AN ABSTRACT OF THE THESIS OF

RICHARD LEWIS WILMOT for the DOCTOR OF PHILOSOPHY
(Name of student) (Degree)

in FISHERIES presented on July 16, 1973
(Major) (Date)

Title: A GENETIC STUDY OF THE RED-BAND TROUT (Salmo sp.)

Abstract approved: Redacted for privacy
Raymond C. Simon

A group of trout that reside in streams of the desiccating lake basins of southeastern Oregon differ markedly from other known Salmo. Known commonly as the red-band trout, this fish was subjected to chromosome analysis for comparison with other species of western North American Salmo. The karyotype of the red-banded trout is 2n = 58 composed of 44 metacentrics, 2 metacentrics with satallites, 2 sub-metacentrics and 10 acrocentrics to give 104 chromosome arms. This karyotype is identical to that of the California golden trout, S. aguabonita. A similar karyotype has been found, for trout from the Deschutes River and summer-run steelhead from the Siletz and Clearwater rivers. The chromosomal and distributional data is believed to indicate a widespread golden trout complex composed of the red-band trout, the California golden trout, and the Kern River trout inhabiting the Pacific Coast drainage from British

Columbia to southern California. In the course of this study, a new karyotype was discovered for the Alvord cutthroat trout of 2n = 64 with 40 metacentrics and 24 acrocentrics to give 104 chromosome arms.

Nineteen enzyme systems were analysed by starch-gel electrophoresis in two populations of the red-band trout. The Bridge Creek population displayed very low variability for only malate dehydrogenase (MDH) and phosphoglucomutase (PGM). No variability was found in the Three-Mile Creek population. A new allele, not previously reported was found for MDH in the Bridge Creek population. The high degree of genetic similarity among and between these populations is thought to be the result of selection pressure rather than stochastic processes.

The isolated populations of the red-banded trout appear to be well adapted to their present environment, but without the genetic variability to meet future environmental changes, they may face extinction. Their most immediate threat is man's use of the environment.

A Genetic Study of the Red-Band Trout (*Salmo* sp.)

by

Richard Lewis Wilmot

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

June 1974

APPROVED:

Redacted for privacy

Professor of Fisheries
in charge of major

Redacted for privacy

Head of Department of Fisheries and Wildlife

Redacted for privacy

Dean of Graduate School

Date thesis is presented July 16, 1973

Typed by Opal Grossnicklaus for Richard Lewis Wilmot

ACKNOWLEDGMENTS

I am most grateful to my major professor, Dr. R. C. Simon, for his help and guidance throughout this study, and to the other members of my committee, Drs. P. S. Dawson, G. E. Davis, E. J. Dornfeld and D. C. England.

I am especially grateful to Dr. J. D. McIntyre for his cooperation and help and to Dr. C. B. Bond for his stimulating discussions on the classification and distribution of fishes.

Thanks are due to many fellow students who devoted their time to help collect specimens.

TABLE OF CONTENTS

Chapter		Page
I.	INTRODUCTION	1
II.	DESCRIPTION OF THE AREA	4
III.	MATERIALS AND METHODS	6
	Collection of Specimens	6
	Chromosome Preparations	6
	Enzyme Electrophoresis	10
IV.	RESULTS	12
	Chromosome Studies	12
	Enzyme Electrophoresis	16
V.	DISCUSSION	24
	Distribution and Genetic Variability of the Red-Band Trout	24
	Origins and Affinities of the Red-Band Trout	28
VI.	CONCLUSIONS	43
	LITERATURE CITED	44
	APPENDICES	49
	APPENDIX I	49
	APPENDIX II	51
	APPENDIX III	53
	APPENDIX IV	56
	APPENDIX V	58
	APPENDIX VI	60

LIST OF FIGURES

Figure		Page
1.	Locality covered in this study.	8
2.	Classification of chromosomes used in this study; a) metacentric, b) metacentric with satellites, c) sub-metacentric and d) acrocentric.	9
3.	Somatic chromosomes from cell culture of *Salmo* sp., Bridge Creek, Lake County, Oregon.	14
4.	Meiosis from testes of *Salmo* sp.; a) and b) first division metaphase of bivalents and multivalents; c) polyploid cell displaying many different types of multivalents and d) second division meiosis metaphase displaying a haploid number of 29.	18
5.	Malate dehydrogenase phenotypes--AAB'B'' (a), AAB'B' (b, d and e) and AABB' (c and f)--from white skeletal muscle extracts of Bridge Creek *Salmo* sp.	21
6.	Phosphoglucomutase phenotypes--AB (a) and AA (b)--from white skeletal muscle extracts of Bridge Creek *Salmo* sp.	21
7.	Somatic chromosomes from kidney of *Salmo aguabonita*.	35
8.	Somatic chromosomes from kidney of *Salmo clarki alvordensi* Whitehorse Creek, Malheur County, Oregon.	41

LIST OF TABLES

Table		Page
1.	Enzymes tested for variability in muscle of the red-band trout.	11
2.	Frequencies of malate dehydrogenase (MDH) and phosphoglucomutase (PGM-2) in muscle extracts of red-band trout.	22
3.	Chromosome numbers for some western North American *Salmo*.	32

A GENETIC STUDY OF THE RED-BAND TROUT (*Salmo* sp.)

INTRODUCTION

The western North American *Salmo* are difficult to understand from a taxonomic point of view. Biologists of the late 1800's and early 1900's did not fully realize the phenotypic plasticity of the *Salmo* and assigned species status to almost every local form. Jordan *et al.* (1930) described thirty-two species of western North American *Salmo*. The advent of the biological and polytypic species concepts produced a great effect upon taxonomists and all forms of western North American *Salmo* were brought into two evolutionary groupings -- the rainbow trout, *S. gairdneri*, and cutthroat trout, *S. clarki* (Behnke, 1970). Recent discoveries of several isolated forms of *Salmo* have indicated that the evolutionary history of this group is more complex. The Mexican golden trout, *S. chrysogaster*; the Apache trout, *S. apache*; and the Gila trout, *S. gilae* are species that do not conform morphologically to either the rainbow or cutthroat trout series.

Another group that does not fit into the conventional scheme is known as the red-band trout. This trout was first recognized in the high desert plateau country of southeastern Oregon, and later in tributaries of the Pit and McCloud rivers in northern California

(Behnke, 1970). Called rainbow trout by some local inhabitants and cutthroat by others, these trout possess characteristics of both species. Cope (1889) collected trout from Silver Creek, a small stream flowing into Silver Lake (Lake County, Oregon), and named them S. purpuratus. Snyder (1908), sampled many areas throughout southeastern Oregon and referred to all trout described as S. clarki. Bond (1961) sampled many of these fish and called them fine scaled rainbow trout which superficially resembled cutthroat trout.

The red-band trout has a striking golden hue when first removed from the water. It has large black spots on the entire dorsal surface, a brick-red stripe along the sides onto the operculum, faint cutthroat marks and parr marks in all age classes. The pelvic and pectoral fins have white leading edges and there is an orange tip on the dorsal fin. A color plate by Dymond (1932) of the mountain kamloops trout, S. kamloops whitehousei, superficially resembles the red-band trout. Because of its characteristics and location, the red-band trout may well be a key group in understanding the origins and affinities of western North American Salmo.

The principal objective of this study was to clarify the taxonomic position of the red-band trout within the genus Salmo from western North America. In addition, two hypotheses about the red band trout were tested:

1) Trout populations in streams draining into the dry lake basins of southeastern Oregon are taxonomically similar and of common ancestral origin; and

2) Assuming that these stocks had a common origin, mechanisms which tend to modify genetic structure of populations have produced locally adapted populations.

DESCRIPTION OF THE AREA

Southeastern Oregon is a semi-arid plateau forming the northwest corner of the Great Basin. The Great Basin, an area covering most of Nevada and parts of Oregon, California, Utah and Idaho, consists of a number of internally drained basins that once contained large lakes following the Pleistocene glaciation. Faulting and warping of the earth's crust have produced alternating eras of mountain building when volcanic activity spewed lava across the land. Intervening periods of relative calm allowed erosion of the mountains into grassy plains and rolling hills, followed by periods of upheaval and volcanic activity. The climate varied from temperate to tropical and precipitation from light to very heavy. This produced a corresponding change in vegetation from sagebrush to grassy plains, to temperate forests of Douglas fir and Redwoods, to tropical forests typical of present day Central America. River systems have been disrupted and rearranged many times. The essential physical features of the land today are the result of forces in the late Pleistocene age.

During the million or so years of the Pleistocene, the climate was cooler and less aris (Hubbs and Miller, 1948). Great ice sheets advanced over the Canadian border and glaciers formed in the higher mountains of the Great Basin. There were four, and possibly five such periods during this era. Vegetation, climate and animal life during the interglacial

periods were probably similar to today. Each interglacial period produced a greater inflow of water into the basins counteracting evaporation and producing large lakes. The second and third glacial periods especially produced large lakes forming connections with basins that did not exist following the last glacial period. Connections were formed with the major river systems of the west including the Columbia, Colorado, Snake, and probably the Pit, Sacramento and Klamath.

Based on pollen profile analysis, Hansen (1947) divided the period following the last great glaciation into four main periods. The first was the Pluvial Lakes (10-15,000 years ago) when temperatures were cooler than present and water flow into the basins greater than evaporation. The second period (Anathermal), from 8-10,000 years ago, was a time of increasing temperature. The third period (Altithermal), 4-8,000 years ago, had decreased inflow and a corresponding increase in temperature, causing desiccation of the Great Basin lakes. Many lakes probably disappeared altogether. The last 4,000 years have been erratic, but the trend appears to be a return to cooler and moister conditions producing a rebirth of some basin lakes. Today, very few of the remaining lakes support salmonids. [For a detailed discussion of the geological and climatological history of the Great Basin see: Antev (1938), Blackwelder (1948), Hubbs and Miller (1948) and Hansen (1961)].

