UNIVERSITY OF ALBERTA

A Molecular Study of Possible Introgressive Hybridization between Westslope Cutthroat Trout and Rainbow Trout

by

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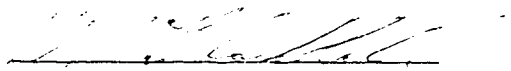

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled 'A Molecular Study of Possible Introgressive Hybridization between Westslope Cutthroat Trout and Rainbow Trout' submitted by William J. Blackhall in partial fulfillment of the requirements for the degree of Master of Science in Zoology.

C. Strobeck, supervisor

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ABSTRACT

Conflicting evidence from molecular studies has raised questions concerning the phylogenetic relationships among some subspecies of cutthroat trout (*Oncorhynchus clarki*) and the rainbow trout (*O. mykiss*). The phylogenetics of these taxa is examined by molecular techniques to find evidence of a hybrid origin of the westslope cutthroat trout (*O. c. lewisi*) or of introgressive hybridization with rainbow trout. DNA sequence data from intron 4 of the two genes coding for growth hormone were collected from westslope cutthroat trout, Yellowstone cutthroat trout (*O. c. bouvieri*), rainbow trout (one gene only), coho salmon (*O. kisutch*), and chinook salmon (*O. tschawytscha*). Intron 4 of both loci from all taxa except the rainbow trout was amplified using the polymerase chain reaction, cloned into the bacteriophage vector M13, and sequenced. The intron from rainbow trout was sequenced directly from the amplification product of the polymerase chain reaction. Phylogenetic relationships were determined by both cladistic and phenetic analyses. No clear evidence of a hybrid origin of westslope cutthroat trout nor of introgressive hybridization between westslope cutthroat trout and rainbow trout was found. The two loci of the gene coding for growth hormone, a result of gene duplication from a tetraploidization event, were found to be evolving independently. The establishment of disomic inheritance of the two loci appears to predate the divergence of *Salmo* and *Oncorhynchus*. The two loci also appear to be evolving at different rates in different lineages. Both cladistic and phenetic analyses from this study indicate that the rainbow trout is

a sister species of the cutthroat trout, rather than of the Pacific salmons as suggested by morphological data.

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INTRODUCTION

Studies of protein electromorphs by Leary *et al.* (1985; 1987) found that westslope cutthroat trout (*Oncorhynchus clarki lewisi*) were genetically more similar to rainbow trout (*O. mykiss*) than to Yellowstone cutthroat trout (*O. c. bouvieri*). A preliminary study of a gene coding for growth hormone conducted in our laboratory found a restriction site shared by westslope cutthroat trout and rainbow trout but not by Yellowstone cutthroat trout nor the Atlantic salmon (*Salmo salar*). These findings raised questions about the phylogeny and possibly the origin of westslope cutthroat trout. This study is an examination of the phylogenetic relationships of these taxa to determine if westslope cutthroat trout, through hybridization, is a sister taxon of rainbow trout.

Hybridization in Fishes

The production of new taxa by the hybridization of existing taxa has important evolutionary consequences. Hybridization between taxa can theoretically produce new taxa in two ways: instantaneously, and by introgression, where backcrossing with either or both parental taxa follows the initial hybridization event. In both cases, reproductive isolation must arise genetically, behaviourally, ecologically, or geographically (Dobzhansky *et al.* 1977) to maintain the hybrids' taxonomic status.

Speciation by hybridization and the incorporation of genetic variation by introgressive hybridization is more common in plants than

in animals (Mayr 1963 1970; White 1978; Grant 1981) and is believed to be more common in fishes than in other vertebrates (Lagler *et al.* 1977· Campton 1987). The higher frequency of hybridization in fishes may be due to external fertilization, weak behavioural isolating mechanisms, competition for limited spawning habitat, and the frequency of secondary contact between recently evolved forms (Campton 1987). Secondary contact, when taxa that were once separated are brought together, may be more common in northern latitudes where ecological, climatic, geological, and glacial disturbances are more extreme (Hubbs 1955; Campton 1987). In recent times, secondary contact has increased due to man-made environmental disturbances and to the widespread introduction of nonnative fishes (Hubbs 1955; Campton 1987; Behnke 1992).

Most proposed hybrid taxa in fishes are unisexuals (reviewed in Dawley 1989; Vrijenhoek *et al.* 1989), although the taxonomic status of unisexuals is under debate. Among bisexual species, a few suspected hybrid taxa have been studied (Smith 1966; Menzel 1977; Echelle and Echelle 1978; Stauffer *et al.* 1979; Smith *et al.* 1983; DeMarais *et al.* 1992), but most have been questioned or refuted (Crabtree and Bluth 1987; Echelle *et al.* 1987; Meagher and Dowling 1991).

Introgressive hybridization in fishes not leading to the production of new taxa is more common (see Smith 1992 for examples). Verspoor and Hammar (1991) believe that introgression, where it occurs, may be more important than mutation as a source of new genetic variation, which may become incorporated by genetic drift or selection (Barton and Hewitt 1985). If a population or species incorporates selectively important novel genes, its adaptive niche and thus its evolution may change (Verspoor and Hammar 1991).

The occurrence of hybridization in fishes and the viability of the offspring are proportional to the genetic similarity of the parents (Hubbs 1955; Hubbs and Drewry 1959). Taxa separated for 2-5 million years or longer retain the ability to hybridize, even when possessing marked morphological differences (Smith 1992). In the family Salmonidae, hybridization between naturally sympatric taxa is rare due to ecological and behavioural isolating mechanisms (Behnke 1972). Where distributions have been altered through introductions or man-made environmental disturbances, however, hybridization has become common (Campton 1987; Behnke 1992). Artificial-breeding experiments show that intrageneric and even some intergeneric crosses in salmonids produce viable offspring (see for example Chevassus 1979). Also, salmonids are autotetraploids (Ohno *et al.* 1969; Ohno 1970; Allendorf and Thorgaard 1984), and survival of hybrids is often enhanced in polyploids (Scheerer and Thorgaard 1983; Gyllensten *et al.* 1985) due to the redundancy of duplicated genes.

Many studies have documented natural hybridization among the species of *Salmo* and the species of *Oncorhynchus*. These include hybridization between Atlantic salmon and brown trout (*Salmo trutta*) (Payne *et al.* 1972; Solomon and Child 1978; Beland *et al.* 1981; Semenova and Slyn'ko 1988; Verspoor 1988; Garcia de Leaniz and Verspoor 1989; Hurrell and Price 1991; Jansson *et al.* 1991), coho salmon and chinook salmon (Bartley *et al.* 1990), pink salmon (*O. gorbuscha*) and chum salmon (*O. keta*) (Hunter 1949; Smith 1992), rainbow trout and cutthroat trout (Schreck and Behnke 1971; Allendorf and Phelps 1981; Busack and Gall 1981; Williams and Bond 1983; Leary *et al.* 1984; Campton and Utter 1985; Martin *et al.* 1985; Marnell *et al.* 1987; Allendorf and Leary 1988;

Bartley and Gall 1991), rainbow trout and Gila (*O. gilae gilae*) or Apache (*O. g. apache*) trout (Rinne and Minckley 1985; Loudenslager *et al.* 1986; Carmichael *et al.* 1993), cutthroat trout and Apache trout (Carmichael *et al.* 1993), Apache trout and Gila trout (Miller 1972), various subspecies of rainbow trout (Schreck and Behnke 1971; Allendorf *et al.* 1980; Campton and Johnston 1985), and various subspecies of cutthroat trout (Gyllensten *et al.* 1985; Marnell *et al.* 1987).

Systematics of Oncorhynchus

The family Salmonidae consists of three subfamilies distributed throughout the northern hemisphere: Coregoninae (whitefishes), Thymallinae (graylings), and Salmoninae (lenok, mekous, belvica, huchen, taimen, chars, salmons, and trouts) (Norden 1961; Sanford 1990). The genus *Oncorhynchus* comprises the Pacific salmons (six extant species) and Pacific trouts (four extant species). The terms "salmon" and "trout" historically refer to differing life histories and not to phylogenetic relationships (Stearley and Smith 1993). Both groups spawn in freshwater, but, generally, salmon spend part of their lives in the sea whereas trout do not. However, some salmon do not go to sea, and some trout do. Also, salmon tend to spawn only once, whereas trout spawn more than once. Until 1989, systematists classified the Pacific trouts in the genus *Salmo*, which included the Atlantic salmon and the brown trout. Smith and Stearley (1989) considered the osteological and biochemical evidence and recommended that the Pacific trouts be grouped with the Pacific salmons and placed in the genus *Oncorhynchus*. This recommendation was adopted by the American Fisheries Society's Committee

on Names of Fishes (Robins *et al.* 1991). The species affected by the name change are cutthroat trout, rainbow trout, Mexican golden trout (*O. chrysogaster*), Gila trout, and Apache trout.

The systematics of *Oncorhynchus* has received much attention. Studies have used morphological (Behnke 1972; Sanford 1990; Stearley and Smith 1993), cytogenetic (Simon 1963; Behnke 1970; Gold *et al.* 1977; Loudenslager and Thorgaard 1979; Gorshkov and Gorshkova 1981; Phillips and Ihssen 1985; Hartley 1987), ontogenetic (Pavlov 1980), muscle myogen and blood protein (Tsuyuki and Roberts 1966), allozyme (Utter *et al.* 1973; Loudenslager and Kitchin 1979; Loudenslager and Gall 1980; Loudenslager *et al.* 1986; Leary *et al.* 1987), retroposon (Kido *et al.* 1991; Koishi and Okada 1991), DNA hybridization (Mednikov and Akhundov 1975), DNA restriction site (Berg and Ferris 1984; Wilson *et al.* 1985; Thomas *et al.* 1986; Gyllensten and Wilson 1987; Ginatulina *et al.* 1988; Grewe *et al.* 1990; Phillips *et al.* 1992; Shed'ko 1992), and DNA sequence (Thomas and Beckenbach 1989; McVeigh and Davidson 1991; Shedlock *et al.* 1992) data. Behnke (1988; 1992) reviewed the systematics of cutthroat and rainbow trout, recognizing 14 subspecies of cutthroat trout and 6 subspecies of rainbow trout.

