretical pairing model fits the observed data in the F_2 even if it can be granted that the model is not mechanically implausible. Before proceeding, it should be stated that pairing via chains and rings is more than convenient fiction in view of the fact that it constitutes the normal pairing associations in several plant genera including Rhoeo and Oenothera (Darlington, 1937).

The observed data on F_2 frequency of metacentrics, acrocentrics and arm numbers were compared with normal expectations erected about the observed means and extending over the observed ranges of variability. The

results of these comparisons are contained in Table 8 and illustrated by arm number distribution in Figure 28.

Table 8

Comparison of observed chromosome distribution in F_2 hybrids to that expected of a normal distribution

variable	observed range and mean	standard deviation	degrees freedom	computed Chi-square	rejection level .05
arm number	97 - 113 $\bar{x}$ = 104.18	3.36	6	8.44*	12.59
diploid number	56 - 69 $\bar{x}$ = 59.18	2.65	5	7.04*	11.07
metacentric number	38 - 52 $\bar{x}$ = 44.64	2.63	6	4.13*	12.59
acrocentric number	6 - 26 $\bar{x}$ = 14.14	3.77	5	8.28*	11.07

*signifies that rejection level is not exceeded, thus distributed in a normal fashion (procedure of Snedecor, 1956)

Since the observed data agree with normal expectations in each case, it seems reasonable to assume that the pairing model offers a satisfactory explanation of variability as a consequence of random assortment. It should be emphasized that this randomness follows very extensive mating of homologous elements.

The departure of the mean chromosome number in F_2 blastulae from that expected on the basis of F_1 constitution has not been reflected upon in considerations of pairing and assortment. Since no data are at hand to support speculation, other than the obvious fact that the discrepancy exists, further treatment of this matter seems more appropriate under topics of discussion and will be considered in that section.

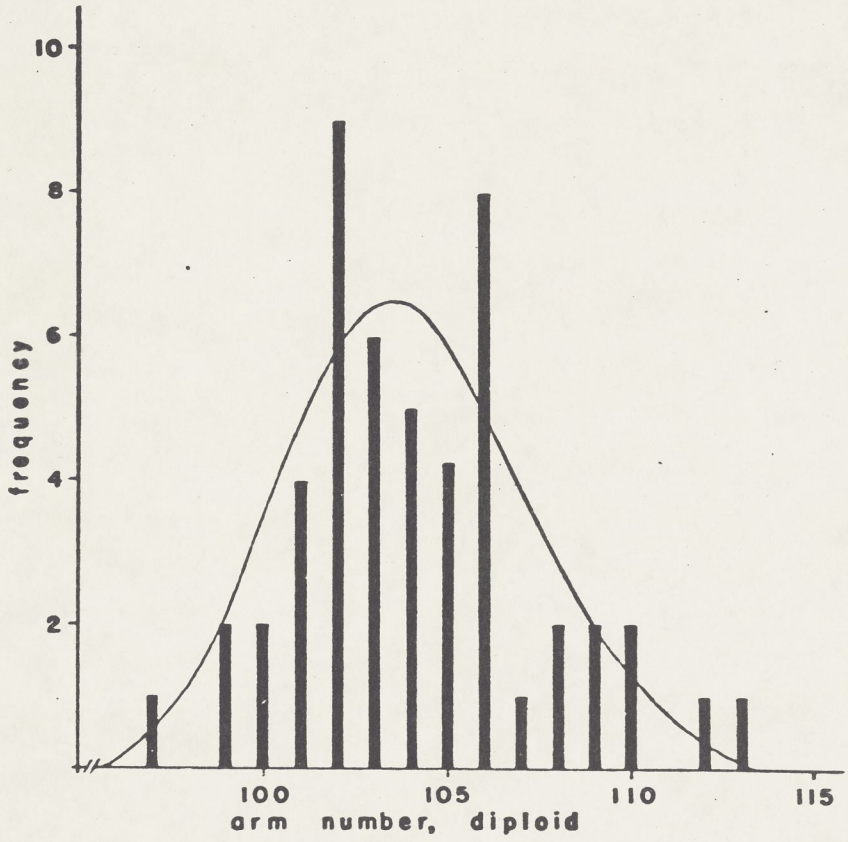


Figure 28

Observed (bars) and expected (curve) distribution of chromosome arm numbers in F_2 blastulae.