MATERIALS AND METHODS

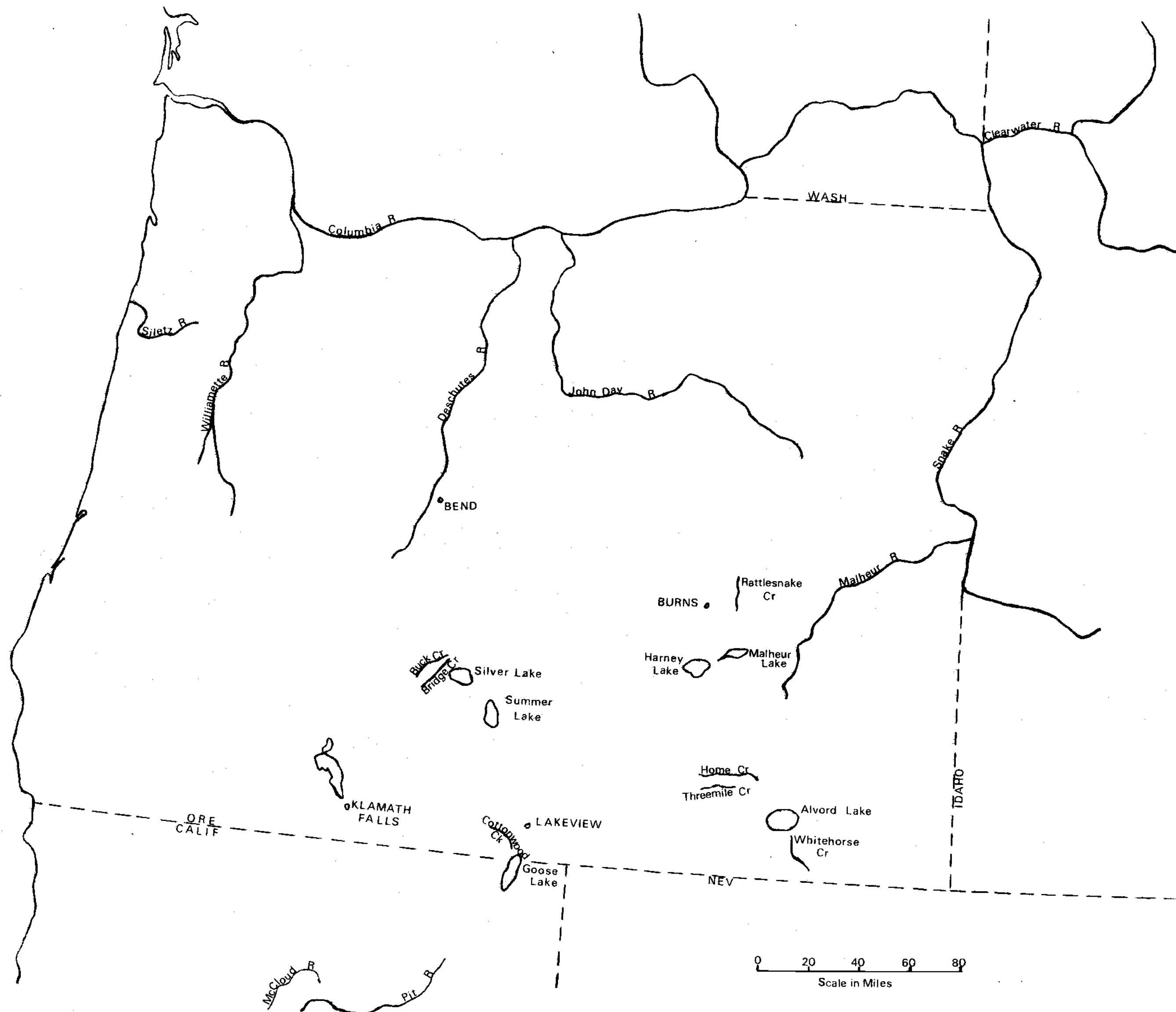
Collection of Specimens

Red-band trout were collected by electrofishing and by hook and line. A total of 51 fish from Bridge Creek and 72 from Three-Mile Creek were collected for karyotyping and electrophoretic examination of enyzmes. Samples for karyotyping from other locations (Fig. 1) ranged from 6 to 24. Specimens were transported alive to Oregon State University and held in live tanks until processed. Alvord trout from Whitehorse Creek (Malheur County, Oregon) were provided by Mr. Robert Borovicka of the Bureau of Land Management, California golden trout by California Fish and Game and the Oregon Game Commission supplied Deschutes River anadromous and native trout, Siletz River summer-run trout and Clearwater River, Idaho summer-run trout. Samples of a golden trout brood stock at the National Marine Fisheries Service, Montlake Laboratories in Seattle were provided by James Mighell.

Chromosome Preparations

Chromosome preparations were obtained by four different methods: 1) primary cell cultures started from air-bladder, kidney or spent ovarian tissues by methods modified from Robers (1963) and Fryer *et al*. (1965); 2) cultures of peripheral blood leukocytes

Figure 1. Locality covered in this study.



Clearwater R
Columbia R
WASH
Siletz R
Willamette R
Deschutes R
John Day R
Snake R
BEND
Rattlesnake Cr
Malheur R
BURNS
Buck Cr
Bridge Cr
Silver Lake
Harney Lake
Malheur Lake
Summer Lake
Home Cr
Threemile Cr
IDAHO
KLAMATH FALLS
ORE
CALIF
Cottonwood Ck
LAKEVIEW
Alvord Lake
Whitehorse Cr
Goose Lake
NEV
0 20 40 60 80
Scale in Miles
McCloud R
Pit R

(Heckman et al., 1971); 3) tissue squashes of kidney, liver, gill, spleen and testes (Ohno, 1965) and 4) kidney tissue rubbed through a fine mesh screen (78 micron) and treated by methods described for blood culture (McGregor, 1970). Details of the four methods are given in Appendices I-IV. The chromosomes were divided into four major types; metacentrics, metacentrics with satellites, sub-metacentrics and acrocentrics (Fig. 2).

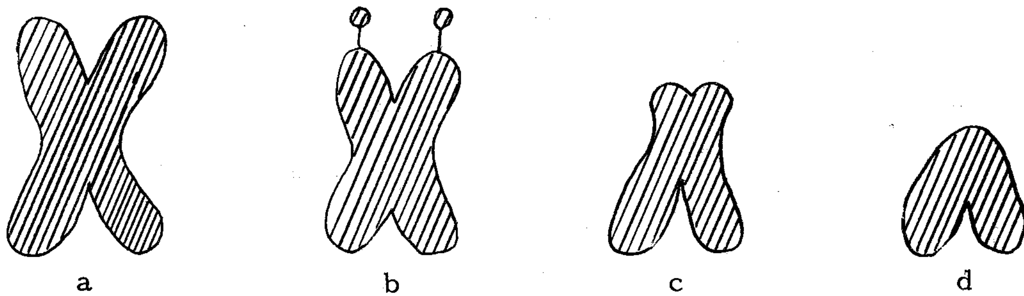


Figure 2. Classification of chromosomes used in this study; a) metacentric, b) metacentric with satellites, c) sub-metacentric and d) acrocentric.

A method was attempted whereby a more complete degree of homology could be ascertained between chromosomes using the various chromosome banding techniques performed on many mammalian species (Casperson et al., 1970; Drets and Shaw, 1971; Lamholt and Mohr, 1971; Patil et al., 1971; Schnedl, 1971; Seabright, 1972; Wang and Fedoroff, 1972; Kato and Yosida, 1972 and Utakoji, 1972). These techniques generally involve the selective removal of chromosomal proteins with chemicals.

Chromosome preparations were examined with a Zeiss WL research microscope and neofluar objectives and 8X eyepieces. Photographs were taken on Kodak Panotomic-X film.

Enzyme Electrophoresis

Nineteen enzyme systems were tested for variability by starch-gel electrophoresis. Tissue samples were taken from frozen specimens and mascerated with a glass rod in 1:2 volumes of 2% phenoxyethanol. A gel-buffer system described by Ridgeway *et al*. (1970) was used for all but one enzyme system (Appendix V). A system devised by Bailey and Wilson (1968) was used for malate dehydrogenase (Appendix VI). Stains for the various enzymes are described by Shaw and Prasad (1970). The nineteen enzymes tested are listed in Table 1.

Table 1. Enzymes tested for variability in muscle of the red-banded trout.