Figure 1 shows the natural distributions of rainbow trout, westslope cutthroat trout, and Yellowstone cutthroat trout (from Behnke 1992). The range of rainbow trout extends from northern Mexico to Alaska, mainly west of the continental divide. Rainbow trout have crossed the divide at only two locations: into the headwaters of the Athabasca River system in Alberta and the Liard River system in British Columbia. Westslope cutthroat trout are found on both sides of the continental divide, mainly in Montana and Idaho, but also in

southeastern British Columbia, southwestern Alberta, and northwestern Wyoming. Yellowstone cutthroat trout are also found on both sides of the continental divide, mainly in Montana, Idaho, and Wyoming, but also in northeastern Nevada and northwestern Utah. Of these three taxa, only rainbow trout and westslope cutthroat trout are presently naturally sympatric, in three river drainages of Oregon and Idaho (Behnke 1988). More extensive sympatry between rainbow trout and westslope cutthroat trout, however, likely occurred in the past (Behnke 1992).

Molecular Analysis

In the past, the detection of hybridization in fishes has assumed morphological intermediacy of the hybrids. Recent studies, however, have shown that hybrids of salmonids are often not morphologically intermediate between the parental taxa (Leary *et al.* 1983; 1985). Introgressive hybridization is also difficult to detect morphologically. Introgressed individuals may possess much genetic material (up to 10%) from another taxon yet be morphologically indistinguishable from the backcrossed parent (Leary *et al.* 1984). For these reasons, the molecular detection of hybridization is justified.

Hybridization can be detected at the molecular level in three ways: protein electrophoresis, restriction fragment analysis, and DNA sequencing. Protein electrophoresis detects genetic differences by measuring the mobilities of variant proteins in an electric field. This technique can examine many loci easily, quickly, and inexpensively. It does, however, have drawbacks. Much genetic variation is not translated into proteins, many proteins are not easily acquired for study, and

amino acid substitutions that do not alter mobility of the electromorphs go undetected, making analysis unreliable.

The analysis of restriction fragment length polymorphisms (RFLPs) detects changes in the DNA itself. Any part of the genome is, in principle, amenable to RFLP analysis. This technique exploits the ability of restriction enzymes to recognize specific short sequences of nucleotides and then to cut the DNA at those sites. Mutations in the DNA can thus eliminate restriction sites and create new ones. These changes produce fragments of different lengths, which can be detected by electrophoresis, when the same region of DNA from different organisms is subjected to enzymatic digestion. However, like protein electrophoresis, RFLP analysis has drawbacks. Restriction sites are relatively rare, so much of the DNA is not examined, and much of its variation remains undetected. Also, the loss of a restriction site can result from different mutations, so identical fragment patterns may represent nonhomologous differences in the DNA. The only way to detect all genetic variation and to verify homology is to sequence the DNA.

DNA sequencing has become common in the last decade. The advent of polymerase chain reaction (PCR) technology has revolutionized DNA sequencing by allowing fast and easy isolation and amplification of specific segments of DNA. These PCR products can then be sequenced directly, or they can be cloned and then sequenced. The segment of DNA chosen for sequencing depends on the amount of differentiation between the organisms being studied. Highly conserved regions are best suited for comparisons across broad taxonomic categories. For closely related species or subspecies, highly variable regions are best. Mitochondrial DNA (mtDNA), which mutates 5-10 times faster than nuclear DNA (Brown

1985), is a common choice for phylogenetic studies of closely related taxa. Furthermore, mtDNA's maternal mode of inheritance is useful for detecting introgressive hybridization: if one taxon is found to possess the mitochondrial genome of another taxon, a case of hybridization is supported. If hybridization is unidirectional, though, with backcrossing to the maternal taxon only, hybridization can go undetected. In such a case, the paternal contribution can only be discovered by examining nuclear DNA.

Many nuclear genes in eukaryotic organisms are interrupted by noncoding regions called introns. Because introns are not translated into proteins, they are under few selective constraints and can thus accumulate nucleotide substitutions at a faster rate than the coding regions of the genes (Kimura 1983). (Introns accumulate nucleotide substitutions at a slower rate than do pseudogenes, which are under no known selective constraints, indicating that introns are selectively constrained to some degree. Known functions of introns include coding for proteins, regulator proteins, endonucleases (Lewin 1990), and enhancers (Emorine *et al.* 1983). Intron sequences are also involved in the splicing mechanism (Li *et al.* 1985).) This property makes them useful for phylogenetic studies of closely related taxa. Comparing the two loci coding for growth hormone in Atlantic salmon, the substitution rate of the introns is about 2.6 times that of the exons. I have chosen to sequence intron 4 (Figure 2a) of the two loci that code for growth hormone, for two reasons. First, intron 4 is sufficiently long (about 1100 base pairs) to be likely to harbour phylogenetically pertinent information. Second, the sequences have been published for both loci from Atlantic salmon (Johansen *et al.* 1989; Male *et al.* 1992) and for

one locus from rainbow trout (Agellon *et al.* 1988).

Growth hormone regulates somatic growth in all vertebrates (Donaldson et al. 1979) and may participate in the adaptation to seawater in salmonids (Miwa and Inui 1985). In salmonids, the hormone is a polypeptide 210 amino acids long, including a signal sequence of 22 amino acids (Male *et al.* 1992). Salmonids possess two genes coding for growth hormone, the ancestor of the family having undergone a doubling of its chromosome complement (Ohno *et al.* 1969). Both genes contain six exons interrupted by five introns (Figure 2a) (Male *et al.* 1992).

Study Objectives

Most systematic studies conclude that cutthroat and rainbow trout are distinct and sister taxa. In studies of protein electromorphs, however, Leary *et al.* (1985; 1987) found westslope cutthroat trout to be genetically more similar to rainbow trout than to Yellowstone cutthroat trout. Also, Stearley and Smith (1993) proposed rainbow trout to be a sister taxon of the Pacific salmons rather than of the cutthroat trout, contrary to common opinion. The main objective of this study is to test the hypothesis of a hybrid origin of westslope cutthroat trout or of introgressive hybridization between westslope cutthroat trout and rainbow trout by using molecular techniques. Secondarily, the data generated by this study may help to resolve to which clade--the cutthroat trouts or the Pacific salmons--the rainbow trout belongs.

The strategy used is to compare the DNA sequences of intron 4 of the growth hormone genes from westslope cutthroat trout, Yellowstone cutthroat trout, and rainbow trout. Atlantic salmon, coho salmon, and

chinook salmon serve as outgroups for the phylogenetic analysis. Evidence of a hybrid origin for westslope cutthroat trout involving rainbow trout or of introgression between rainbow trout and westslope cutthroat trout would appear as a higher frequency of shared derived characters in the sequences of these taxa relative to Yellowstone cutthroat trout.

MATERIALS AND METHODS

Samples

All samples of cutthroat trout were supplied as tissue by Tom Hurd of Parks Canada. Westslope cutthroat trout came from Marvel Lake, Banff National Park. Westslope cutthroat trout are native to Marvel Lake, but the lake has also been stocked with westslope cutthroat trout (McAllister *et al.* 1981). Yellowstone cutthroat trout came from Taylor Lake, Banff National Park. These fish are not native to Taylor Lake (see Figure 1) (McAllister *et al.* 1981). A protein electrophoretic analysis of fish from these two lakes (McAllister *et al.* 1981) confirmed the genetic purity of the two subspecies. Also, an electrophoretic analysis of the DNA of the Yellowstone cutthroat trout was identical to an analysis of samples of pure Yellowstone cutthroat trout from the Yellowstone River State Trout Hatchery, Big Timber, Montana supplied by F.W. Allendorf and R.F. Leary. These studies provided me with confidence of the genetic purity of these two subspecies. I used two fish of each subspecies for the collection of sequence data. A single blood sample of hatchery rainbow trout was supplied by Bruce Wakeford of the Department of Zoology, University of Alberta. The fish originated from Circle M Fish Farm, St. Paul, Alberta and is of mixed and uncertain ancestry. Blood samples of coho and chinook salmon were supplied by Dave Hunchak from Vancouver Island. One fish of each species was used for the collection of sequence data.

Extraction of Total DNA

For tissue samples, one-half gram of tissue was frozen in liquid nitrogen, ground to a fine powder with mortar and pestle, placed into a 1.5 ml microcentrifuge tube, and kept at -20°C until time of extraction. When ready, the tissue sample was suspended in 1 ml of phosphate-buffered saline (PBS) (see Appendix A for all unspecified solutions). For blood samples, 200 µl of whole blood were added to 1 ml of PBS in a 1.5 ml microcentrifuge tube. The tissue and blood samples were then loaded onto an Applied Biosystems GENEPURE™ 341 Nucleic Acid Purification System. To lyse the cells and digest and solubilize proteins, 3.35 ml of 2X lysis buffer (see GENEPURE manual for solution descriptions) and 0.54 ml (0.36 ml for blood samples) of 1X proteinase K were added. Three extractions, two with 6.75 ml of 70% (v/v) phenol/water/chloroform and one with 4.6 ml of chloroform, separated the nucleic acids from the rest of the cellular material. The DNA was precipitated with 225 µl of 3M sodium acetate and 7.56 ml of 100% isopropanol and then washed once with 10.88 ml of 80% ethanol. The DNA was collected on a filter which was placed into a 2 ml Nalgene cryovial™ containing 0.5-2.0 ml of 1X TE, depending on the amount of DNA collected. The resuspended DNA was then ready for amplification.