DISCUSSION

Limitations of the present study

The use of very early embryos for purposes of chromosome study is potentially hazardous with relatively small samples such as those relied upon in the present study because abnormal embryos often experience little difficulty until gastrulation, which appears to be an early "critical period" (i.e., Hyman, 1926; Hinrichs, 1925; Stockard, 1921). Since 10% mortality to the time of hatching is not considered to be atypical of good hatching success, it seems probable that this study has included some embryos which would have subsequently perished. Chromosome abnormalities can be expected to account for some embryonic mortality, thus some conclusions could be affected by inclusion of observations on pathological material. Attempts have been made to exclude atypical embryos from consideration by application of criteria for selecting chromosome complements; however, the inclusion of some atypical embryos is probably inevitable. More extensive use of juvenile or adult tissues should circumvent this possibility of confusion in future studies.

The results of the present study, while perhaps less than absolute, are nonetheless considered to have attained a reasonable level of reliability. This statement is supported by the confirmatory data obtained from cornea chromosomes and from diakinesis observations in rainbow trout and in O. gorbuscha. A more serious limitation may be the fact that relatively few populations have been studied to date. Literally dozens of populations (some distinct species) have never been studied cytologically. It seems highly desirable to obtain information on populations of

unstudied salmonoid genera, i.e., Brachymystax, Hucho, Plecoglossus, Stenodus, Prosopium, etc.

The very minute dimensions of chromosomes in the male germ line seems to preclude extensive study of maturation divisions in the species observed. While such study is not impossible, it may be misleading if relied upon exclusively as evidenced by the recent reports of Nogusa (1960).

Even though some pitfalls may be encountered in observations of chromosomes of very early embryos, such studies are very valuable in the absence of data on meiotic chromosome pairing and segregation. Evaluation of species relationships by studies of chromosomes in F_2 blastulae are in fact improved by inclusion of embryos which would subsequently die. By this inclusion, extensive indirect information can be obtained on meiotic chromosome segregation, while confining studies to surviving hybrids may be confusing due to elimination of some segregation combinations through mortality.

Speciation and changes in chromosome number

Many salmonid species possess chromosome arm numbers of about 100 while diploid numbers vary from 52 to 84 in species studied to date (Table 9). The stability of arm number in the presence of different diploid numbers dictates a principal mechanism responsible for changes in diploid number, namely centric "fusions" (joining of two acrocentrics by unequal reciprocal translocation). Centric fusions result in decreases of diploid number accompanied by increase in number of metacentric chromosomes. While fracture of metacentric chromosomes to form rod-like chromosomes is by no

Table 9

Summary of chromosome constitution in various species of Oncorhynchus, Salmo and Salvelinus

species	2n	metacentric	acrocentric	arms
Salvelinus				
alpinus (1)	80	16	64	96
fontinalis (1, 2)	84	16	68	100
namaycush (3)	84	16	68	100
Salmo				
trutta (1, 2)	80	16	64	96
g. irideus (2, 4, 5, 6)	60	44	16	104
salar (1)	60	12	48	72
salar (2)	56	16	40	72
g. gairdneri (8)	60	44	16	104
c. clarkii (8)	70 (68?)	36	34	106
c. lewisi (6, 8)	64	42	22	106
aguabonita (8)	58	48	10	106
Oncorhynchus				
keta (9)	74	28	46	102
tshawytscha (9)	68	36	32	104
kisutch (9)	60	52	8	112
nerka (9)	56	46	10	102
gorbuscha (9)	52	52	(0)	104
masou (8)	63 (70?)	?	?	104

Author Key, Table 9

(1) Svardson, 1945

(2) Wright, 1955

(3) Wahl, 1960

(4) Lieder, 1956

(5) Bungenberg DeJong, 1955

(6) Simon and Dollar, 1963

(7) Boothroyd, 1959

(8) present study

(9) Simon, 1963

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means impossible, such fracture would be accompanied by a reduction in arm number since acentric fragments are lost. An evolutionary direction is thus imposed which essentially limits arm rearrangements to events which reduce, rather than increase, chromosome number if a stable number of chromosome arms is to be retained.