Enzyme Tested	Bridge Creek	Three-Mile Creek
Aldolase	0	0
Fructose 1, 6 Diphosphatase	0	0
Leucine Aminopeptidase (Intestine)	0	0
Galactose 6 Phosphate Dehydrogenase	0	0
Glucose 6 Phosphate Dehydrogenase	0	0
Glutamate Dehydrogenase	0	0
Glyceraldehyde 3 Phosphate Dehydrogenase	0	0
Alpha Glycerophosphate Dehydrogenase	0	0
Hexose 6 Phosphate Dehydrogenase	0	0
Isocitrate Dehydrogenase	0	0
Lactate Dehydrogenase (also heart and liver)	0	0
6 Phosphogluconate Dehydrogenase	0	0
Malate Dehydrogenase	0	0
Sorbitol Dehydrogenase (liver)	+	0
Adenylate Dehydrogenase	0	0
Creatine Kinase	0	0
Glucosephosphate Isomerase	0	0
Hexokinase	0	0
Phosphoglucomutase	+	0

0 - no variability

+ - variability

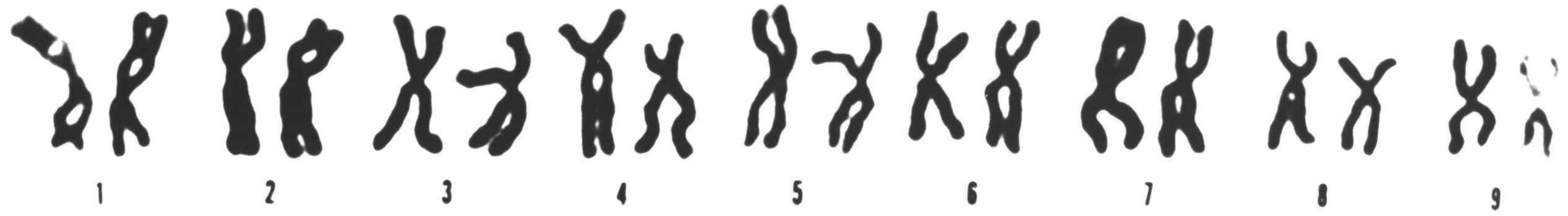
RESULTS

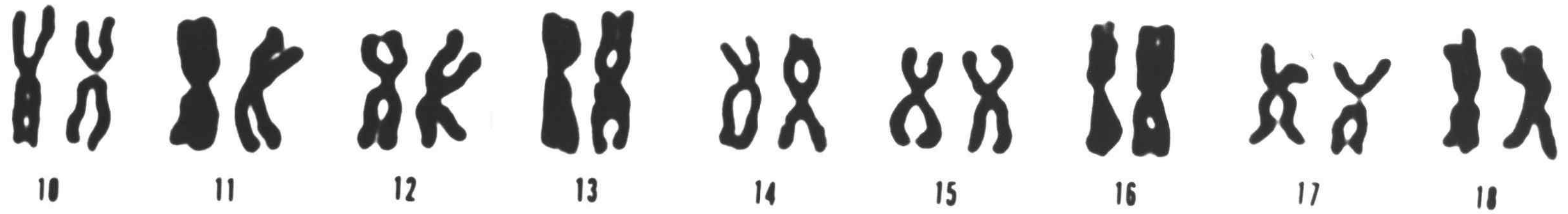
Chromosome Studies

The karyotype shown in Fig. 3 was common to specimens collected from Bridge, Buck, Cottonwood, Home, Three-Mile and Rattlesnake Creeks. The karyotype consisted of a diploid chromosome number of 2n=58 with 44 metacentrics, 2 metacentrics with satellites, 2 sub-metacentrics and 10 acrocentrics. The pair of chromosomes with short second arms (pair 21) are considered as one armed chromosomes for the determination of total arm number. Accordingly, this produces an arm number of 104 for the red-band trout which is identical to *S. gairdneri*. This karyotype was found in squashes of kidney, liver, spleen and gill tissues; monolayer cultures of air-bladder and spent ovarian tissue and suspension cultures of peripheral blood leukocytes. In hatchery rainbows (called *S. irideus* by Ohno, 1965) different chromosome numbers were found in different organs of the same individual. The arm number did not vary from 104 and the different number of chromosomes was interpreted as the result of "Robertsonian" rearrangements. This phenomenon was not found in any populations of red-band trout.

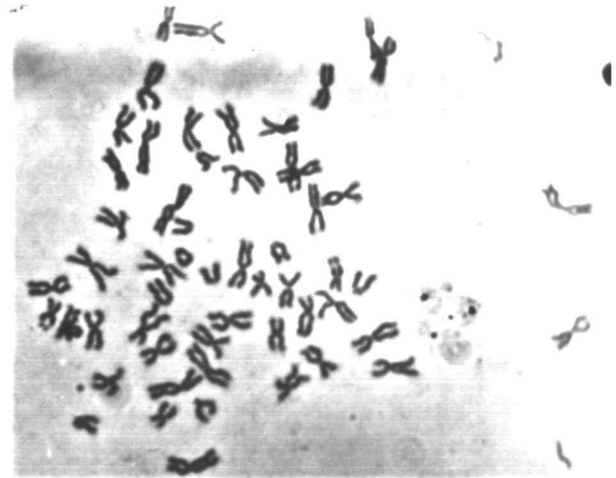
Red-band trout were found in six locations in southeastern Oregon (Fig. 1) and their identity was confirmed by chromosome analysis. Bridge and Buck creeks (Lake County) flow northeast out

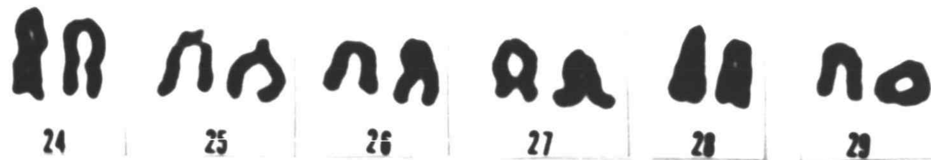
Figure 3. Somatic chromosomes from cell culture of *Salmo* sp., Bridge Creek, Lake County, Oregon.


1 2 3 4 5 6 7 8 9


10 11 12 13 14 15 16 17 18

19 20 21 22 23




24 25 26 27 28 29

of Fremont National Forest into Palina Marsh, a remnant of Pluvial Silver Lake. Palina Marsh will not support trout and the two streams are effectively isolated from one another. Red-band trout are the only salmonid species found in Bridge Creek, while Buck Creek also supports a large population of brook trout (Salvelinus fontinalis) resulting from plants made some years ago. Cottonwood Creek (Lake County), flowing southeast out of Cottonwood Meadow Lake towards Goose Lake, contained red-band trout when first sampled in 1968. Cottonwood Meadow Lake was stocked with over 10,000 hatchery rainbow in 1969 (Koski, 1970). Sampling in the next two years failed to produce any red-band trout. Home and Three-Mile Creeks (Harney County) flow west out of the Steens Mountains into marshes on the floor of Catlow Valley. These two creeks, separated by only a few miles, are completely isolated from one another. Rattlesnake Creek (Harney County) flows south from Malheur National Forest into a marsh north of Malheur Lake.

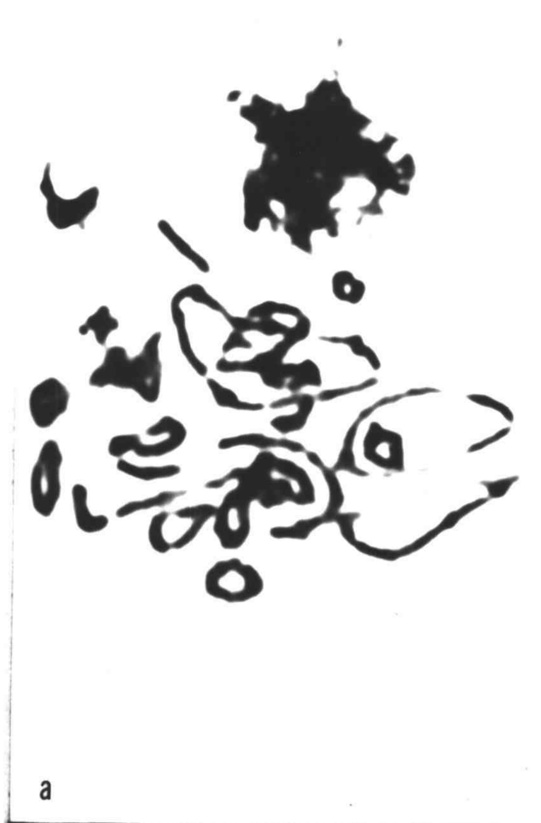
Red-band trout have also been found in tributaries of the Pit and McCloud rivers in northern California (Behnke, 1970) and were karyotyped by Miller (1972). Behnke (personal communication) examined preserved specimens of trout collected throughout southeastern Oregon and by meristic comparisons concluded that they were red-band trout or red-band-rainbow trout hybrids.

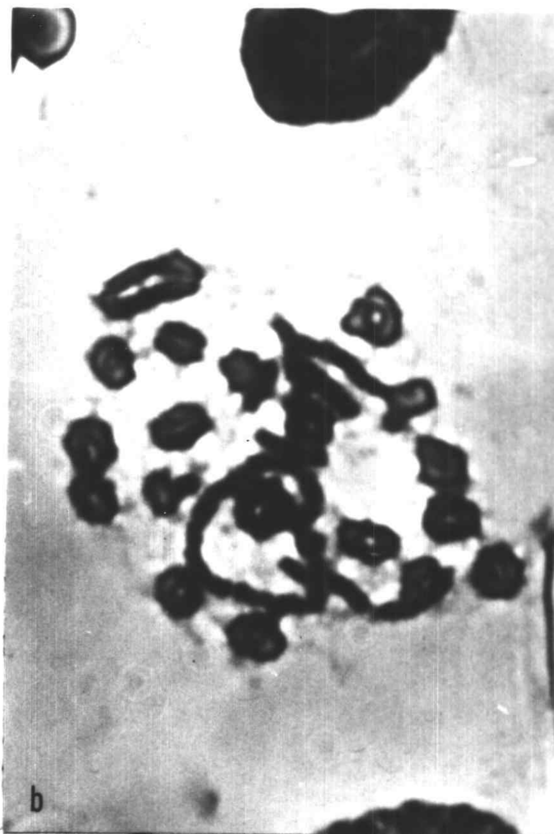
The number of bivalents in meiosis from red-band trout testes varied from 21 to 29 and the number of multivalents from 0 to 8 (Fig. 4a and 4b). A polyploid cell showing the various type of multivalents formed in red-band trout meiosis is shown in Fig. 4c. Some configurations are quadravalents while others are very complex multivalents. A second meiotic division metaphase showing a haploid chromosome number of n=29 is shown in Fig. 4d.