Amplification of Growth Hormone Intron 4

Intron 4 of the genes for growth hormone was amplified using the polymerase chain reaction. In preparation for cloning, two primers were constructed, each containing a *Bam*HI and a *Sal*I restriction site. CST

75 (5' ACAGGATCCAGTCGACATCAATGTGCTCATCAAG 3') anneals to the 3' end of exon IV (Figures 2b, 2c). CST 74 (5' ACAGTCGACAGGATCCTTCTTGAAGCAGGC CAGCAG 3') anneals near the 3' end of exon V of GHII. These two primers thus amplify intron 4 of GHII. To amplify intron 4 of GHI, two other primers were constructed, each containing only one restriction site. CST 106 (5' AAAGTCGACATCAATCTGCTCATCAAG 3') anneals to the homologous region of GHI as does CST 75 and contains a *SalI* restriction site. CST 112 (5' TGCGGATCCTTCTTGAAGCAGGCCAACAA 3') anneals to the homologous region of GHI as does CST 74 and contains a *Bam*HI restriction site. Replacing the 3' terminal G of CST 74 with an A in CST 112 was sufficient to selectively amplify the intron of the GHI locus (Figure 2c).

Each PCR amplification reaction contained approximately 100 ng of template DNA; 10 µl of 10X reaction buffer; 1.5 mM $MgCl_2$ (1.0 mM for GHI); 2 mM each of dATP, dCTP, dGTP, and dTTP; 20 pM of CST 75 (or CST 106 for GHI) and 20 pM of CST 74 (or CST 112 for GHI); 1 unit of *Taq* polymerase; and sterile distilled water to a final volume of 100 µl. Reactions were overlaid with 50-75 ml of mineral oil to prevent evaporation. The amplification reactions were performed on a Perkin Elmer Cetus 480 Thermal Cycler™ programmed to the following conditions: an initial 5 minute denaturing step at 94°C; 30 amplification cycles with a denaturing temperature of 94°C for 15 seconds, an annealing temperature of 56°C for 30 seconds, and an extension temperature of 72°C for 2 minutes; and a termination step at 72°C for 10 minutes. Ten microlitres of the above reactions were electrophoresed in a 1% agarose gel containing ethidium bromide (0.5 µg/ml) in 0.5X TBE running buffer, and the products visualized by illumination of the gel with UV light.

Cloning of Intron

The use of the bacteriophage vectors M13 mp18 and M13 mp19 allowed the directional cloning of PCR products. The vectors were prepared following the protocol of Sambrook *et al.* (1989). The *E. coli* strain used was DH5αF'. Approximately 10 µg each of M13 mp18 and M13 mp19 DNA were digested at 37°C with 30 units of *SalI* in 30 µl of 10X digestion buffer H and 270 µl of sterile distilled water in a 1.5 ml microcentrifuge tube. After 3 hours, the DNA was purified by adding 300 µl of phenol/chloroform, vortexing for 1 minute, and centrifuging at 13,000 rpm in a microcentrifuge for 3 minutes. The aqueous phase was transferred to a new tube, and 150 µl of 7M ammonium acetate and 600 µl of 95% ethanol were added. The solution was briefly vortexed to mix, microcentrifuged at 13,000 rpm for 10 minutes, the supernatant removed, and the DNA pellet air-dried for 10 minutes. The DNA was resuspended in 100 µl of 1X TE. To this solution were added 30 µl of digestion buffer B, 170 µl of sterile distilled water, and 50 units of *BamHI*. The second digestion proceeded for 3 hours. A phenol/chloroform extraction and ethanol precipitation was performed as described above. The pellet of DNA was resuspended in 100 µl of 1X TE.

The PCR products were prepared for cloning in a similar way. The remaining 90 µl of the PCR reaction (approximately 1 µg of DNA) was extracted with 90 µl of phenol/chloroform and precipitated with 45 µl of 7M ammonium acetate and 180 µl of 95% ethanol. The dried pellet of DNA was resuspended in 10 µl of 1X TE. The resuspended DNA was digested with 10 µl of digestion buffer H, 80 µl of sterile distilled water, and 20 units of *SalI* at 37°C for 2 hours. The DNA was extracted with 100 µl

of phenol/chloroform and precipitated with 50 µl of 7M ammonium acetate and 200 µl of 95% ethanol. The DNA was microcentrifuged, the supernatant removed, and the pellet air-dried. The dried pellet was resuspended in 10 µl of 1X TE. The second digestion, with 20 units of *Bam*HI in digestion buffer B, as well as the phenol/chloroform extraction and ethanol precipitation proceeded as described above. The dried pellet was resuspended in 10 µl of 1X TE.

Before the PCR inserts were ligated into the M13 vectors, the inserts were electrophoresed in a 1% low-melting-point (LMP) agarose gel containing ethidium bromide (0.5 µg/ml) in 1X TAE running buffer, and visualized by illumination with UV light. The bands of DNA were cut from the gel and placed in 0.5 ml microcentrifuge tubes. The tubes were heated to 65°C for 10 minutes prior to performing the ligation reactions.

The amplified intron 4 of the growth hormone locus II from both trout taxa was inserted into both M13 mp18 and M13 mp19. Locus II from both salmon taxa was inserted into M13 mp18 only. The intron of locus I from all fish except coho salmon was inserted into M13 mp18 only. The ligation reactions were as follows: 1 µl vector DNA, 5 µl insert DNA, 1 µl 10X ligase buffer, 1 µl ligase, and 2 µl H_2O for a final volume of 10 µl. These reactions were incubated overnight at 16°C and diluted 1:5 in 10X TE.

Transformations were performed using a Bio-Rad *E. coli* Pulser™ with cuvettes having a 0.2 cm gap. The applied pulse was 2.5 KV for 5 msec. One microlitre of the diluted ligation reactions was added to 80 µl of electrocompetent cells (see the Bio-Rad manual for the preparation of electrocompetent cells) and pulsed once. The cells were immediately

suspended in 1 ml of cold SOC medium and then transferred to a culture tube and placed on ice. The cells were allowed to recover by incubating in a rotary shaker at 37°C and 225 rpm for 30 minutes. After the recovery period, 10 µl of X-gal (100 mg/ml), 4 µl of IPTG (isopropyl-β-D-thiogalactpyranoside)(200 mg/ml), and 3 ml of top agar were added to the cells before plating on LB agar plates. The plates were incubated at 37°C overnight.

About 12 clear plaques, which indicate transformed cells, from each transformation were transferred to 1 ml of LB medium in a 1.5 ml microcentrifuge tube, briefly vortexed, and allowed to stand at room temperature for a minimum of 2 hours. Using 5 µl of this suspension as template, PCR reactions were performed to confirm the transformation of vectors with inserts. The products obtained from these reactions were then digested with CfoI and electrophoresed on a 6% polyacrylamide gel to confirm the identity of the insert.

Production of Single-stranded DNA (ssDNA)

Small-scale preparations of ssDNA from M13 vectors containing inserts followed the protocol of Sambrook *et al.* (1989) and yielded 5-10 µg of recombinant DNA from each clone.

Sequencing of ssDNA

All sequencing of ssDNA employed the Promega *fmol*™ DNA Sequencing System. This system uses the Sanger dideoxy chain termination method of sequencing in combination with thermal cycling using *Taq* polymerase and

a primer end-labeled with [γ-^{32}P]ATP (see the kit's manual for the protocol). The amplification reactions were conducted on a Techne PHC-2 Dri-Block® thermal cycler. An annealing temperature of 56°C and a final extension step at 70°C for 10 minutes were the only deviations from the manual's protocol. Reactions were stored at -20°C until needed.

Two sets of primers, one for each strand of DNA, were synthesized for the sequencing reactions. Each set consisted of five primers designed from the published sequence for GHII of rainbow trout (Agellon *et al.* 1988) and spaced 200-250 bases apart along the intron (Figure 2b). DNA cloned into M13 mp18 was amplified by the following primers (sequentially from the 5' end of the intron): CST 32 (5' GTGGGCATCAATGTG CTCATCAAG 3'), CST 104 (5' CCCAGCATGCTCTACTACAGGTAG 3'), CST 111 (5' GAG TTTCAGGCCACTGTATTTGGG 3'), CST 113 (5' GTGGGGGCATTACTAAAAAATGTC3'), and CST 114 (5' ACTATGCTTTCCTAGTTAGAAAGC 3'). DNA cloned into M13 mp19 was amplified by the following primers (sequentially from the 3' end of the intron): CST 36 (5' ATCCAGGCTCAGTACGCCATCCTG 3'), CST 103 (5' AGAGGCATA CGTGGTCCTACACTA 3'), CST 108 (5' CCTATACAGTCGCCCTGTAGAGGG 3'), CST 115 (5' ATAAGGCCTTATTTTGAGGTGTAGC 3'), and CST 116 (5' TGCAAAAACACAGATATTAA AAACAATCC 3').