These considerations appear to indicate that higher chromosome numbers of those species within Salmoninae are more closely akin to a primitive condition. Specialization is thus typified by reduction in chromosome number. The accompanying reduction in numbers of centromeres would appear to impose a measure of limitation upon the variability of the species as a consequence of increased genetic linkage. An increase in linkage could clearly be advantageous by assuring obligatory (non-random) segregation of complementary genes which were originally located on

separate, non-homologous, acrocentric chromosomes. The pink salmon, O. gorbuscha, could thus be termed highly specialized since all chromosomes appear to be metacentric. The rigidly fixed, two-year life cycle of this species would seem to document such a concept (Neave, 1958). Most authors agree that the Pacific salmon are more specialized than the trouts on the basis of anatomical-morphological characters. Both groups are further considered to be less primitive than the members of Salvelinus. Data on chromosome numbers and direction of change in numbers are in good agreement with these postulates.

Species differences are suggested to be a consequence of shuffling of very similar chromosomal information. This has been based on the general correspondence of chromosome arm number and on the success of artificial hybrids. The overall validity of this concept appears to be damaged by the deviant numbers of chromosome arms in O. kisutch and S. salar (110 and 72 respectively). While these species appear to cripple an "equal arms" relationship theory, they may be seen to agree very well with such a theory upon more detailed examination. Figure 29 presents a comparison of "pair" lengths in the somatic chromosome complements of the two deviant species cited above and of the more typical (104 arms) S. gairdneri. The comparison is facilitated by the fact that all three species possess diploid numbers of 60.

Lengths of S. salar chromosomes have been estimated from Figure 12 of Svardson (1945, p. 45). Measurements of O. kisutch chromosomes are from Simon (1963) while those of S. gairdneri are original data obtained for the present comparison. Since lengths of the longest pair in the three species differ by about 4 microns (probably reflecting different cleavages),

the percent of total length has been utilized for individual chromosomes. The fact that Boothroyd's (1959) results on S. salar do not agree numerically with the diploid determination of Svardson for that species, is deemed to be of little consequence in this application since both authors report the same arm number. Actual measurements of individual pairs in the somatic complement of each species are contained in Appendix I. It should be noted that similarities in total length of the pairs in each species are in reality quite close, thus comparisons of percentage-lengths are not entirely artificial. Total lengths of the entire complement of each species are: O. kisutch, 414.4; S. salar, 386.5 and S. gairdneri, 468.8 to the nearest tenth-micron.

One prominent feature of the comparisons in Figure 30 is seen in the unique occurrence of acrocentric chromosomes in categories including chromosomes of length equal to, or greater than 3% of the total complement length. Acrocentrics are absent in these size groups in the remaining two species. It should be emphasized that the total numbers of chromosomes in the three larger groups are very nearly equal in each case (19, 19, 18) suggesting that the total bulk of hereditary material is much the same.

It thus seems reasonable to explain the discrepant arm number in S. salar as a consequence of centromere shifts (pericentric inversions) which have resulted in terminalization of centromeres in 13 originally-metacentric chromosomes of the haploid set. If these 13 "long" acrocentrics are assumed to be equivalent to "long" metacentrics of similar length, then it may be stated that in this special case the 13 acrocentrics are the equivalent of 26 arms. If the arm number in the diploid complement of S. salar is adjusted accordingly (72 plus 26 = 98) the