Enzyme Electrophoresis

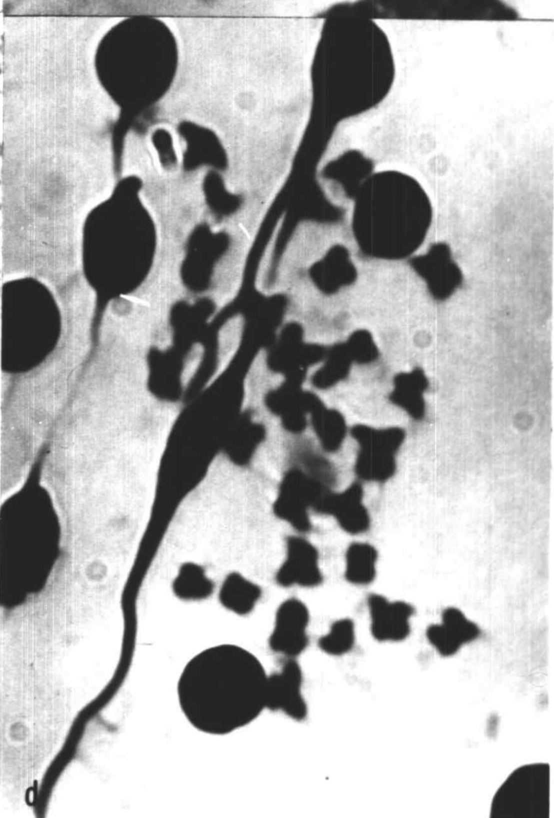
Genetic variation was found only in the Bridge Creek population, and then only for malate dehydrogenase (MDH) and phosphoglucomutase (PGM-2). The nomenclature of Bailey *et al*. (1969) for *S. gairdneri* was used for the interpretation of the MDH isozymes. They postulated two types of subunits (A and B), the products of two separate loci, which formed three dimeri c forms of MDH (AA, AB and BB). They found some individuals to have six forms of MDH rather than three. It was concluded that this resulted from individuals heterozygous for an allelic form of the B locus (B'). The six dimeric forms are then AA, AB, BB, AB', BB' and B'B'. The A subunit is the slowest migrating form and B' is the fastest. These same results were found for the red-band trout; but in addition, a third subunit was found for muscle MDH in two specimens. It is a faster form than the B' subunit and does not appear to join with either

Figure 4. Meiosis from testes of _Salmo_ sp.; a) and b) first division metaphase with bivalents and multivalents; c) polyploid cell displaying many different types of multivalents and d) second division meiosis metaphase displaying a haploid number of 29.


a


b

c


d

A or B' subunits. The occurrence of this third type of subunit presented the problem of deciding whether it was a third allele of the B locus or a new locus. When this form appeared, the B subunit did not appear, supporting the hypothesis of a third allele. A cross was made between a female displaying the third subunit and an AAB'B' male. Only six progeny survived for testing and all possessed the third subunit. Assuming the third subunit is an allele of the B locus, the cross was between an AAB'B' male and an AAB'B'' female. The gametes produced by the male are all AB' and the female gametes are 50% AB' and 50% AB''. Half the progeny should be AAB'B' and the other half AAB'B''. If this third subunit is a result of a new locus, the cross was between an AAB'B' male and an AAB'B'CC female. The male gametes again are all AB' and the female gametes all AB'C. All the progeny would then be AAB'B'C and all would show the presence of the third subunit. Since all the progeny possessed the third subunit, and this subunit does not join with any other type, a strong case can be made for a new locus. If it were a new allele B'', the survivors among the progeny makes it possible that all AAB'B' phenotypes perished. Unless some other factor is involved, it would be difficult to argue for selective mortality against the AAB'B' progeny since this is the dominant phenotype in the Bridge Creek population. In spite of the mating results, I concluded that this third subunit is the result of a new allele at the B locus for two reasons: 1) when this

subunit appeared the B subunit did not; and 2) the emergence of a new allele is not a particularly rare or dramatic event whereas the emergence of a new locus is both of these. Only more matings would solve this question beyond doubt.

Three muscle MDH genotypes were found in the Bridge Creek population (AAB'B', AABB' and AAB'B'') and only one in the Three-Mile Creek population (AAB'B'). The three muscle MDH phenotypes of the red-band trout are shown in Fig. 5. Table 2 shows the observed and expected calculations for the phenotypic and gene frequencies for muscle MDH in the two populations assuming Hardy-Weinberg equilibrium. The B and B'' alleles are absent from the Three-Mile Creek population and it appears as if the B' allele has become fixed in the population. In Bridge Creek, the gene frequencies are: B=0.07, B'=0.93 and B''=0.03.

PGM is a dimeric enzyme found in two groups of bands in extracts of liver of the Atlantic herring (Lush, 1969). PGM-1 is the faster migrating form and PGM-2 the slower one. PGM-1 is faint or absent in muscle extracts of Pacific herring, but PGM-2 stains very dark (Utter, 1972). PGM-2 was found to be polymorphic in Pacific herring and presumed to be the result of two alleles at a single locus (A and B) expressed as three phenotypes, AA, AB and BB. The AA band was the fastest migrating and BB the slowest. This same system was found to be operating in muscle extracts of

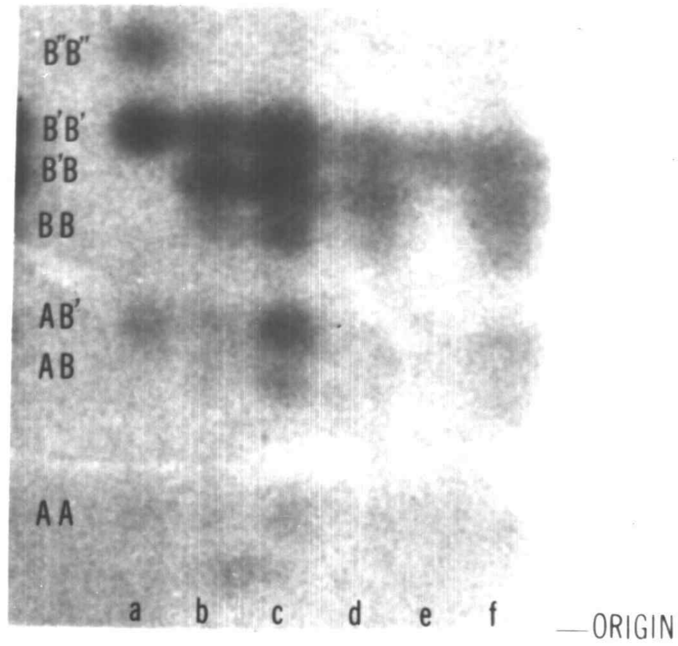


FIGURE 5
MALATE DEHYDROGENASE PHENOTYPES - AAB'B" (a), AAB'B' (b,d and e) and AABB' (c and f) - FROM WHITE SKELETAL MUSCLE EXTRACTS OF BRIDGE CREEK <u>SALMO</u> <u>sp</u>.

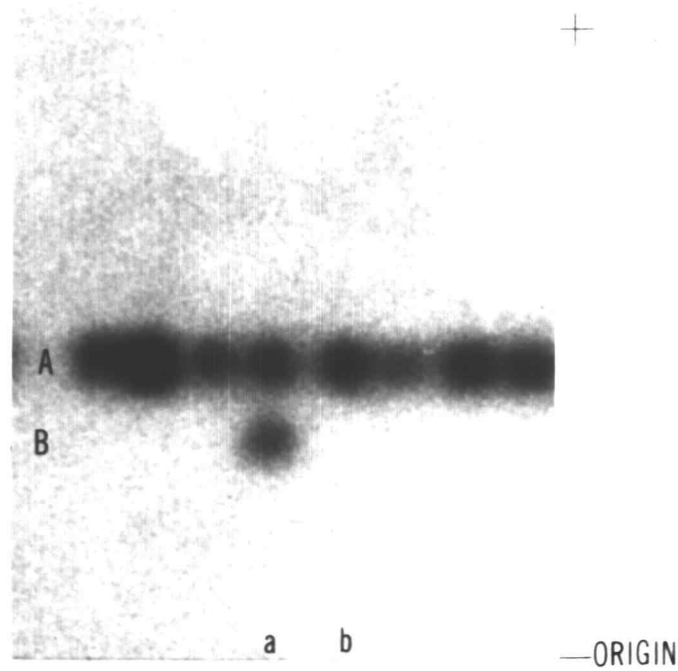


FIGURE 6
PHOSPHOGLUCOMUTASE PHENOTYPES - AB (a) AND AA (b) - FROM WHITE SKELETAL MUSCLE EXTRACTS OF BRIDGE CREEK <u>SALMO</u> <u>sp</u>.

Table 2. Frequencies of malate dehydrogenase (MDH) and phosphoglucomutase (PGM 2) in muscle extracts of red-banded trout.