All sequencing gels were 8% polyacrylamide denaturing gels prepared fresh using 18 ml of 5X TBE, 18 ml of 40% acrylamide, 37.8 g of urea, and 21.5 ml of distilled water. This solution was filtered through a Whatman™ No. 1 filter and degassed for 15 minutes. Polymerization was induced by adding 25 µl of Temed (N,N,N'N'-(Tetramethylethylene-diamine) and 900 µl of 10% ammonium persulphate and was allowed to proceed for 1 hour. The sequencing plates (33.5 cm X 39.5 cm) were mounted on BRL Model S2™ sequencing rigs, the buffer

chambers each filled with 450 ml of 1X TBE (diluted from the same 5X stock solution used for making the gels), and the gels prerun at 60 watts for 10-15 minutes using a Bio-Rad Power Supply Model 3000/300™ or an ISCO Electrophoresis Power Supply Model 494™. The sequencing reactions were heated to 70°C for 2 minutes prior to loading. Three microlitres of the reactions were loaded and electrophoresed for about 4 hours. A second loading of 2.5 µl was electrophoresed for another 2 hours. Gels were transferred to Whatman™ paper or a discarded X-ray film, covered with plastic wrap, and placed in an X-ray cassette. A sheet of Fuji Medical X-ray Film RX™ (35 cm X 43 cm) was laid on top of the gel and exposed overnight in a -70°C freezer. Films were developed, dried, and analyzed. I sequenced six clones each of both loci of westslope cutthroat trout, Yellowstone cutthroat trout, coho salmon, and chinook salmon. From these six clones, a consensus sequence was determined.

Sequencing of Double-stranded DNA (dsDNA)

The only dsDNA sequenced was the GHI locus of rainbow trout. PCR products were electrophoresed on a 1% agarose gel and the bands of DNA were cut out of the gel. The DNA was electroeluted from the gel in 0.5% TBE buffer at 115 volts for 30 minutes and trapped in 75 µl of 7M ammonium acetate. The ammonium acetate and 125 µl of buffer were removed and placed in a 1.5 ml microcentrifuge tube, to which 1.0 ml of 95% ethanol was added. The tube was microcentrifuged at 13,000 rpm for 20 minutes, the supernatant removed, and the pellet of DNA resuspended in 20 µl of 1X TE. Approximately 250 ng of DNA were used as template

for the sequencing reactions. The sequencing reactions were performed with the same primers as were used for ssDNA sequencing, following the protocol supplied by Applied Biosystems Inc., and were electrophoresed on an Applied Biosystems 373A DNA Sequencer™. Both strands of DNA were sequenced. The sequences were analysed using the SeqEd™ programme supplied by Applied Biosystems Inc.

Phylogenetic Analysis

The published sequences, the consensus sequences obtained from the clones, and the sequence of GHI of rainbow trout were aligned manually and were arranged for analysis into two data sets. Data Set 1 (Table 1) consists of GHI sequences followed by GHII sequences; this arrangement allows the analysis of the six taxa rather than the twelve loci. Data Set 2 consists of the GHI sequences aligned with the GHII sequences.

Cladistic analyses were performed with the computer programme PAUP 3.1.1 (Swofford 1993). Characters were unordered, reversible, and unweighted. For Data Set 2, the large number of "taxa" (12) made an exhaustive search unfeasible, so a branch-and-bound search was performed. An exhaustive search was performed on the 6 taxa of Data Set 1. Bootstrap analyses with a branch-and-bound search used 1500 replicates for Data Set 1 and 500 replicates for Data Set 2.

Phenetic analyses were performed with the computer programme package PHYLIP 3.3 (Felsenstein 1990). Corrected DNA distance values (Table 2) based on Kimura's (1980) "2-parameter" model were calculated using the programme DNAdist. A transition:transversion ratio of 1:1 was used. Kimura distance values represent the mean number of nucleotide

substitutions per site. A bootstrap analysis of Data Set 2 using 100 replicates was generated by DNAdist and further analysed by the programme Fitch, which uses the Fitch-Margoliash tree-building methods. The programme Consense was then used to create a majority-rule consensus tree. Only Data Set 2 was analysed in this manner because Data Set 1 contained too many nucleotide sites for analysis.

RESULTS

Growth Hormone Sequence Evolution

Intron 4 of both loci of the growth hormone gene was sequenced for westslope cutthroat trout, Yellowstone cutthroat trout, coho salmon, and chinook salmon. Only intron 4 of GHI was sequenced for rainbow trout. Table 1 shows the sequence data (Data Set 1) for the above taxa and loci, the published sequence data for both loci of Atlantic salmon (Male *et al.* 1992; Johansen *et al.* 1989), and the published sequence data for GHII of rainbow trout (Agellon *et al.* 1988). Table 2 shows the same data but with GHI sequences aligned with GHII sequences. This arrangement of the data aids in determining the ancestry of the characters: GHI and GHII of Atlantic salmon can, in effect, be used as two outgroups.

Within the genus *Oncorhynchus*, a total of 209 base pair changes were found at 199 sites, with 105 and 104 of the changes found in GHI and GHII, respectively. Only 19 of the 209 base pair changes, plus 3 insertion/deletion sites, were shared between taxa and, therefore, phylogenetically informative. Table 3 shows the character states and positions of the informative sites that involve base pair changes. Table 4 shows the insertion/deletion data. The three informative insertions/deletions are at positions 274-298, 1484-1491, and 1966-1967. (Unless otherwise stated, all sequence positions refer to those of Table 1.)

The overall transition:transversion ratio was 92:117, with ratios of 49:56 for GHI and 43:61 for GHII. Many small and large deletions and

insertions were found throughout the introns, with some shared between taxa and some unique to a single taxon (see Table 1). The lengths of the introns of GHI and GHII, respectively, in number of base pairs, are: Atlantic salmon, 1117 (Male *et al.* 1992) and 1176 (Johansen *et al.* 1989); coho salmon, 735 and 1087; chinook salmon, 1160 and 1118; rainbow trout, 898 and 1115 (Agellon *et al.* 1988); westslope cutthroat trout, 1116 and 1112; and Yellowstone cutthroat trout, 1040 and 1118. The first 218 base pairs of the intron in rainbow trout GHI were found to be deleted, suggesting a possible alteration in the structure and thus the function of GHI in rainbow trout.

A cladistic analysis of Data Set 2, where GHI sequences are aligned with GHII sequences, indicates that the two loci coding for growth hormone are evolving independently. Figure 3 shows a 50% majority-rule consensus tree constructed by PAUP using 500 branch-and-bound bootstrap replicates and mid-point rooting. The numbers at the nodes represent the confidence values of the branching pattern. Two main clades are apparent, each representing one locus.

The Kimura distances (Table 5) between GHI and GHII in each taxon (range 0.1029 to 0.1390) are greater than the distances within each locus between *Salmo* and *Oncorhynchus* (range 0.0705 to 0.0806 for GHI; range 0.0588 to 0.0711 for GHII). These distance values also suggest that the two loci have been diverging longer than the taxa have, assuming a constant rate of nucleotide substitution.

Table 5 also shows that the two loci are accumulating mutations at different rates in different lineages. For example, a comparison between Atlantic salmon and the *Oncorhynchus* taxa shows that the distance values for GHI are higher than those for GHII (range 0.0705 to

0.0806 for GHI; range 0.0588 to 0.0711 for GHII). Comparing coho salmon to the Pacific trouts, however, the distance values for GHII (range 0.311 to 0.383) are higher than those for GHI (range 0.204 to 0.321). Comparisons of distance values involving chinook salmon are higher for GHI in all comparisons except that with rainbow trout, where the distance value for GHII is higher.

Introgressive Hybridization

Figure 4 shows a 50% majority-rule consensus tree constructed by PAUP from Data Set 1 and the insertion/deletion data using 1500 branch-and-bound bootstrap replicates and rooted using Atlantic salmon as an outgroup. The numbers at the nodes indicate the confidence values of the branching pattern. This analysis recognizes the Pacific trouts as a monophyletic clade but cannot resolve the branching pattern within the trouts and does not recognize the Pacific salmons as a monophyletic clade.

An exhaustive search of this data set by PAUP without bootstrapping found two most parsimonious trees each requiring 259 mutational steps, five trees requiring 260 mutational steps, and two trees requiring 261 mutational steps. No trees were found requiring 262 or 263 mutational steps. One of the most parsimonious trees is identical in topology to Figure 4. The other differs only in grouping the Pacific salmons together in a monophyletic clade. A 50% majority-rule consensus tree of the nine most parsimonious trees constructed by PAUP is shown in Figure 5.

Figure 6 shows a majority-rule consensus tree constructed by the

PHYLIP programme Consense from Data Set 2 using 100 bootstrap replicates. The topology of the tree is similar to that in Figure 3. The numbers at the nodes represent the confidence values of the branching pattern. Most confidence values are lower than those in Figure 3.

Table 1 shows that rainbow trout share one derived character with westslope cutthroat trout (position 1212) and two derived characters with Yellowstone cutthroat trout (positions 2165 and 2285). The derived nature of these characters can be seen in Table 2. The synapomorphy between rainbow trout and westslope cutthroat trout is at position 38 of Table 2. Both loci of Atlantic salmon have a cytosine at this site, indicating that a cytosine is probably the ancestral state. In *Oncorhynchus*, this cytosine has changed to a thymine in GHI, except in rainbow trout where this site is missing. In GHII, the cytosine has changed to an adenine in rainbow trout and westslope cutthroat trout. The first of the two synapomorphies between rainbow trout and Yellowstone cutthroat trout is at position 1025 of Table 2. Adenine is obviously ancestral and has been changed to cytosines in GHII of these two taxa. The second synapomorphy is at position 1145 of Table 2. Thymine is ancestral and has been changed to cytosines in GHII of the two taxa. PAUP, however, recognizes a third site (position 2323 of Table 1; position 1183 of Table 2) to justify a rainbow-Yellowstone cutthroat clade (see Figure 4 and the GHII locus in Figure 3). This site has a thymine in rainbow trout GHII and a cytosine in Yellowstone cutthroat trout, with adenine being ancestral. The reason why PAUP recognizes this site as a synapomorphy is unclear. The two cutthroat trout share one derived character--a two base insertion at positions

1966-1967. The derived nature of this insertion can be clearly seen in Table 2 at positions 824 and 825.