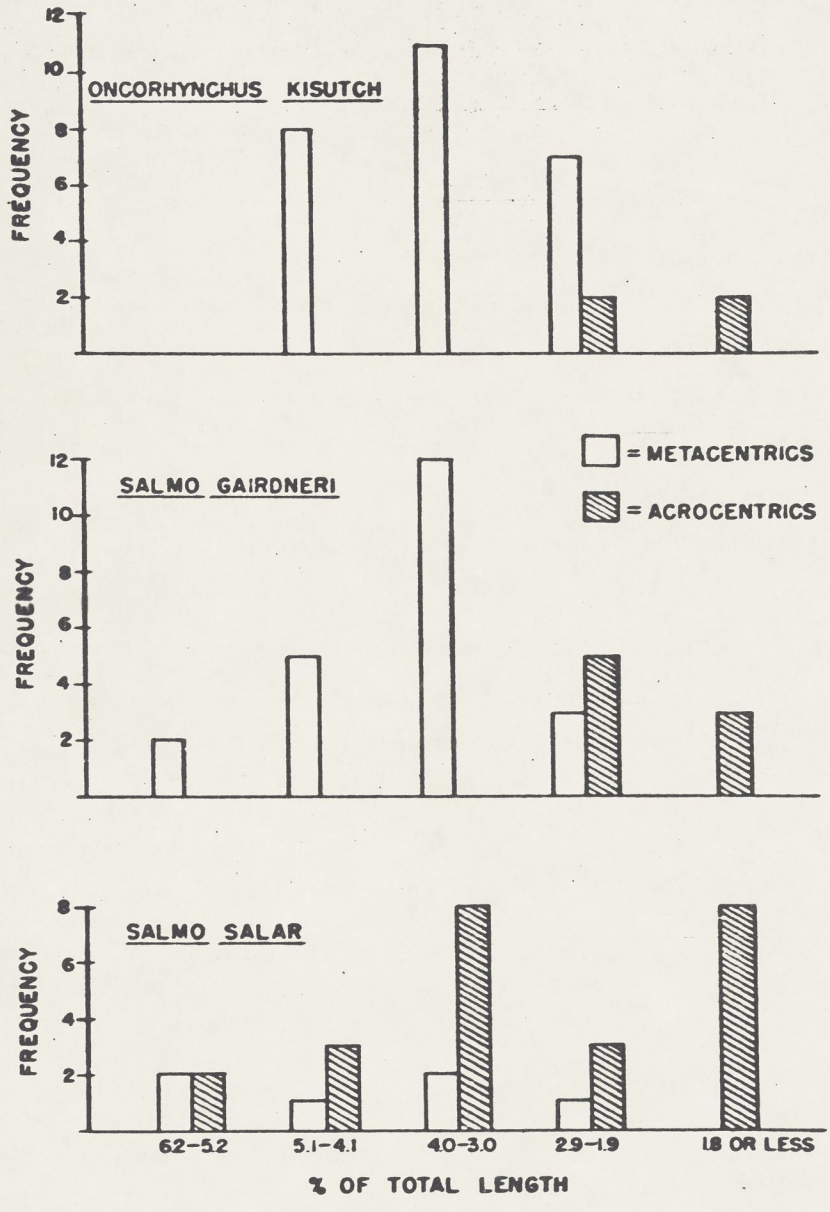

Figure 29

Comparison of chromosome lengths in three salmonid species.

seemingly aberrant nature of this species is greatly minimized.

The discrepant arm number in O. kisutch can likewise be brought into much better agreement by assuming that the arbitrary decision of Simon (1963) is unrealistic in the inclusion with metacentrics of any chromosome which possesses a second arm that measures one micron or more. In blastular chromosomes at least, the thermal "age" of the embryo will determine the absolute length of chromosome arms. In the case of chromosomes in the diploid complement of O. kisutch six are definitely borderline with short arms very close to one micron in length. Had the study of embryonic chromosomes in that species been conducted one or two cleavages later, the arm number would likely have been reported as 10^4, since the length of chromosomes diminishes rapidly during early cleavages.

The net result of the above considerations has suggested that the short arms in subtelocentric chromosomes of S. clarkii clarki should be considered to be borderline with respect to length and do not warrant equal status with truly metacentric chromosomes.

It is obvious that decisions are artificial as to what constitutes a significant arm length. These decisions are nonetheless necessary in comparing the species of the three genera with which the present study is concerned. Despite vagarities of interpretation it can be stated that the several species studied to date are remarkably similar with respect to total hereditary material. Species divergence has apparently progressed by a major process of shuffling the linkage relationships of the ancestral complement. The direction of change favors strengthened linkage with coincident reduction in diploid number.

The above considerations lead naturally to the indicting of the

genetic phenomena of linkage and position effect to be in part responsible for difference in species. Chromosome data indicate that linkage is most complete in the pink salmon (arm number divided by centromere number = 2.0) and least complete in Salvelinus fontinalis, namaycush and alpinus, and in Salmo trutta (arm number divided by centromere number = 1.19 - 1.25). These extremes in linkage coincide very nearly or precisely with the extremes in meristic distributions of pyloric caecae, vertebrae, gill rakers, lateral line scales and horizontal scale rows (Rounsefell, 1962). It would be foolish to ignore other mechanisms which can influence genetic variability (i.e., crossing-over, segmental inversions and mutations) but the present methods have not been selected to measure these contributing factors. The effects of mutations, etc., do not appear to have been sufficient to obscure the effects of linkage and position effect which have resulted from translocations of whole chromosome arms.

Possibilities of chromosome rearrangement are by no means exhausted for the pink salmon, as evidenced by a consideration of the chromosomes of S. salar. This latter species is unique in possessing numerous long acrocentrics which appear to have been produced by alterations of centromere position in chromosomes which were previously metacentric. This avenue of change is largely unexplored by most salmonines and is at least potentially available to all remaining species. Further, there would appear to be no mechanical restriction to prevent further joining of "long" acrocentrics to form metacentrics equivalent to four original arms, so that a salmonid (present or future) with ca. 25 chromosomes is by no means impossible.