Location	MDH phenotypes[a]			Allele frequency		N	X^2	P of X^2
	B'B'	B'B	BB	B'	B			
Bridge Creek	51 (50.99)	8 (7.71)	0 (0.29)	0.93	0.07	59	0.3	$0.7<P<0.5$
Three-Mile Creek	72	0	0	1.0	0	72	-	-

Location	PGM-2 phenotypes[a]			Allele frequency		N	X^2	P of X^2
	AA	AB	BB	A	B			
Bridge Creek	0 (0.04)	3 (2.96)	56 (55.99)	0.03	0.97	59	0.04	$0.9<P<0.8$
Three-Mile Creek	0	0	72	0	1.0	72	-	-

[a]Expected frequencies are in parenthesis.

the red-band trout. Two phenotypes were found in the Bridge Creek population, AB and AA, whereas only AA was found in the Three-Mile Creek population (Fig. 6). The observed and expected phenotypic and gene frequency calculations are shown in Table 2. The allelic frequencies in the Bridge Creek population are 0.97 for A and 0.03 for B. The A allele is fixed in the Three-Mile population.

In the nineteen enzymes tested by starch-gel electrophoresis, only the one new allele was discovered. Otherwise, it was impossible to distinguish a red-band trout from S. gairdneri, S. aguabonita or S. clarki. Utter (personal communication) could not find any differences between the enzymes of the red-band trout and S. gairdneri.

DISCUSSION

Distribution and Genetic Variability of the Red-Band Trout

The finding of a common karyotype in the various populations of trout throughout southeastern Oregon confirms the hypothesis that they are relicts of a previously widespread group. Gross chromosomal changes have not occurred in the period since they were isolated from one another, 4-8,000 years ago. Certain chromosomal changes, such as whole arm exchanges or inversions, could have taken place in some populations but they were not recognized if present with my techniques.

That extensive arm rearrangement is going on in the red-band trout populations is shown by the many complex multivalents formed in meiosis. Why this is not associated with different diploid chromosome numbers in different organs of the same individual, as was the case for _S. irideus_ (Ohno, 1965), is not known.

The finding of no genetic variation in the Three-Mile population, and very low levels in the Bridge Creek population, was an unexpected result. Most natural populations investigated have shown a high degree of polymorphism. Prakash _et al._ (1969) investigated central, marginal and isolated populations of _Drosophila pseudoobscura_ in North and South America. In the central and marginal populations, 38-42% of

the loci were estimated to be polymorphic and an average individual was heterozygous for 10-12% of the loci. A small, isolated population in South America was estimated to have 0.25% of its loci polymorphic and 5.0% of the loci were heterozygous in an average individual. The low variability found in the isolated population, which is still large compared to the red-band trout, was explained as the result of a small founder population--a form of random genetic drift which would produce a similar reduction in heterozygosity. The only other natural population of fishes that have been studied that approached the degree of homozygosity of the red-band trout are the cave dwelling fish of Mexico. Astyanax mexicanus (Avise and Selander, 1972) and some runs of Pacific salmon (Utter et al., 1973). The explanation given for the low variability in the cave fish was stochastic processes (random genetic drift), in that the population densities ranged from 200 to 500 individuals. In the case of Three-Mile Creek, the population density was estimated to be near 10,000 fish and over 200,000 fish for Bridge Creek--surely large enough to rule out the effects of stochastic processes.

The red-band trout cannot be considered the result of a small founder stock. The present day populations are the remains of a once large population that dwindled gradually until approximately 4,000 years ago when they probably started a gradual increase again. The low variability could be explained as the result of the populations passing through a "bottleneck" when temperatures in the Great Basin

reached a maximum and, moisture a minimum approximately 4-8,000 years ago. The red-band trout populations could have been reduced to very small numbers causing the loss of many alleles. Selection pressure has probably been so intense and removed variation introduced by mutation. Other examples postulated as resulting from random drift, founder populations or bottlenecking have been reported (Avise and Selander, 1972 and Webster _et al._, 1972).

In light of the nearly identical homozygosity in the two widely separate populations of red-band trout, stochastic processes are not thought to have been the overriding factor. Stochastic processes should lead to different results in the two populations. It may be argued that results from two populations are not enough to rule out stochastic processes and that by chance alone, I could have chosen two populations with nearly identical gene pools. Because of the present-day population size and similar homozygosity, I conclude that selection pressure was the dominant factor producing the small amount of genetic variation. Intense selection can easily over-ride the effects of small population size. Even in cases of balanced polymorphism (overdominance), Robertson (1962) and Nassar (1970) have shown theoretically that when the equilibrium gene frequency ($\hat{p}$) reaches extreme values, fixation and loss are accelerated. Overdominance is an extremely efficient method of retaining variability within a

population when $\hat{p} > 0.2$ or $\hat{p} < 0.8$. When $\hat{p} < 0.2$ or $\hat{p} > 0.8$, overdominance will not resist drift, but accelerates fixation or loss.

The present status of the populations seems to be good in spite of the lack of variability. The red-band trout populations are well adapted to their environment and are maintaining a population size apparently large enough to rule out effects of inbreeding. What of their future? Geographical isolates have three fates according to Mayr (1963). Without genetic variability maintained in the population, they lack the ability to adjust genetically to changes in the environment and face extinction. This is not inevitable for there are a number of examples in nature of small isolates that are specifically adapted to their particular environment and have maintained themselves for long periods of time. Nevertheless, extinction is a possibility the small isolate must continually face. The second possibility is a sudden extension of their niche favoring an expansion of the population. This possibility provides a great potential for evolutionary change and speciation. A third fate of the isolate is to merge with another closely related population reestablishing gene exchange. The consequences of this is to retard differentiation and evolutionary change.

Perhaps the greatest danger facing these isolated populations of trout is man's intrusion upon their environment through activities such as stocking hatchery reared fish, spraying of insecticides

and herbicides, scouring streambeds for gravel, diversion of water for irrigation and other forms of water pollution.

Origins and Affinities of the Red-Band Trout

The use of the karyotype to determine relatedness has been criticized because chromosomal homology in different genomes cannot be determined and the mechanisms involved in the direction of change in chromosome number are not fully understood (Atchley, 1973). In the case of western North American *Salmo*, it is intuitively obvious that a fair degree of homology exists between the genetic material of most forms because of the ease of hybridization between them with little or no loss of viability in the F_1 generation. Two methods were attempted by which homology between karyotypes could be established. The first was a search for marker chromosomes--chromosomes with a distinctive morphological shape that could be recognized from one karyotype to another. Two such chromosome types were found in the red-band karyotype--a metacentric pair with satellites, and an acrocentric pair with short second arms (submetacentrics). Two such pairs of chromosomes out of many chromosomes are most likely unreliable evidence on which to base homology.

The metacentric pair with satellites appears common to most, if not all, western North American *Salmo*. My own work shows this pair to be in the karyotypes of the red-band trout, *S. gairdneri*,

S. aguabonita, S. clarki clarki, S. clarki henshawi and S. clarki alvordensi. It is difficult to verify the existence of this pair in the karyotype for S. apache described by Miller (1972). It is difficult to see the satellites with any regularity except in very clean and sharp preparations. I have not observed any karyotypes of S. clarki lewisi except those from blastula where these satellites do not seem to show up in any species. The presence of this pair in the karyotype of many species of Salmo establishes its stability and importance and its absence in any group would indicate a major divergence from the main stream of Salmo.

The pair of sub-metacentric chromosomes was found in the red-band trout, S. gairdneri, S. clarki henshawi and S. aguabonita, but not in the S. clarki alvordensi. Simon (1963) also found this pair in S. gairdneri and S. clarki lewisi. Miller's (1972) karyotype for S. apache does not show this pair of chromosomes. It cannot be stated with certainty that this pair of chromosomes is homologous in every species where it shows up, but they are all approximately the same size.

The second method whereby homology between karyotypes could be established was the chromosome banding technique mentioned previously. Unfortunately, I could not obtain consistent results with any method, or any variation of those methods. Faint banding was attained in some instances and I believe usable results are possible

with further work.

Generalized inferences only can be made about the direction of change in chromosome number. Within Salmoninae, species with higher chromosome numbers appear to be more primitive (Simon, 1964). The rational behind equating specialization with a reduction in chromosome number is that an increase in linkage of adaptive gene complexes on the same chromosome assures non-random segregation. Classical cytogenetics has held that it is possible for two acrocentric chromosomes to join by centric fusion, forming one metacentric and reducing the haploid number by one. On the other hand, metacentrics are supposed to break on either side of the centromere resulting in one piece without a centromere which is therefore lost, causing a decline in arm number. The loss of such a large mass of genetic material would most likely be lethal. Accordingly, it is generally accepted that only events which reduce, rather than increase chromosome number can be tolerated.

I do not believe the above scheme is necessarily applicable in salmonids. The work of Ohno *et al.* (1965) indicates the apparent ease with which the chromosome arms of *S. irideus* (*S. gairdneri*) can dissociate and reunite. This happens when metacentrics are actually splitting within the centromere producing two functional acrocentrics. Meiosis in most *Salmo* species produces complex configurations that could only result from large scale arm rearrangements by

both centric fusion and splitting of metacentrics within the centromeres. At the same time, few, if any, unbalanced gametes are formed. If acentric chromosomes were produced, they would be lost in segregation and produce many unbalanced gametes. It is not uncommon to get better than 90% survival of salmonid progeny under hatchery conditions.

Chromosome numbers within and between the genera of Salmoninae seem to support an argument for reduced chromosome numbers in the more advanced forms (Simon, 1964). Table 3 lists the chromosome numbers for the western North American _Salmo_. Within the genus _Salmo_, the picture is not clear and only a very general statement can be made. _S. clarki_ is considered by most authors to be the most primitive form based on distribution and the presence of basibranchial teeth. Based on the same two factors, this would make _S. gairdneri_ the most recent or specialized form. Coastal and inland _clarki_ have higher chromosome numbers (2n=70 and 2n=64 respectively) and _S. gairdneri_ has 2n=60. While the other forms of _Salmo_ appear to be intermediate between _clarki_ and _gairdneri_, there is not a straight line reduction in chromosome numbers.