The confidence values for the branching patterns within the trouts in Figures 3 and 4 are low. The lack of synapomorphies among the trout taxa accounts for the uncertainty in the branching pattern of the trouts. Felsenstein (1985) states that three synapomorphies, in a data set with no character conflicts, are necessary to achieve 95% confidence in a branch point.

Deletions within the data set may be obscuring the analysis and interpretation of the data. For example, the 218 base pair deletion at the beginning of GHI in rainbow trout may have eliminated synapomorphies between rainbow trout and westslope cutthroat trout at positions 15 and 80, assuming the deletion to have occurred after any possible introgression. The low number of actual and possible synapomorphies, however, renders such a conjecture unwarranted.

Position of Rainbow Trout within Oncorhynchus

The sequence data generated by this study provides good support for the lower branches of the phylogenetic trees as analysed by PAUP. All trees suggest that rainbow trout is the sister species of cutthroat trout. This finding contradicts Stearley and Smith's (1993) assertion, based on an analysis of morphological characters, that the rainbow trout is a sister species of the Pacific salmons. Of the informative sites shown in Table 3, four represent shared derived characters between rainbow trout and both subspecies of cutthroat trout (positions 488, 520, 1637, and 2333). The derived nature of these characters can be

seen in Table 2 at positions 511, 547, 491, and 1193, respectively.

In addition, three other sites that are not in Table 3 appear to represent derived characters shared by rainbow trout and both cutthroat subspecies (positions 484, 612, and 739). At the first of these sites (position 511 of Table 2), adenine appears to be ancestral but has been changed to cytosine in GHI of Atlantic salmon and to thymine in GHI of the three trout taxa. The next site (position 639 of Table 2) has an adenine as ancestral, which has been changed to a cytosine in GHI of chinook salmon and to thymine in GHI of the three trout taxa. The last site (position 770 of Table 2) has cytosine as ancestral, which has been changed to a thymine in chinook salmon and to an adenine in the three trout taxa. Three further positions (159, 784 and 899) may represent apomorphies shared by the trout taxa that have been obscured by deletions. The apparent synapomorphy between rainbow trout and the cutthroat subspecies at position 473 (see Table 3) is actually a symplesiomorphy, since adenine is ancestral (see position 500 of Table 2). Of the three informative sites in the insertion/deletion data (Table 4), two provide good evidence that rainbow trout is phylogenetically closer to the cutthroat trouts than to the Pacific salmons (positions 274-298 and 1484-1491). The derived nature of the first site (positions 274-298 of Table 2) can be seen as a deletion in GHI of the trout taxa. The second site (positions 337- 344 of Table 2) represents an eight nucleotide insertion in GHII of the trout taxa, and an independent four nucleotide insertion in GHII of Atlantic salmon. Synapomorphies involving insertions or deletions are less likely to represent homoplasies than are synapomorphies at single nucleotide sites, so these two insertion/deletion synapomorphies, along with the

above nucleotide synapomorphies, provide strong support for a rainbow-cutthroat clade.

One region of the sequence data for GHI was problematic and created uncertainties in sequence alignment. This region (positions 243-273 of both Tables 1 and 2) contains deletions of thymines and insertions of adenines. Due to the variability of this region, it was not included in the insertion/deletion data and was therefore not included in the analysis of the data. The deletion of thymines and the insertion of adenines in the Pacific salmons, however, may be viewed as support for a monophyletic clade for the Pacific salmons.

Other support for a Pacific salmon clade includes two informative sites that represent shared derived characters between the two species (positions 899 and 1198 of Table 1; positions 951 and 24 of Table 2). A chinook salmon-Pacific trout clade, however, is supported by four shared derived characters. Chinook salmon and the Pacific trout share the same characters at positions 846, 1013, 1374, and 1753. That these characters are derived can be seen from Table 2 (positions 879, 1068, 205, and 607, respectively). The resolution of the phylogenetic relationship between the Pacific salmons and the Pacific trouts must await further investigation.

DISCUSSION

Growth Hormone Sequence Evolution

Polyploidy, the possession of more than the normal diploid complement of chromosomes, is more common in the lower than the higher vertebrates (Ohno 1970). Newly arisen polyploids can give rise to new taxa. Autotetraploidy, where a genome is doubled, is rare in nature. Backcrossing with the parental taxon can occur, but since the new gametes are diploid and the parental gametes are haploid, the offspring are triploid and usually sterile due to the inability of chromosomes to form pairs during meiosis. Only when autotetraploids mate with each other can the lineage continue. However, some gametes may be missing one or more chromosomes or may contain one or more extra chromosomes due to irregular chromosome assortment during meiosis (White 1978). Thus, the loss of chromosomes could accompany the process of diploidization of a tetraploid species.(Ohno 1970). Tetraploidy is not believed to be an important factor in evolution beyond the species level, but entire tetraploid families are known (Allendorf and Thorgaard 1984).

An autotetraploidization event may have given rise to the family Salmonidae (Ohno *et al.* 1969), but estimates of the time of the event vary. Allendorf and Thorgaard (1984) suggested a conservative estimate of 25-100 million years ago based on fossil evidence. The oldest known salmonid fossil, however, dates from 40-50 million years ago (Wilson 1977). From studies of the divergence of duplicated lactate dehydrogenases, Lim *et al.* (1975) estimated the tetraploidization event to have occurred 80-100 million years ago.

Loci duplicated by autotetraploidization, however, do not begin to diverge immediately. A newly arisen autotetraploid possesses four rather than two homologous chromosomes, which join to form tetravalent elements during meiosis. Recombination within tetravalents prevents the accumulation of sequence differences between duplicated loci. Sequence divergence can only begin with the establishment of disomic inheritance, when duplicated loci are located on chromosomes that no longer pair and recombine during meiosis. This process of diploidization is still occurring in salmonids. In rainbow trout, 30% of ancestral loci show no evidence of duplicate gene expression, where one locus has been silenced or has acquired a new function; 46% are duplicated and show evidence of divergence; and 24% are isoloci, showing no evidence of divergence (Allendorf and Thorgaard 1984). The loci coding for growth hormone appear to belong to the second category.

The formation of two main clades, one for GHI loci and one for GHII loci (Figure 3), indicates that the duplicated loci coding for growth hormone have attained disomic inheritance in the study taxa. The Kimura distances (Table 5) between GHI and GHII in each taxon (range 0.1029 to 0.1390) are greater than the distances within each locus between *Salmo* and *Oncorhynchus* (range 0.0705 to 0.0806 for GHI; range 0.0588 to 0.0711 for GHII). These distance values suggest that the two loci have been diverging longer than the genera have, assuming a constant rate of nucleotide substitution, and thus that the attainment of disomic inheritance occurred prior to the split of *Salmo* and *Oncorhynchus*. The independent establishment of disomic inheritance of these loci by *Salmo* and *Oncorhynchus* after divergence, however, cannot be ruled out.

The assumption of a constant rate of substitution, though, may not be valid. If the common ancestor of *Salmo* and *Oncorhynchus* had attained disomic inheritance for the genes coding for growth hormone, a constant rate of substitution would produce equal distance values between GHI and GHII of all taxa, and equal distance values between *Salmo* and all *Oncorhynchus* taxa for each locus. These distance values are not equal, indicating that the rate of substitution differs between the two loci and between *Salmo* and *Oncorhynchus*.

The distance values between GHI and GHII range from a low of 0.1029 in Atlantic salmon to a high of 0.1390 in Yellowstone cutthroat trout. A comparison of the distance values between *Salmo* and the *Oncorhynchus* taxa for each locus shows that GHI is accumulating substitutions (range 0.0705 to 0.0806) at a faster rate than GHII (range 0.0588 to 0.0711). After a gene is duplicated, one copy may escape selective constraints and thus accumulate substitutions at a higher rate (Li *et al.* 1985). As long as a functional gene product continues to be made, one copy of the gene is free to change. The possibility that the rate of substitution is slowing in one copy, however, cannot be ruled out.

A further discrepancy in distance values is seen within *Oncorhynchus*. In all comparisons between taxa except those involving chinook salmon, the rate of substitution is higher in GHII than in GHI. In chinook salmon, GHI appears to be accumulating substitutions at a higher rate than GHII. The distance values in Table 5 may indicate that GHI is evolving faster than GHII in Atlantic salmon and chinook salmon, and GHII is evolving faster than GHI in coho salmon and the Pacific trouts. Different loci may thus be primarily responsible for the

production of growth hormone in different species. Varying rates of substitution of the two loci in different lineages will confound any phenetic analysis of the phylogenetic relationships within this group of fishes. A cladistic analysis of the sequence data may therefore be more reliable for phylogenetic purposes.

Two recent studies (Forbes *et al.* 1994; Du *et al.* 1993) have introduced an additional complication. Forbes *et al.* (1994) reported the existence of an allele of GHII in coho and chinook salmon that is sex-linked, found only in males, and thus probably resides on the Y chromosome. Du *et al.* (1993) also found a sex-linked version of GHII in male coho and chinook salmon that they have characterized as a pseudogene. The latter study found no evidence of this pseudogene in Atlantic salmon, three species of Pacific salmon (pink, sockeye, and chum salmon), and rainbow trout. These findings have important implications for molecular phylogenetic studies of salmonids, pointing out the need to fully characterize the molecules, and the fish, used in such studies.