The potential for variability to meet changes in natural selection

is suggested to be highly pregnant in salmonine fish at a level of gross changes in chromosome morphology. The question emerging from considerations of change in chromosome numbers is: why is such a spectrum of change evident? A survey of other animal groups indicates that numerical differences of the order observed are not unusual in closely-related groups (i. e., White, 1945 on insects; Matthey, 1949 on reptiles; Chu and Giles, 1957 on primates). Chromosome polymorphism appears to be especially great in Drosophila. Da Cunha and Dobzhansky (1954) have provided evidence that the extent of chromosome polymorphism in Drosophila willistoni is correlated in direct manner with the environmental polymorphism embraced by that species. They believe that chromosome polymorphism is increased in a complex environment with many ecological niches and that this polymorphism provides a flexible adaptive system to cope with environmental variability. Chromosomal polymorphism has been observed to be typical of populations which occupy the central portion of the range of species distribution, while marginal populations usually display limited polymorphism. Stone (1955) has indicated that marginal populations cannot sacrifice the advantages of free recombination in the face of harsh environmental conditions which they encounter. While the chromosomal polymorphism in Drosophila is chiefly accounted for by segmental inversions which suppress free crossing-over, the result is no different than that produced by the reduction in chromosome numbers observed in Salmoninae. The effect of either type of polymorphism is to restrict recombination possibilities. Carson (1955) has provided an explanation of the "evolutionary dilemma" of overspecialization, that is to say, restriction of recombination. He states that "the inversion systems of species with extensive central polymorphism,

like D. robusta and D. willistoni, have a means of escape from the specializing tendency which a high amount of chromosomal polymorphism confers on a population. This escape mechanism consists of relatively homozygous marginal populations where recombinations of polygenes throughout the genome is permitted on a large scale."

These considerations appear to apply very closely to the conditions of chromosomal polymorphism displayed in the arm-rearrangements of salmonines. In this group, however, the terms anadromous and lacustrine could be substituted for central and marginal respectively. It would seem that the anadromous habit could be interpreted as a device to enable circumvention of the limitations imposed by exclusive residence in freshwater environments, such as seasonal temperature fluctuation and restricted food supply. Those species which spend the bulk of their life in freshwater appear to be subject to more rigorous environmental conditions than are their anadromous relatives. The chars are only weakly anadromous, if at all, while many of the trouts and Pacific salmon spend an appreciable portion of their lives in saltwater (Rounsefell, 1958). The chars also exhibit the least linkage (have the highest chromosome numbers) in contrast to extensive chromosome polymorphism in Salmo and Oncorhynchus (Table 8).

Chromosomal polymorphism would thus appear to enable the specialization (restriction of random recombination) necessary for adaptation to a variety of ecological situations. A consideration of interbreeding may reflect further upon possible sources of chromosome rearrangements, as well as offer a hope of judging relationships. Any understanding of chromosome pairing in gamete maturation of hybrids should contribute to additional clarification of these interwoven topics.

Potential importance and consequences of hybridization

Hybridization may be suspected as possibly important in evolution of salmonid species in view of the general success of artificial hybrids. Natural hybrids provide a criterion for judging the distinctness of species. This measure of species boundaries has been elevated to a position of prime importance by proponents of more recent species concepts (i.e., Huxley, 1940; Mayr, et al., 1953; Simpson, 1961; Mayr, 1963). Natural hybrids between S. clarkii lewisi and S. gairdneri are known to be fairly common where these species are mixed by transplantation in the case of inland populations, or by natural coexistence in the case of the coastal cutthroat and steelhead (Dymond, 1932; Hanzel, 1959; Madsen, 1936; Miller, 1950; Miller and Alcorn, 1946; Needham and Gard, 1959; Simon, 1946). The authors of the latter paper have considered that S. clarkii may be considered a subspecies of S. gairdneri upon further study. This conclusion was questioned (Simon and Dollar, 1963) on the basis that chromosome numbers suggested the inland cutthroat to be more primitive, thus in an evolutionary sense, the rainbow was thought to be in subspecific position. Despite this minor objection, the informal proposal of Needham and Gard appears to be reasonable.