All species of _Salmo_ appear to possess the metacentric pair of chromosomes with satellites. The sub-metacentric pair is found in all except _S. apache_ and the _S. clarki alvordensi_. The karyotype

Table 3. Chromosome numbers for some western North American Salmo.

Species	2N	M	MS	SM	A	Arm No.	Reference*
S. clarki clarki Coastal cutthroat	70	36	2	34	0	106	2, 6
S. clarki lewisi Inland cutthroat	64	38?	2?	2?	22	106	2, 3
S. clarki ? alvordensi Alvord cutthroat	64	38	2	0	24	104	1
S. gairdneri Rainbow trout (including winter steelhead)	60	42	2	2	14	104	2, 3, 5, 6
S. aguabonita Golden trout	58	44	2	2	10	104	1, 4
S. species Red-band trout	58	44	2	2	10	104	1, 4
Deschutes River summer steelhead and resident trout, Siletz River summer steelhead and Clear-water River, Idaho summer steelhead	58	44	2	2	10	104	1
S. apache Apache trout	56	50?	0?	0?	6?	106	4

[a]Metacentrics [b]Metacentrics with satellites [c]Sub-metacentrics [d]Acrocentrics

*Authors have not classified the chromosomes as in this study.

REFERENCES

1 This study
2 Simon, 1964
3 Simon and Dollar, 1963
4 Miller, 1972
5 Ohno et al., 1965
6 Wilmot, Unpubl. data

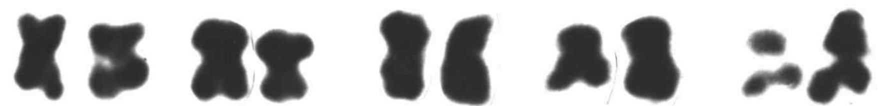
of the red-band trout could easily be generated from the S. gairdneri karyotype by centric fusion of four acrocentrics to form two metacentrics. The reverse procedure is also possible. The red-band trout karyotype could also be formed from the S. clarki lewisi karyotype by a two step procedure outlined by Simon and Dollar (1965) for the evolution of S. gairdneri from S. clarki lewisi. This process involves centric fusion of four acrocentrics and inversion in a metacentric converting it to a large acrocentric. These changes are postulated to take place in the haploid state and result in an increase of one metacentric, from 21 to 22, and a decrease of three acrocentrics, from 11 to 8--the haploid karyotype of S. gairdneri. A large pair of acrocentrics that could have been formed from metacentrics does exist in the karyotype of S. gairdneri. The reverse procedure, the evolution of the gairdneri karyotype to the clarki lewisi karyotype, is not considered a likely possibility in that it would require an increase in arm number.

When this study was first initiated, the red-band trout was assumed to have a unique karyotype and a distribution limited to a few small streams in southeastern Oregon. Subsequent karyotyping of S. aguabonita in this study and by Miller (1972) has shown it to possess a chromosome complement identical to the red-band trout. The karyotype of S. aguabonita, obtained from a brood stock maintained by the National Marine Fisheries Service, Montlake Biological

Figure 7. Somatic chromosomes from kidney of *Salmo aguabonita*.

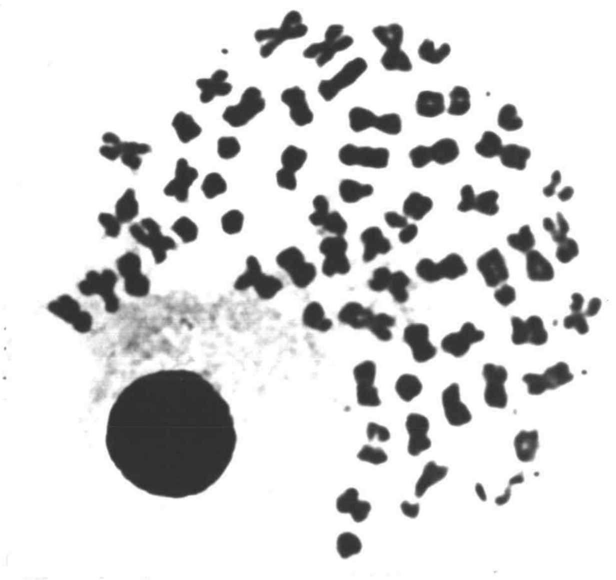
1 2 3 4 5 6 7 8 9

10 11 12 13 14 15 16 17 18



19 20 21 22 23

24 25 26 27 28 29



Laboratories, Seattle, is shown in Fig. 7. This stock came from Wyoming and is assumed to have originated in California (James Mighell, personal communication). The golden trout karyotype is identical to the red-band trout (i.e. 2n=58 consisting of 44 metacentrics, 2 metacentrics with satellites, 2 sub-metacentrics and 10 acrocentrics to give 104 chromosome arms). A consistent count could not be obtained from golden trout from Soda Springs Creek in California but varied between 2n=58 and 2n=60 indicating hybridization with known introductions of S. gairdneri.

Anadromous and resident trout from the Deschutes River in central Oregon were also karyotyped. The resident trout of the Deschutes River are known commonly as the "Deschutes Red-Sides." The karyotype of both forms of Deschutes River trout were identical to the red-band and golden trout karyotypes. Chromosome preparations were made from kidney squashes and leukocyte cultures from six resident trout and eight anadromous trout. These results are not too surprising considering an old river channel runs from the Deschutes River watershed to the Fort Rock Basin, which in turn was connected to the Silver Lake Basin. This old river channel is now buried in places by ancient lava flows.

The results from the Deschutes River led to a check of summer-run steelhead trout from the Siletz River on the Oregon coast and from the Clearwater River in Idaho. Time precluded a thorough survey and

only one specimen from each location was karyotyped. Both specimens possessed karyotypes identical to the red-band trout. Winter steelhead trout from the Siletz and Alsea Rivers display a normal *S. gairdneri* karyotype (i. e. 2n=60 consisting of 42 metacentrics, 2 metacentrics with satellites, 2 sub-metacentrics and 14 acrocentrics. This karyotype has been reported for *S. gairdneri* in other studies (Bungenberg de Jong, 1955; Simon, 1964 and Ohno *et al.*, 1965), but they did not use the same chromosome classification.

This study has shown the red-band trout to be a widespread group with a common karyotype, rather than a small relict population. It gives evidence supporting the idea of a golden trout complex proposed by Schreck and Behnke (1971) and Legendre *et al.* (1972) composed of the red-band trout, California golden trout, Kern River trout, Gila trout and possibly the Mexican golden trout and the Apache trout. The latter study was a computer taxonomy investigation of western North American *Salmo* that linked the Gila trout, Kern River trout, Apache trout and the red-band trout. The next closest link was to *S. aguabonita* and then to *S. clarki*. The links of *S. gairdneri* and the Mexican golden trout were more remote. These investigators interpret this data to propose a cutthroat like progenitor for the golden trout complex. The chromosome data neither confirms nor denies this belief, but as was discussed previously the karyotypes of the golden trout and the red-band trout are much easier to generate

from S. gairdneri than from S. clarki.

My own speculations on the origins of this golden trout complex favor a rainbow type progenitor and I propose the following broad scheme for western North American Salmo. The original salmonid type invaded western North America migrating all the way to the east slope of the rockies. Subsequent geological forces isolated an inland form from those left on the Pacific coast--i.e. inland cutthroat and coastal cutthroat. A latter invasion from Asia of a rainbow type invaded the Pacific coast of North America sometime just before or during the Pleistocene glaciation and was able to migrate inland only where connections existed with the Pacific Ocean. This latter invasion then gave rise to the S. gairdneri and the golden trout complex. The golden trout complex being the only ones to establish freshwater resident populations.

The occurrence of the 2n=58 karyotype in the Columbia River drainage, as well as on the Pacific coast, indicates the presence of three groups of Salmo along the Pacific coast, rather than two groups. Historically, it has been believed that only S. gairdneri and S. clarki clarki inhabited this area, but two groups of gairdneri have been discussed for years. Schutz (1935) discussed the characteristics of a fine scaled race of anadromous rainbows in the Columbia River, and Behnke (1965) found rainbow in the Columbia and Frazier rivers possessing 10 to 20 more scales in the lateral line than coastal

rainbows. Many populations had bright coloration and slight cutthroat marks. All of these characteristics are features used to distinguish the red-band trout from gairdneri. Behnke further reports that Livingston Stone, a fish culturist for the U. S. Fish Commission in the 1880's, found two forms of trout in the McCloud Rivers system of California. One was a fine scaled trout found in tributary streams and the other, a large coarse scaled trout found in the main river. Jordan and Henshaw (1878) and Wales (1939) also made reference to these two groups in the McCloud River. A similar situation was noted by Snyder (1908) in the Pit River and Burney Creek in California. Dymond (1932) described a form of gairdneri from the Selkirk Mountains of British Columbia displaying coloration and markings very similar to populations of the headwaters of the Pit and McCloud rivers.

I believe that two races, or species, occur sympatrically along the Pacific coast. The rainbow or steelhead group (gairdneri, 2n=60) is the coarse scaled trout that enters freshwater in December and January to spawn soon after. The fine scaled group (summer-run steelhead and resident trout, 2n=58) enter freshwater in March and April and hold there until the following spring to spawn. Most likely, man's intrusion has caused interbreeding in many locations. This hypothesis should be easily resolved with a thorough karyotyping of trout along the Pacific coast of North America. An understanding

Figure 8. Somatic chromosomes from kidney of *Salmo clarki alvordensi* Whitehorse Creek, Malheur County, Oregon.