The sequence of GHII from chinook salmon in the present study is derived from the functional GHII gene and not from the pseudogene. Intron 4 of the pseudogene in chinook salmon contains an insertion of approximately 700 base pairs (Du *et al.* 1993), and none of my clones possessed this insertion, the clones having been derived from inserts selected by size. The phylogenetic comparisons presented in this study thus represent those between homologous genes and remain valid.

Introgressive Hybridization

Cladistic analyses of both data sets found no significant evidence of a hybrid origin of the westslope cutthroat trout or of introgressive hybridization between rainbow trout and westslope cutthroat trout. A bootstrap analysis of Data Set 2 (Figure 3) could not resolve the phylogenetic relationships at the GHI locus and grouped rainbow trout with Yellowstone cutthroat trout at the GHII locus. The position of rainbow trout is questionable because the confidence value at this node is low (57%). A bootstrap analysis of Data Set 1 (Figure 4) gave similar results. The branching patterns and confidence values from these analyses represent the low number of synapomorphies within the Pacific trouts.

Two apomorphies are shared between rainbow trout and Yellowstone cutthroat trout (positions 2165 and 2285) and one apomorphy is shared between rainbow trout and westslope cutthroat trout (position 1212), all at the GHII locus. The low number of informative sites in the data afforded by intron 4 of the genes coding for growth hormone will result in a poor resolution of phylogenetic relationship.

Gyllensten and Wilson (1987) also failed to find evidence of introgression between rainbow trout and westslope cutthroat trout. Their study examined restriction fragment length polymorphisms of the mitochondrial genome of rainbow trout, westslope cutthroat trout, and Yellowstone cutthroat trout. No synapomorphies were found linking rainbow trout with either subspecies of cutthroat trout. The study by Leary *et al.* (1987) was extended by examining more loci (R.N. Williams, personal communication reported in Phillips and Pleyte 1991). The

results agreed more with the mtDNA data (Gyllensten and Wilson 1987) that found no evidence of introgression. The restriction fragment length polymorphism found by our laboratory that was shared by rainbow trout and westslope cutthroat trout but not by Yellowstone cutthroat trout was also shared by coho salmon and chinook salmon. Therefore, the RFLP is not a synapomorphy linking rainbow trout and westslope cutthroat trout. The paucity of molecular evidence of introgression between rainbow trout and westslope cutthroat trout supports the conclusion of no introgression.

Position of Rainbow Trout within Oncorhynchus

Traditional taxonomists have considered rainbow trout to be the closest sister species of cutthroat trout (reviewed in Behnke 1992). Molecular studies, including the present study, tended to support this relationship. In 1993, however, Stearley and Smith examined 119 morphological characters and found no synapomorphies linking rainbow trout and cutthroat trout. Stearley and Smith (1993) found rainbow trout to be the closest sister species of the Pacific salmons (Figure 7). Molecular and morphological evidence appeared to be in conflict.

Stearley and Smith (1993) reviewed the molecular evidence supporting a rainbow-cutthroat clade. Wilson *et al.* (1985) had found three mtDNA restriction sites shared by rainbow trout and cutthroat trout, but a subsequent study (Thomas *et al.* 1986) that included Pacific salmons showed that the salmons also shared these sites. Gyllensten and Wilson (1987) had found 19 mtDNA restriction fragments unique to rainbow and cutthroat trout. Of these 19 fragments, 13 were generated by

enzymes also used by Thomas *et al.* (1986), and with this inclusion of data from Pacific salmons, only one of the 13 fragments represented a possible synapomorphy linking the trouts. Stearley and Smith concluded that the data from these studies of mtDNA could not support a common ancestry of the trouts separate from the salmons.

Other molecular studies have examined the relationships of the Pacific trouts and Pacific salmons. The study of Thomas and Beckenbach (1989), which sequenced 2214 base pairs of mtDNA from rainbow and cutthroat trout and four species of Pacific salmon, found 21 characters shared by the trouts and salmons. The lack of an outgroup in their study, however, rendered the data unsuitable for phylogenetic purposes, because the ancestral or derived states of these characters were unknown. Phillips *et al.* (1992) used restriction enzymes to examine ribosomal DNA from rainbow trout, cutthroat trout, six species of Pacific salmon, Atlantic salmon, brown trout, six species of char, and huchen (*Hucho perryi*). The Pacific trouts shared only one restriction site not shared by the Pacific salmons, but this site was also shared by two species of char. Finally, Shedlock *et al.* (1992) sequenced the mitochondrial control region from rainbow trout, cutthroat trout, six species of Pacific salmon, Atlantic salmon, and Arctic grayling (*Thymallus arcticus*). Although the cladistc analysis from this data grouped rainbow trout with cutthroat trout, with a bootstrap confidence value of 52%, an examination of the sequences used in the analysis revealed no synapomorphies to support such a grouping. Again, molecular evidence supporting a rainbow-cutthroat clade is weak or lacking.

The present study, however, does support a rainbow-cutthroat clade. A cladistic bootstrap analysis of Data Set 2 (Figure 3) shows

that the three trout taxa form clades for both GHI and GHII, with confidence values of 93% and 94%, respectively. A cladistic bootstrap analysis of Data Set 1 (Figure 4) also supports a rainbow-cutthroat clade with a confidence value of 97%. A phenetic analysis of Data Set 2 (Figure 6) groups the three trout taxa for both GHI and GHII as well, albeit with lower confidence values. These results are in direct conflict with those of Stearley and Smith (1993).

The published molecular studies discussed above failed to provide clear evidence that rainbow trout is a sister species of cutthroat trout. These studies, however, also failed to provide evidence that rainbow trout is the closest sister species of the Pacific salmons. The possibility arises that rainbow trout is a species of fish possessing (at least some) trout genes in a salmon-like body and may itself thus represent a case of introgression. The finding by Leary *et al.* (1984) that introgression of less than 10% of genetic material from another taxon has little detectable effect on morphology is consistent with this possibility. Further studies of nuclear genes will be required to clarify this problem.

The proposed phylogeny of the taxa examined in this study is shown in Figure 8. Rainbow trout belongs in the clade that includes the two subspecies of cutthroat trout. A monophyletic clade for coho and chinook salmon is not supported, in agreement with Stearley and Smith (1993) (see Figure 7). The trichotomy within the clade formed by the trouts is due to the insufficient number of synapomorphies in my data for resolving the true phylogenetic relationships within the Pacific trouts.

Methodology

The poor resolution of the branching pattern within the trouts is due to a lack of informative sites in the data. Sequencing other introns of the genes coding for growth hormone should increase the resolution. Different genes have diverged to different extents in salmonids (Lim and Bailey 1977), so other loci may prove to be more variable and thus more informative phylogenetically.

Including other salmonid taxa would also help to improve the study. The coastal cutthroat trout is naturally sympatric, and is known to hybridize (Campton and Utter 1985), with rainbow trout. Stearley and Smith's (1993) study not only found the positioning of rainbow trout within *Oncorhynchus* to be problematic, but also that of the Mexican golden trout and the Gila and Apache trouts, compared to conventional classification (see Behnke 1992). Including these taxa could help to resolve the evolutionary history of the Pacific trouts. Including the four other species of Pacific salmon would help to determine which characters are ancestral and which are derived.

The determination that GHII is sex-linked in coho and chinook salmon (Forbes *et al.* 1994; Du *et al.* 1993) illustrates the importance of identifying the sex of the fish used in phylogenetic studies. I did not do so and was fortunate that intron 4 of the GHII pseudogene is larger than in the functional gene. Other sex-linked genes, or other segments of GHII, may not be so easily identified.

Conclusions

The sequencing of intron 4 of the two genes coding for growth hormone found no incontrovertible evidence of a hybrid origin of westslope cutthroat trout or of introgressive hybridization between westslope cutthroat trout and rainbow trout. The low degree of nucleotide substitution of the introns studied was insufficient to resolve the phylogenetic relationships of the three taxa of trout. The examination of other introns of these genes may help to clarify the relationships. Contrary to the morphological evidence, rainbow trout was found to be a sister species of cutthroat trout rather than of the Pacific salmons. The duplicated genes coding for growth hormone are evolving independently and appear to have attained disomic inheritance prior to the divergence of *Salmo* and *Oncorhynchus*. The two loci also appear to be evolving at different rates in different lineages.

Table 1: Data Set 1 -- Published and consensus sequence data of growth hormone intron 4.