A discussion of chromosome segregation to F_2 hybrids of Oncorhynchus may suggest a means by which species relatedness may be clarified, if the common circumstance of natural hybrids (fertile) requires augmentation.

Segregation of chromosomes to F_1 gametes was reflected in the chromosome constitution of O. keta x O. gorbuscha F_2 as previously noted. A pairing model was postulated with the cytological knowledge that

extensive pairing of some sort took place during meiosis in the F_1 male. While the validity of the species concerned is not in question, the irrelationship is nonetheless directly commented upon by pairing of meiotic chromosomes. The chum salmon (O. keta) and pink salmon (O. gorbuscha) have been stated to be very closely related with respect to temperature tolerance (Brett, 1952), distribution (Neave, 1958), scale characters (Kobayasi, 1955), habitat preference (Hoar, 1958) success of artificial hybrids (Foerster, 1935), anadromy (Rounsefell, 1958) and in the advanced state of gonad maturity attained at sea (Rounsefell, op. cit.).

Hoar, 1958, considered the pink to have been split from the chum salmon in a fourth and final step of Pacific salmon evolution. If the chromosome complement of the chum at the time of this split was similar to its present condition, then all metacentrics of the chum would be individually homologous to metacentrics of the pink. In addition, the remaining metacentrics of the pink would each be homologous to two acrocentrics from the chum. This condition could be expected to result in a variable diploid number ranging ca. $\pm$ 6 about a mean of 63 but with stable arm number, if and when hybrids persisted into and beyond the F_2. Such a close compatibility does not seem to be the case. Either the chum, the pink, or both appear to have undergone changes in the combinations of arms which constitute their metacentrics. Variable arm numbers in hybrid generations beyond the F_1 seem to provide a measure of the immediacy of species divergence, presuming the specifications of the pairing model are accurate. It should be evident that similar results could ensue following absence of pairing and subsequent random movement of univalents. In either case, the measure is one of divergence. Numerous univalents have not

been observed in the chum-pink hybrid testis, and are not believed to contribute any appreciable amount of the observed variability in arm number. The implications of these considerations are:

(a) The pink did not evolve from the chum per se, but diverged at some more remote time from a common ancestor which was karyotypically different from either species of the present;

(b) the numerous similarities of the two species might be suspected to have resulted from parallel adaptation to a shared and similar habitat; this speculation requires further clarification by observing chromosome segregation of other species when crossed with pink and/or chum salmon.

The departure of the F_2 diploid number and modal numbers of metacentric and acrocentric chromosomes remains a puzzle. Only three of 50 embryos displayed numbers equal to or greater than the F_1 constitution (63, 64 and 69). Two possibilities might account for this behavior:

(a) preferential viability (or vitality) of gametes with increased numbers of metacentrics;

(b) centric fusions may have occurred during or following gamete formation in the F_1.

The results of these possibilities are indistinguishable; indeed both could be operative. The consequences are identical in reducing the diploid number without discarding genetic information in the course of a single hybrid generation.

"Centric fusions" might thus include three entirely different processes which are virtually identical in net result:

(a) reciprocal translocation of whole chromosome arms,

(b) hybrid averaging of the contribution of parents with different chromosome numbers,

(c) preferential viability of gametes with increased numbers of metacentrics (in the context of hybridization but distinct from hybrid averaging).

In theory, hybridization could account for many of the chromosomal alterations which appear to be the basis of speciation in salmonid fishes. The question of paramount importance is: How important has hybridization been in actuality? A definitive answer is well beyond the present data. Stebbins (1959, 1963) has indicated that hybridization seems to be of minor significance under stable environmental conditions; however, he cautions (1963) that ". . . the presence of a hybrid swarm in a new, unstable environment, can have profound effects on major evolutionary trends far out of proportion to the low frequency with which they occur." New and unstable environments recalls the apparent link between chromosomal polymorphism and anadromy. Might the relief from environmental rigors availed through anadromous habit confer the luxury of polymorphism, or could simple mobility in dispersion favor hybridization with polymorphism as a consequence? The choice is one of hybrid averaging versus reciprocal translocation and in an evolutionary view the distinction may be entirely obscured. Future studies of chromosome segregation to F_2 hybrids would appear to offer a potential hope for resolving the question.