1

2

3

4

5

6

7

8

9

10

11

12

13

14

15

16

17

18

19

20

21

22

23

24

25

26

27

28

29

30

31

32

of the origins and relationships of these two groups may be facilitated by a study of populations above and below barrier falls existing on a number of coastal streams.

Two specimens *S. clarki alvordensi* were karyotyped from Whitehorse Creek in the Alvord Basin. Chromosome preparations were made only from kidney and display a diploid number of 2n=64 consisting of 38 metacentrics, 2 metacentrics with satellites, and 24 acrocentrics (Fig. 8). The pair of sub-metacentrics does not appear in this karyotype. The total arm number is 104, which is the same as *gairdneri* and the red-band trout. Hubbs and Miller (1948) believed the Alvord Basin has been isolated since Prepluvial times when it apparently was connected with the Lahontan Basin through the Thousand Creek system of Nevada. Lahontan cutthroat trout, *S. clarki henshawi*, could have invaded the Alvord Basin at this time and evolved into a distinctive form in the long interval. The existence of the Alvord trout karyotype adds more complexity to the evolutionary history of *Salmo* and points up the need of a through karyotyping of western North American *Salmo*. A detailed picture of the distribution of karyotypes may help us to clarify the evolutionary history of *Salmo*.

CONCLUSIONS

The chromosome and distribution data lead me to believe that the red-band trout is part of a widespread golden trout complex originating from a rainbow trout type progenitor that includes the California golden trout, the Kern River trout and the red-band. The Mexican golden trout and Gila trout may also belong to this group, but without more information I would not speculate on their affinities. If my hypothesis is correct, this complex (2n=58) will be found in the drainages flowing into the Pacific Ocean, from British Columbia south to at least the Kern River drainage in California.

If the red-band trout is as widespread as this study suggests, then we may not be in danger of extinguishing this species. On the other hand, these isolated populations are well adapted to environmental conditions that other populations may not tolerate. I have seen hatchery fish that were stocked in small streams of southeastern Oregon die in late summer when stream flows are greatly reduced, and water temperatures are very high. The red-band trout survives, not only under these conditions, but under circumstances that most likely were more severe in the past. We could very well lose a unique and beautiful creature that could not be replaced.

LITERATURE CITED

Antev, E. 1938. Rainfall and tree growth in the Great Basin. Carnegie Inst. Wash. Publ. 469.

Atchley, W. R. 1972. The chromosome karyotype in estimation of lineage relationships. Syst. Zool. 21:199-209.

Avise, J. C. and R. K. Selander. 1972. Evolutionary genetics of cave-dwelling fishes of the genus Astyanax. Evol. 26:1-19.

Bailey, G. S. and A. C. Wilson. 1968. Homologies between isoenzymes of fishes and those of higher vertebrates. J. Biol. Chem. 243:5843-5853.

Bailey, G. S., G. T. Cocks and A. C. Wilson. 1969. Gene duplication in fishes: Malate dehydrogenases in salmon and trout. Biochem. Biophys. Res. Comm. 34:605-612.

Behnke, R. J. 1965. A systematic study of the family Salmonidae with special reference to the genus Salmo. Unpubl. Ph. D. Thesis. Univ. Calif., Berkeley. 273 pp.

____________ 1970. The application of cytogenetic and biochemical systematics to phylogenetic problems in the family Salmonidae. Trans. Am. Fish. Soc. 99:237-248.

Blackwelder, E. 1948. The Great Basin, with emphasis on glacial and postglacial times. Bull. Univ. Utah, Biol. Ser. 10(7):3-16.

Bond, C. E. 1961. Keys to Oregon freshwater fishes. Ore. St. Univ. Ag. Exp. Stat., Tech. Bull. 58:42 pp.

Bungenberg de Jong, C. M. 1955. Cytological studies on Salmo irideus. Genetica 27:472-483.

Casperson, T., L. Zech and C. Johansson. 1970. Analysis of human metaphase chromosome set by aid of DNA-binding agents. Exp. Cell Res. 62:490-492.

Cope, E. D. 1889. The Silver Lake of Oregon and its region. Am. Nat. 23:970-982.

Drets, M. E. and M. W. Shaw. 1971. Specific banding patterns of human chromosomes. Proc. Nat. Acad. Sci. 68:2073-2077.

Dymond, J. R. 1932. The trout and other game fishes of British Columbia. Biol. Bd. Canada 32:51 pp.

Fryer, J. L., A. Yusha and K. S. Pilcher. 1965. The in vitro cultivation of tissue and cells of Pacific salmon and steelhead trout. Ann. N. Y. Acad. Sci. 126:566-586.

Hansen, H. P. 1947. Postglacial forest succession, climate, and chronology of the Pacific Northwest. Trans. Am. Philos. Soc. 37:1-130.

_______________ 1961. Cycles and geochronology. Occ. Papers Calif. Acad. Sci. 31:1-24.

Heckman, J. R., F. W. Allendorf and J. E. Wright. 1971. Trout leukocytes: Growth in oxygenated cultures. Sci. 173:246-247.

Hubbs, C. L. and R. R. Miller. 1948. Correlation between fish distribution and hydrographic history in the desert basins of western United States. Bull. Univ. Utah, Biol. Ser. 10(7): 17-166.

Jordan, D. S. and H. W. Henshaw. 1878. Report upon the fishes collected during the years 1875, 1876, and 1877, in California and Nevada. Ann. Rept. U. S. Geog. Surv. w. 100th meridian (Wheeler Survey), (Append. NN of Ann. Rept. Chief of Engineers for 1878):187-200.

_______________, B. W. Evermann and H. W. Clark. 1930. Check-list of the fishes and fishlike vertebrates of North and Middle America north of the northern boundary of Venezuela and Columbia. Append. 10, Rept. U. S. Comm. Fish. for 1928, 67 Opp.

Koski, R. O. 1970. Stocking of trout and salmon by watershed, 1969. Oregon State Game Comm., Fish. Div. 49 pp.

Legendre, P., C. B. Schreck and R. J. Behnke. 1972. Taximetric analysis of selected groups of western North American Salmo with respect to phylogenetic divergences. Syst. Zool. 21:293-307.

Lomholt, B. and J. Mohr. 1971. Human karyotyping by heat-giemsa staining and comparison with fluorochrome techniques. Nat. New Biol. 234:109-110.

Lush, I. E. 1969. Polymorphism of a phosphoglucomutase isoenzyme in the herring (Clupea harengus). Comp. Biochem. Physiol. 30:391-395.

Mayr, E. 1966. Animal species and evolution. Belknap Press, Cambridge, Mass. 797 pp.

McGregor, J. F. 1970. The chromosomes of the maskinonge (Esox masquinongy). Can. J. Genet. Cytol. 12:224-229.

Miller, R. R. 1972. Classification of the native trouts of Arizona with the description of a new species, Salmo apache. Copeia 1972(3):401-422.

Nassar, R. F. 1970. Effects of selection and drift on the dynamics of finite populations IV. Gene frequency dependent selection. Theor. Appl. Genet. 40:245-250.

Ohno, S., C. Stenius, E. Faisst and M. T. Zenzes. 1965. Post-zygotic chromosomal rearrangements in rainbow trout (Salmo irrideus, Gibbons) Cytogenet. 4:117-129.

Patil, S. R., S. Merrick and H. A. Lubs. 1971. Identification of each human chromosome with a modified giemsa stain. Sci. 173:821-822.

Prakash, S., R. C. Lewontin and J. L. Hubby. 1969. A molecular approach to the study of genic heterozygosity in natural populations IV. Patterns of genic variation in central, marginal and isolated populations of Drosophila pseudoobscura. Genetics 61: 841-858.

Ridgway, G. J., S. W. Sherburne and R. D. Lewis. 1970. Polymorphism in the esterases of Atlantic herring. Trans. Am. Fish. Soc. 99:147-151.

Roberts, F. L. 1964. A chromosome study of twenty species of Centrarchidae. J. Morphol. 115:401-418.

Robertson, A. 1962. Selection for heterozygotes in small populations. Genetics 47:1291-1300.

Schnedl, W. 1971. Analysis of the human karyotype using a reassociation technique. Chromosoma 34:448-454.

Schreck, C. B. and R. J. Behnke. 1971. Trouts of the upper Kern River Basin, California, with reference to systematic and evolution of western North American Salmo. J. Fish. Res. Bd. Canada 28:987-998.

Schutz, L. P. 1935. Species of salmon and trout in the northwestern United States. Proc. Fifth Pac. Sci. Cong. 5:3777-3782.

Seabright, M. 1972. The use of proteolytic enzymes for the mapping of structural rearrangements in the chromosomes of man. Chromosoma 36:204-210.

Shaw, C. R. and R. Prasad. 1970. Starch gel electrophoresis of enzymes - a compilation of recipes. Biochem. Genet. 4:297-320.

Simon, R. C. and A. M. Dollar. 1963. Cytological aspects of speciation in two North American teleosts, Salmo gairdneri and Salmo clarki lewisi. Can. J. Genet. Cytol. 5:43-49.

Simon, R. C. 1964. Cytogenetics, relationships and evolution in Salmonidae. Unpubl. Ph. D. thesis, Univ. Wash., 72 pp.

Snyder, J. O. 1908. Relationships of the fish fauna of the lakes of southeastern Oregon. Bull. U.S. Bur. Fish. 27:69-102.

Utakoji, T. 1972. Differential staining patterns of human chromosomes treated with potassium permanganate. Nat. 239:168-169.

Utter, F. M. 1972. Phosphoglucomutase and esterase polymorphism in Pacific herring in Washington waters. In: The stock concept in Pacific Salmon, H. R. MacMillan lectures in fisheries. R.C. Simon and P. A. Larkin, eds., pp. 191-197. Instit. Animal Res. Ecol., Univ. B.C., Vancouver, B. C.