Sequence data GHI:

```
                 1
Atlantic s.      GTAAAGAAAG GAGGGAGAAC AATGACCATT TGTGGTGCCA CACTTTGTGC
coho s.          .......... .......... .......... ....T..T.. ..........
chinook s.       .....T..T. ...A...... .......... ......CT.. ..........
rainbow t.       ---------- ---------- ---------- ---------- ----------
Yellowst. c.t.   .......... .......... .......... .......T.. ..........
westslope c.t.   .......... ....T..... .......... .......T.. ..........

                 51
Atlantic s.      ACTGTAAACC CCAAGGCATT TTTAACTCAA ATACTTCTAG TAAGTTGAAC
coho s.          .........T .......... .......... .......... ..........
chinook s.       .........T .......... .......... .......... ..........
rainbow t.       ---------- ---------- ---------- ---------- ----------
Yellowst. c.t.   .........T .......... .......... .......... ..........
westslope c.t.   .........T .......... .........C .......... ..........

                 101
Atlantic s.      TCAAAGTCAA TGAAAAGTCA TTATTACTTA AAATGTTTAT GTGGTACTGG
coho s.          .......... ...C..A..C .....G.... .......... ..........
chinook s.       ....G..... ......A..C .....G.... .......... ..........
rainbow t.       ---------- ---------- ---------- ---------- ----------
Yellowst. c.t.   .......... ......A..C .....G.... .......... ..........
westslope c.t.   .......... ......A..C .....G.... .......... ..........

                 151
Atlantic s.      CTCAAAACTA AATGAGAAGT GACATCAACA CAATTTTTTA AAGTTATAAC
coho s.          .......... .......... C.......TG .........T ..........
chinook s.       .......... .......... C.......TG .......... ..........
rainbow t.       ---------- ---------- ---------- ---------- ----------
Yellowst. c.t.   ........C. .......... C.......TG .......... ..........
westslope c.t.   ........C. .......... C.......TG .......... ..........

                 201
Atlantic s.      AAATTAACTT TTTATCCAGC ATGCTCTACT GCAGGTAGAT TTTTT-GGAA
coho s.          .....C...C ..-.C.A... .......... .......... ..------..
chinook s.       .....C.... ..-.C.A... .......... .......... ..------..
rainbow t.       ---------- ---------. .......... .......... G....T--..
Yellowst. c.t.   .....C.... ..-.C.A... .......... T......... .....---..
westslope c.t.   .....C.... ..-.C.A... .......... .......... .....---..

                 251
Atlantic s.      ---------- ---------- -TTGTTTTTA ACTATCTGTG TTTT-GCATG
coho s.          ATTTAAAAAA AAAA------ --......... ..G....... ....T.....
chinook s.       AAAAAAAAAA AAAAAAAAAA GAC....... .TG....... ....T.....
rainbow t.       A--------- ---------- ---------- ---------- --------..
Yellowst. c.t.   AA-------- ---------- ---------- ---------- --------..
westslope c.t.   AA-------- ---------- ---------- ---------- --------..

                 301
Atlantic s.      TACAGGACAT TGAGTGATTG ATTCATCGTA TGCTACACAA AGATATATAA
coho s.          .....A.... .........C ......TT.. .......... ..........
chinook s.       .....A.... .......... ......TT.. .......... ..........
rainbow t.       .....A.... .......... ......TT.. .......... ..........
Yellowst. c.t.   .....A.... .......... ......TT.. .....G.... ..........
westslope c.t.   .....A.... .......... ......TT.. .......... ..........
```

Table 1 (cont.):

```
                351
Atlantic s.     CATACATTTT TCAACATTTT CACAAAGATG AATAAGTTAC CAGAATTTTG
coho s.         .......G.. .....G.... .......... ..C....... ----------
chinook s.      .......G.. .....G.... ..T....... ..C....... T.........
rainbow t.      .......G.. .......... .......... ..C....... ..........
Yellowst. c.t.  .......G.. .......... .......... ..C....... ..........
westslope c.t.  .......G.. .......... .......... ..C....... ..........

                401
Atlantic s.     CAAACCCGAC TTGCAGGCCT GATGTGGCCT -TAAACTATG AGTTTCAGGC
coho s.         ---------- ---------- ---------- ---------- ----------
chinook s.      .....T.A.. .......... .......... G..T..CG.. ..........
rainbow t.      .....T.A.. .......... .......... G..T..C... ..........
Yellowst. c.t.  .....T.A.. .......... .......... G..T..C... ..........
westslope c.t.  .....T.A.. .......... .......... G..T..C... ..........

                451
Atlantic s.     CACTGTATTA GGGTACACGT ACGCCTCAAA ATACGGTCTT ATGACATATG
coho s.         ---------- ---------- ---------- ---------- ----------
chinook s.      .......... .....A.GC. .......... ...A...... ........A.
rainbow t.      .......... .....A.GC. ..A....... ...T...A.. ........A.
Yellowst. c.t.  .......... .....A.GC. ..A....... ...T...A.. ........A.
westslope c.t.  .......... .....A.GC. ..A....... ...T...A.. ........A.

                501
Atlantic s.     TAATGTATTG TTATAAAGAG TTGAATTACA ATGATAATAT TTGCCTAGGA
coho s.         ---------- ---------- ---------- ---------- ----------
chinook s.      .......... ..G....... .......CT. .......... ..........
rainbow t.      .......... ..G......C .......CT. .......... ..........
Yellowst. c.t.  .......... ..G......C .......CT. .......... ..........
westslope c.t.  .......... ..G......C .......CT. .......... ..........

                551
Atlantic s.     ATTAACTTGA AGGCCACAGG ACTGAAAATG AATGACAAC- --CATGTCTC
coho s.         ---------- ---------- ---------- ---------- ----------
chinook s.      ...C...... .......... .......... .........A GC........
rainbow t.      ...C...... .......... .......... .........A GC........
Yellowst. c.t.  ...C...... .......... .......... .........A GC........
westslope c.t.  ...C...... .......... .......... .........A GC........

                601
Atlantic s.     TGTTACTAAC AAATACAGTC ATGGGTGATA ACTACAATTC ACTCAAAAAG
coho s.         ---------- ---------- ---------- ---------- ----------
chinook s.      ...C...... .C........ .......... ......C..T ..........
rainbow t.      ...C...... .T........ .......... ......C... ..........
Yellowst. c.t.  ..AC...... .T........ .......... ......C... ..........
westslope c.t.  ...C...... .TG....... .......... ......C... ..........

                651
Atlantic s.     GCCAGGCACA CTGGGAAATG ATATTGGGGA CGTGGCTTAG TGAGGGCATT
coho s.         ---------- ---------- ---------- ---------- ----------
chinook s.      .......... .........T .....T.A.. .......... ..G.......
rainbow t.      .......... .........T .....T.A.. .......... ..G.......
Yellowst. c.t.  .......... .........T .....T.A.. .......... ..G.......
westslope c.t.  .......... .........T .....T.A.. .......... ..G.......
```

Table 1 (cont.):

```
                701
Atlantic s.     ACTAAAAAAT GTCAAGCTGA TACAACTCAA ATCTGGACCC TTCACAGGGT
coho s.         ---------- ---------- ---------- ---------- ----------
chinook s.      .......... .......... .......... ........T. A.........
rainbow t.      .......... .......... .......... ........A. A.........
Yellowst. c.t. .......... .......... .......... ........A. A.........
westslope c.t. .......... .......... .......... ........A. A.........

                751
Atlantic s.     GACTA----- ----GAGTAA TGACT----- ---------- ------AACT
coho s.         ---------- ---------- ---------- ---------- ----------
chinook s.      ..A.CTATAG GTTT...... .T...GACTA TAATATCACT TTAAGT....
rainbow t.      ....CTATAG GTTT...... .....GACTA TAACATCACT TTAAGT....
Yellowst. c.t. ....CTATAG GTTT...... .....GACTA TAACATCACT TTAAGT....
westslope c.t. ....CTATAG GTTT...... .....GACTA TAACATCACT TTAAGT....

                801
Atlantic s.     GCAGTCAGAT TCTATATATT AAGTGCAACG GGTTTCCTAA AACGTTTTGA
coho s.         ---------- ...G...... .......... .......... ..A..C....
chinook s.      .. ........ ...G...... .......... .......... ..A..G....
rainbow t.      .......... ...G..C... .......... .......... ..A..G....
Yellowst. c.t. .......... ...G...... .........A .......... ..A..G....
westslope c.t. .......... ...G...... .......... .......... ..A..G....

                851
Atlantic s.     GTAATGACAG CACATTGGGT TTTACAGTGA CATGAAAGTG AAATACCTCT
coho s.         ......G... .........G .......... ........G. ........G.
chinook s.      ......G... .........G .......... ........G. ........G.
rainbow t.      ......G... .........G .......... ........G. ........T.
Yellowst. c.t. ......G... .........G .......... ....------ ----------
westslope c.t. ......G... .........G .......... ........G. ........T.

                901
Atlantic s.     ATGCTTTCCT AGTTAGAAAG CATAGTGTA- GGACCACGTT TGCCTCTTCT
coho s.         .......... .......... .........- .........A ..........
chinook s.      .......... .......... .........A .........A ..........
rainbow t.      .......... .......... .........- .........A ..........
Yellowst. c.t. ---......... .........-- ---------- ---------- ----------
westslope c.t. .......... .......... .........- .........A ..........

                951
Atlantic s.     CAGCAGATCT TTCAGTGCTT TACATTGTGA TGGGGTAAAT AACCTCATCT
coho s.         .......... .....G.... .......... ..T.....C. G....T....
chinook s.      .......... .....G.... .......... ..T.....C. G....T....
rainbow t.      .......... .....G.... .......... ..T.....C. G....T....
Yellowst. c.t. ---------- ---------- --......... ..T.....C. G....T....
westslope c.t. .......... .....G.... .......... ..T.....C. G....T....

                1001
Atlantic s.     ATCATCACTA ATATTGACTA TATCAGTAAC ACCCCATTCA ATGACTGAAT
coho s.         ...------- -.TG...T.. .......G.. .......... ..........
chinook s.      ...------- -.CG...T.. .......G.. .......... ..........
rainbow t.      ...------- -.CG...T.. .......G.. .......... ..........
Yellowst. c.t. ...------- -.CG...T.. .......G.. .......... ..........
westslope c.t. ...------- -.CG...T.. .......G.. .......... ..........
```

Table 1 (cont.):

```
                1051
Atlantic s.     ATCAGCCCAT TCAAGGATAT TTATGCATGC GTCTTTTGCT GTGTGTGCTT
coho s.         ...GC..... .......C.. .........T .......... AC........
chinook s.      ...GC..... .......C.. ....C....T .......... AC........
rainbow t.      ...GC..... .......C.. .........T .......... AC........
Yellowst. c.t.  ...GC..-.. -.--...C.. .........T .......... AC........
westslope c.t.  ...GC..... .......C.. .........T .......... A.........

                1101
Atlantic s.     TCAGAAAGGC CCAATAAACA A\TATTGATA TGCACACATC CACCCCACCA
coho s.         .......... .......... .......... .......... ..........
chinook s.      .......... .......... .......... .......... ..........
rainbow t.      G......... .......... .......... .......... ..........
Yellowst. c.t.  .......... .......... .......... .......... .........
westslope c.t.  .......... .......... .......... .......... ..........

                1151
Atlantic s.     TGCATCTCTC TCTGTCTCCC ACAG
coho s.         .......... .......... ....
chinook s.      .......... .......... ....
rainbow t.      .......... .......... ....
Yellowst. c.t.  .......... .......... ....
westslope c.t.  .......... .......... ....
```