Polymorphisms

"Polymorphism" would appear to apply to more than chromosome variability in salmonids. Variability and intergradation are evident in species comparisons in nearly every, or perhaps all, characters embracing morphology or meristic attributes. In comparisons of species of Salmo, Salvelinus and Oncorhynchus, many characters comprise a continuum distributed as a grand, bell-shaped distribution from one extreme species to another. Within this distribution, each species displays a lesser binomial distribution of its own, but overlapping with neighboring species (see especially the clear depictions of Rounsefell, 1962). As more gaps are filled with larger samples and new populations, the task of identifying discontinuities has grown increasingly difficult. In Oncorhynchus, Salmo and Salvelinus there is an amount of evidence that distinctions are not absolute even between genera.

Morton and Miller (1954) have listed two prime characters to separate Salvelinus from Salmo:

(1) presence of light spots against a dark background (chars) rather than dark spots against a lighter background (trouts).

(2) presence of teeth on the vomer confined to the head of that bone, or on a crest extending posteriorly but not connected to the shaft (chars), while in Salmo the shaft of the vomer bears teeth, as well as the head.

It is interesting to note that the brown trout is intermediate with respect to the first character (since it has both red and black spots). The brown trout is further similar to the chars in autumn spawning habit,

but sharply distinct in regard to vomerine dentition. This character (vomer with toothed shaft) is shared with the genus Oncorhynchus, but is not found in Brachymystax or Hucho. These differences are well illustrated by Norden (1961, Figure 1, p. 733).

The prominence of the vomer has further appeared in assigning generic distinction to the lake-inhabiting char, Salvelinus namaycush, on the basis that on the vomer crest it bears teeth. This distinction (Cristovomer) has been most recently refuted by Morton and Miller (1954), who have shown intergradation in that character between S. namaycush and S. alpinus.

It would appear that no extensive attempt has been made to determine extreme types of vomerine dentition in chars as compared to trouts and Pacific salmons. One might wonder whether the dravings of Gunther (1880, p. 636, Figure 289) are fact or oversight wherein he illustrates a vomer of S. salar with four teeth confined to the anterior portion. Although vomerine teeth are known to be deciduous in that species, no tooth sockets are evident in ventral and lateral views.

Until further studies are made, there appears to be no justification for combining Salmo and Salvelinus, despite overlapping of their chromosome numbers, since significance of this overlap has not been evaluated. There may be reason to consider the overlap important. Simpson (1961, p. 90) has stated, "It would seem that evidence of chromosomal and gene identities would be the most conclusive criterion of homology." However, chromosomal identities are not the same thing as chromosome numbers, as pointed up in considerations of F_2 hybrids.

The question of generic distinction between Salmo and Oncorhynchus has not been challenged since the time of Regan (1914). Most authors

have followed Tchernavin's (1937, 1939) recommendation to retain both genera but have not given much attention to the fact that Tchernavin failed to consider the very species, O. masou, which provided the crux of Regan's argument. Behnke, et al., (1962) have provided a summary of the generic characters of Salmo and Oncorhynchus in a review of O. masou. They conclude that O. masou is intermediate or more like Salmo in regard to counts of anal fin rays, branchiostegal rays, gill rakers and vertebrae. This species is also like Salmo in that the paired dorsal fontanelles in the chondrocranium of adults, although reduced, do not roof over and become obscured as in adult Oncorhynchus. It resembles Oncorhynchus, however, in the rounded shape of the anterior terminus of the ethmoid cartilage (rostrum). In O. tshawytscha, O. keta and O. nerka, the rostrum of some juveniles has been described as bluntly truncate, much as in Salvelinus namaycush. Other juveniles of these species were noted to display a slight notch (Norden, 1961, p. 727). Some males of O. masou are known to survive after spawning in the fashion of Salmo.

The characters noted above are generally considered to be adequate support of generic distinction.

Further features not considered above were employed by Norden in defining Salmo and Oncorhynchus:

Character	Salmo	Oncorhynchus
postorbitals	no contact with preopercle	contact preopercle
supraethmoid	notched posteriorly	deeply notched posteriorly (adult)
opisthotic	not touching prootic	touching prootic

Character (continued)	Salmo	Oncorhynchus
ascending process of premaxilla	present	absent
separation of palatine and vomerine teeth	narrow	wide