Utter, F. M., F. W. Allendorf and H. O. Hodgins. 1973. Genetic variability and relationships in Pacific salmon and related trout based on protein variations. Syst. Zool. (In press).

Wales, J. H. 1939. General report of investigations on the McCloud River drainage in 1938. Calif. Fish and Game 25:272-309.

Wang, H. C. and S. Federoff. 1972. Banding in human chromosomes treated with trypsin. Nat. New Biol. 235:52-53.

Webster, T. P., R. K. Selander and S. Y. Yang. 1972. Genetic variability and similarity in the *Anolis* lizards of Bimini. Evol. 26:523-535.

APPENDICES

APPENDIX I

PREPARATION OF MITOTIC CHROMOSOMES FROM DISPERSED KIDNEY

1. The fish were injected intramuscularly with 0.5% colchicine six to eight hours before sacrificing (0.4 ml per 10 in. fish).
2. The anterior portion of the kidney was removed and rubbed onto a fine mesh screen (78 microns) and washed through into a beaker with 0.05M KCl.
3. The dispersed cells were left in the hypotonic solution for 15 min. at room temperature.
4. The cell suspension was centrifuged for 10 min. at 200 g and the supernatant discarded.
5. Two ml of methanol:glacial acetic acid (3:1) was added without disturbing the button of cells, the tubes stoppered and refrigerated for at least 30 min. at 4°C.
6. After dispersing the cells with a pipette, the suspension was centrifuged and the supernatant discarded.
7. The cells were washed with three changes of methanol:glacial acetic acid and the cells thoroughly dispersed after each change.
8. A clean slide was dipped in chilled, distilled water, the excess water shaken off and one drop of cell suspension put on the slide and allowed to dry.

9. The slides were stained in 1:40 buffered giemsa (pH 7.0) for 5 min., rinsed in tap water and air-dried.
10. A coverslip was mounted with Permount (Fisher Scientific).

APPENDIX II

SQUASH PREPARATIONS OF MITOTIC OR MEIOTIC CHROMOSOMES

1. The fish were injected intramuscularly with 0.5% colchicine six to eight hours before sacrificing (0.4 ml per 10 in. fish).

2a. Kidney, liver, spleen or testes were removed and minced very fine in 0.05M KCl (distilled water for testes).

2b. Before the hypotonic treatment, whole gill arches were placed in 0.85% NaCl and the blood and slime removed.

3. After 15 min., the hypotonic was decanted and replaced with 50% glacial acetic acid.

4. The tissue was allowed to soften and fix for at least 15 min.

5. Small pieces of tissue were placed on a clean slide in a drop of freshly filtered 2% aceto-orcein and mascerated with forceps.

6. A coverslip was placed on top of the tissue, filter paper placed on top of the coverslip and light pressure applied with the thumb.

7. The edges of the coverslip were sealed with a mixture of 50% lanolin and 50% paraffin applied with a hot wire.

To make permanent slides, the above procedure was followed through step five except the aceto-orcein was omitted.

6a. The slides were placed momentarily into a beaker containing methanol and dry ice and the coverslip removed with a scalpel.

7a. The slides were air dried and then hydrolysed in 1N HCl at 60°C for 10 min.

8a. After washing the slides in running tap water for 30 min., they were stained in 1:40 buffered giemsa (pH 7.0) for 5 min.

APPENDIX III

MONOLAYER CELL CULTURE FOR MITOTIC CHROMOSOMES

All of the following procedures were performed aseptically and all glassware, instruments and solutions were sterilized.

1. Excized air-bladder, kidney, liver, spent ovary or developing embryos were placed in a petri dish of culture medium containing 4000 units per ml of antibiotic-antimycotic solution (Gibco).
2. The tissue was minced and placed in a 50 ml screw top bottle containing 20 ml of a 0.25% trypsin mixture and a magnetic stirring bar.
3. After stirring for 30 min. at 4° C, the liquid was discarded and replaced with fresh trypsin.
4. At the end of two hours, the liquid was decanted into centrifuge tubes and fresh trypsin added to the bottle.
5. After two more hours, the liquid was decanted and pooled with the first batch.
6. The trypsin-cell suspension was centrifuged at 200 g for 10 min. and the supernatant discarded.
7. Five ml of culture medium was added to each tube, the button of cells disrupted with a pipette and then centrifuged again. The cells were washed in this manner three times.

8. The number of cells per ml were determined with a hemocytometer and diluted to approximately 1×10^6 cells per ml with culture medium.
9. Leighton tubes containing coverslips were inoculated with 1.5 ml of cell suspension and incubated on a slant for 24 hours at 21°C.
10. After 24 hours, the culture medium was removed and replaced with fresh medium.
11. The tubes were incubated at 21°C for five to seven days, or until a complete monolayer of cells formed on the coverslip.
12. One drop of 0.05% colchicine was added four to six hours before the cells were harvested.
13. The culture medium was removed and replaced with methanol: glacial acetic acid (3:1) for 15 min.
14. The fixative was replaced with freshly filtered 2% aceto-orcein for five min., the coverslip inverted onto a slide and squashed with filter paper and thumb.
15. The edges of the coverslip were sealed with 50% lanolin and 50% paraffin applied with a hot wire.

TRYPSIN MIXTURE (0.25%)

NaCl	800.0 mg
KCl	40.0 mg
Glucose	100.0 mg
Phenol Red	10.0 mg
Trypsin	200.0 mg

Add 40 ml of antibiotic-antimycotic solution, 100X (Gibco) and make up to 100 ml with distilled water and sterilize by filtration.

MEM, MINIMUM ESSENTIAL MEDIUM (EAGLE)

Glucose	500.0 mg
NaCl	3400.0 mg
KCl	200.0 mg
$CaCl_2$	100.0 mg
$MgCl\ 6\,H_2O$	100.0 mg
$NaH_2PO_4\ 2\,H_2O$	75.0 mg
$NaHCO_3$	1000.0 mg
Phenol Red	5.0 mg
TC-Amino Acids Minimal Eagle, 50X (Difco)	10.0 ml
TC-Vitamins Minimal Eagle, 100X (Difco)	5.0 ml
Antibiotic-Antimycotic Solution, 100X (Gibco)	5.0 ml

Make up to 444 ml with distilled water and sterilize by filtration. Then add 56 ml of fetal calf serum to make a total of 500 ml.

APPENDIX IV

LEUKOCYTE CULTURE FOR MITOTIC CHROMOSOMES

1. Blood was withdrawn by heart puncture into a syringe containing 0.1 ml heparin (1000 units per ml), the needle capped and allowed to set inverted at room temperature for one hour.
2. The needle was bent at a right angle with the cap and four to five drops of the leukocyte-plasma suspension added to each tube of culture medium.
3. The tubes were incubated five days at 21° C with the caps loose to allow the CO_2 to escape.
4. One drop of 0.05% colchicine was added six to eight hours before harvesting the cells.
5. Proceed as in steps three through ten in Appendix I.

<u>Culture Medium</u>

MEM, Minimum Essential Medium (Eagle) for suspension cultures (Gibco) with Spinner Salts with L-Glutamine without Sodium Bicarbonate	10.7 g/liter
Antibiotic-Antimycotic Solution, 100X (Gibco)	10.0 ml
Heparin	100 units/ml

Sterilize by filtration and add fetal calf serum to make a 20% solution. Dispense 4 ml per 10 ml screw cap tubes and add one drop of phytohemaglutinin to each tube. Store frozen until use. Phytohemaglutinin is not necessary if the concentration of leukocytes is high. The exact concentration has not been determined.

Preparation of the Phytohemaglutinin

1. Five grams of red kidney beans (*Phaseolus vulgaris*) were washed in water and then ground in 50 ml of normal saline.
2. The mixture was then set in the refrigerator overnight at 4° C.
3. The mixture was centrifuged at 15, 000 g for 20 min., the supernatant sterilized by filtration and stored frozen.

APPENDIX V

STARCH-GEL ENZYME ELECTROPHORESIS SYSTEM
(Ridgeway *et al.*, 1970)

1. A 12% gel was made with hydrolysed potato starch (Connaught Lab.).
2. The starch was mixed thoroughly with gel buffer in a vacuum flask and heated over a bunsen burner with constant stirring to obtain a clear, viscus mixture.
3. After degassing, the gel was poured into a glass mold and allowed to cool to room temperature.
4. The gel was cooled to 4° C and one side of the glass mold removed.
5. Filter paper wicks saturated with sample were inserted in a slit cut approximately 5 cm. from the cathode end.
6. Electrophoresis was performed at 4° C at 300 volts for 10 min., the filter paper wicks removed and the run continued until the ion front had moved approximately 9 cm. past the slit (3 to 4 hours).
7. The gel was cut into three slabs with monofilament fish line and each slab stained for a separate enzyme.

Electrode Buffer

0.06M LiOH
0.30M H_3BO_3

Gel Buffer

0.03M Tris
0.005M Citric Acid
One ml of electrode buffer per 100 ml of gel buffer.

APPENDIX VI

STARCH-GEL ENZYME ELECTROPHORESIS SYSTEM FOR MALATE DEHYDROGENASE
(Bailey and Wilson, 1968)

This system makes a pH 6.0 gel and is prepared in the same manner as listed in Appendix V. Gels are run 24 hours at 4° C at 300 volts, sliced in half and stained for MDH activity.

Electrode Buffer

0.2M Citric Acid	90 ml
0.2M Disodium Phosphate	320 ml
Distilled Water	1870 ml

Gel Buffer

Dilute the electrode buffer 3:10 with distilled water.