This comparison does not include O. masou and cannot be presumed to reflect prime generic characters until that species has been substantially represented. According to Norden (p. 729), "The supraethmoid, present in all salmonines, has the greatest taxonomic significance of all the dorsal roofing bones." The most striking differences are to be found in large Oncorhynchus. The size differences in supraethmoids of O. gorbuscha and S. gairdneri illustrated in Norden's plate 12 (p. 783) appear to be trivial (nor did he claim otherwise) when it is considered that the adult pink salmon (only heads studied, no body size given) could be expected to be about twice as large as the largest rainbow (11 inches) of that author's series. The striking extent of notching of the supraethmoid of O. gorbuscha is implied by Norden to be moderated in smaller specimens and would thus approach the moderately-notched condition typical of Salmo. Further, the shape of the ethmoid cartilage, apparently diagnostic of adult Oncorhynchus, is found in juvenile salmon (O. keta, nerka, tshawytscha) to be intermediate between Salmo and adult Oncorhynchus (Norden, 1961). This character must also be considered to be correlated with secondary changes. Thus the azygous rostrum in adult Oncorhynchus which Tchernavin (1937, 1938) denoted to typify the genus would appear to be a further reflection of growth, and/or sexual change.

In summary, generic differences between Oncorhynchus and Salmo appear to fall into one of three categories:

1. INTERGRADING due to the intermediate nature of O. masou
2. INADEQUATELY TESTED by failure to include O. masou
3. SECONDARILY CORRELATED with maturity and/or growth

1 and 2 do not offer valid bases for generic distinction. The third category includes an aggregate of correlated characters. Mayr, et al. (1953, p. 124) indicate that characters which vary as a unit (correlated characters of the present discussion) should be weighted as though they were a single character. The alterations of Oncorhynchus which accompany sexual maturity would appear to represent a constellation of such characters. It could be considered that secondary sex changes represent a character diagnostic for Oncorhynchus; however, O. masou is intermediate with respect to persistence of dorsal fontanelles and hence is intermediate concerning the remaining correlated characters if they are to be treated as a unit.

No character in the trouts and Pacific salmon appears to be mutually exclusive to either group.

If continued retention of Oncorhynchus is advocated, the severe logical problem must be faced of having one or more "generic" characters apply to species of a different genus.

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APPENDIX I

Chromosome Pair Lengths (microns) and Per Cent of Total Complement Length in Three Species (*S. salar* from Svardson, 1945, *O. kisutch* from Simon, 1963

Pair number	*O. kisutch* microns	% total	*S. gairdneri* microns	% total	*S. salar* microns	% total
1	20.6	4.97	28.5	6.08	24.6	6.36
2	20.0	4.83	24.5	5.25	21.8	5.64
3	20.0	4.83	21.0	4.48	16.4	4.24
4	18.6	4.49	21.0	4.48	15.5	4.01
5	18.0	4.34	21.0	4.48	15.5	4.01
6	18.0	4.34	20.0	4.27	11.0	2.85
7	18.0	4.34	19.5	4.16	23.6	6.11
8	18.0	4.34	19.0	4.05	21.0	5.34
9	16.0	3.86	19.0	4.05	17.2	4.45
10	16.0	3.86	17.5	3.73	16.4	4.24
11	16.0	3.86	18.0	3.84	16.4	4.24
12	15.0	3.62	18.5	3.95	15.5	4.01
13	15.0	3.62	17.0	3.63	14.6	3.78
14	14.0	3.38	17.0	3.63	14.6	3.78
15	14.0	3.38	16.5	3.52	14.6	3.78
16	14.0	3.38	16.0	3.41	13.6	3.52
17	14.0	3.38	15.0	3.20	13.6	3.52
18	13.0	3.14	15.0	3.20	12.8	3.31
19	13.0	3.14	15.0	3.20	11.8	3.05
20	12.0	2.90	13.0	2.77	8.2	2.12

APPENDIX I (continued)

pair number		O. kisutch microns	% total	S. gairdneri microns	% total	S. salar microns	% total
21		11.6	2.80	12.0	2.56	8.2	2.12
22		11.0	2.65	9.0	1.92	7.2	1.86
23		10.6	2.56	11.0	2.35	7.2	1.86
24		9.6	2.32	12.0	2.56	8.2	2.12
25		8.6	2.08	10.0	2.13	7.2	1.86
26		8.6	2.08	10.0	2.13	7.2	1.86
27		10.0	2.41	9.5	2.03	6.4	1.66
28		8.6	2.08	8.0	1.71	5.4	1.40
29		7.6	1.83	8.0	1.71	5.4	1.40
30		5.0	1.21	7.3	1.56	5.4	1.40
	Totals	414.4	100.02	468.8	100.04	386.5	99.99

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