Sequence data GHII:

```
                1175
Atlantic s.     GTAAAGAAAG GAGGGAGAAC AATGACCATT TGTGGTGCCG CACTTTGTGC
coho s.         ....T.GT-- ---------. ...T...... .......... ..........
chinook s.      ....T.GT-- ---------. ...T...... .......... ..........
rainbow t.      ....T.GT-- ---------. .......... .......A.. .........CG
Yellowst. c.t.  ....T.GT-- ---------. ......A... ........T. ..........
westslope c.t.  ....T.GT-- ---------. .......... .......A.. ..........

                1225
Atlantic s.     ACTGTAAACC ACAAGGCATT TTTAACTCAA ATACTTCTAG TAAGTTGAAC
coho s.         G--------- --------.. .......... .......... .........G
chinook s.      .--------- --------.. .......... .......... .........G
rainbow t.      .--------- --------.. .......... .......... .........G
Yellowst. c.t.  .--------- --------.. .......... .......... ...A.....G
westslope c.t.  .--------- --------.. .......... .......... .........G

                1275
Atlantic s.     TCAGTCAATG AAAAGTCATT ATTACTTAAA ATGTCTATGT GGTACTGGCT
coho s.         .......... .......... .......CG. .......... ......A...
chinook s.      .......... .......... .......C.. .......... ..........
rainbow t.      .......... .......... .......C.. .......... ..........
Yellowst. c.t.  .......... .......... .......... -......... ..........
westslope c.t.  .......... ......A... .......C.. .......... ..........

                1325
Atlantic s.     CAAATCTAAA TGAGTCACAT TAATGCAATT TTTTTAAGTT ATAACAAATT
coho s.         .......... ....G..... C....AT... ....A..... ..........
chinook s.      .......... .......... C......... ....A..... .........G
rainbow t.      .......... .......... C......... ....A..... ....G....G
Yellowst. c.t.  .......... .......... C......... .......... .........G
westslope c.t.  .......... .......... C......... ....A..... .........G
```

Table 1 (cont.):

```
                1375
Atlantic s.     AACTTTTTAC CCAGCATGCT CTACTACAGG TATATTTTTT -GGAATTGTT
coho s.         .......A.. .......... .......... ..G....... -.........
chinook s.      .......... .......... .......... ..G....... -.........
rainbow t.      .......... .......... .......... ..G....... -.........
Yellowst. c.t.  .......... .......... .......... ..G....... -.........
westslope c.t.  .......... .......... .......... ..G....... T.........

                1425
Atlantic s.     TTTAATATCT GTGTTTTTGC ATGTACTATT TGCATTTTTG CATTGAGTGA
coho s.         .......... .......... .C....AG.A ---------- ..........
chinook s.      .......... .......... ......AG.A ---------- ..........
rainbow t.      .......... .......... ......AG.A ---------- ..........
Yellowst. c.t.  .......... .......... ......AG.A ---------- ..........
westslope c.t.  .......... .......... ......AG.A ---------- ..........

                1475
Atlantic s.     TTGATTGATT ----AATTTT ATGCTTCACA CAGATATATA ACATACATTT
coho s.         ......C..- -------C.. .....A.... .......... ..G.......
chinook s.      .........- -------C.. .....A.... .......... ..G.......
rainbow t.      .......... GATTC..C.. .....A.... .........G ..G.......
Yellowst. c.t.  .......... GATTC..C.. .....A.... .......... .TG.......
westslope c.t.  .........G GATTC..C.. .....A...G .......... ..G.......

                1525
Atlantic s.     TTCTACGTTT TCACAAAGAT AAATAACATA C--------CG GAATTTTGCA
coho s.         ......A... .......... .......... .AAGGAAC.. ..........
chinook s.      ......A... .......... .......... .AAGGTAC.. ..........
rainbow t.      ......A... ........G. .......... .AAGGTAC.. A.........
Yellowst. c.t.  ......A... .......... .......... .AAGGTAC.. ..........
westslope c.t.  .......... .......... .......... .AAGGTAC.. ..........

                1575
Atlantic s.     AACCTACTTG CAGGCCTGAT GTGGCCTGTA AA-CCATGAG TTTCAAGGCC
coho s.         .......... .......... .......... ..A....... .....-....
chinook s.      .......... .......... .......... ..-....... .....-....
rainbow t.      .......... .......... .......... ..-....... .....-....
Yellowst. c.t.  ...T...... .......... .......... ..-....... .....-....
westslope c.t.  .......... .......... .......... ..-....... .....-....

                1625
Atlantic s.     ACTGTATTAG GGTAAAGCTA CACCTCAAAA TAAGGCCTTA TGAGATATGT
coho s.         ........T. .......... .......... ......A... .A........
chinook s.      ........T. .......... .......... G......... .A........
rainbow t.      ........T. ..A....... .......... .......... .A........
Yellowst. c.t.  ........T. ..A....... .......... .......... .A........
westslope c.t.  ........T. ..A....... .......... .......... .A........

                1675
Atlantic s.     AATATATTGT TATAAAGAGT TTAACTATAA TGATAATATT TGCCTAGAAA
coho s.         .......... .......... .......... .......... .......-..
chinook s.      .......... .......... .......... .......... .....G....
rainbow t.      .......... .......... .......... .......... ..........
Yellowst. c.t.  .......... .......... .......... .......... ..........
westslope c.t.  .......... .......... .......... .......... ..........
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Table 1 (cont.):

```
                1725
Atlantic s.     ATCACTTGAT GGCCACAGGA CTGAAAATGA ATGACAACAA ACATGTCTCT
coho s.         .........A .......... .........T .......... ..........
chinook s.      .........A .......... ........T. .......... ..........
rainbow t.      .........A .......... ........T. .......... ..........
Yellowst. c.t.  .........A .......... ........T. .......... ..........
westslope c.t.  .........A .......... ........T. .......... ..........

                1775
Atlantic s.     GTCGCTAACA AATACAGTCA TGGGTGATAA CTCGACAATT CACTCAAAAG
coho s.         ---------- ---------- ------.... ...T...... ..........
chinook s.      ---------- ---------- ------.... ...T...... ..........
rainbow t.      ---------- ---------- ------.... T..T...... ..........
Yellowst. c.t.  ---------- ---------- ------.... ...T...... ..........
westslope c.t.  ---------- ---------- ------.... ...T...... ..........

                1825
Atlantic s.     GCAAGGCACA CTTGGAAATT ATATTGGAGA CATGGCA-TT AGTGCGGGCA
coho s.         .......... .......... .......... T.....--.. ....G...T.
chinook s.      .......... .......... .......... .......--.. ....G.....
rainbow t.      .......... ........G. .......... ....T.--.. ....G.....
Yellowst. c.t.  .......... .......... .......... .......-T.. ....G.....
westslope c.t.  C......... .......... .......... .......--.. ....G.....

                1875
Atlantic s.     TTACTAATAA ATGTCAAGCT GATACCACTC AAATCTCAA- CCTCTACAGG
coho s.         .......A.. .......--- ---------- ---------C ..........
chinook s.      .......A.. .......... .......... .........C ..........
rainbow t.      .......A.. .......... .......... .........C ..........
Yellowst. c.t.  .......A.. .......... .......... .........C ..........
westslope c.t.  .......A.. .......... .......... .........C ..........

                1925
Atlantic s.     GTGACTCTAT AGGTTTGAGT AATGACTATA AAAATCACTT T--AAGTAAC
coho s.         .C........ .......... .......... .......... .--....G..
chinook s.      .C........ .......... .......... .......... .--..A.G..
rainbow t.      .C........ .......... .......... ...-...... .--...CG..
Yellowst. c.t.  .C........ .......... .......... .......... .TT....G..
westslope c.t.  .C........ .......... .......... .......... .TT....G..

                1975
Atlantic s.     TGTAGTCAGA TTCTGTTTAT TAAGTGCAAC GGTTTCCTCA AAAGTTTTGA
coho s.         .......... ......A... .......... .......... ..........
chinook s.      .......... ......A... .......... .......... ..........
rainbow t.      .......... ......A... .......... .......... ..........
Yellowst. c.t.  .......... C.....A... .......... .......... ..........
westslope c.t.  .......... ......A... .......... .......... ..........

                2025
Atlantic s.     GTAATGACAG CACATTGGGG TTTACAGTGT GGTTATTATC TTCCACTGAC
coho s.         .......... .......... ......A... .......... ..........
chinook s.      .......... .......... ......A... ........A. ..........
rainbow t.      .......... .......... ......A... ....G..... ..........
Yellowst. c.t.  .......G.. .......... ......A... .......... ..........
westslope c.t.  .......... .......... ......A... .......... ..........
```

Table 1 (cont.):

```
                2075
Atlantic s.     ATGAAAGTGA AATACAACTA TGCTTTCCTA GTTAGAAAGC ATAGTGTAGG
coho s.         .......... .......... .......... .......... ..........
chinook s.      .......... .......... .......... .......... ..........
rainbow t.      .......... .......... .......... .......... ..........
Yellowst. c.t. .......... .......... .......... .......... ..........
westslope c.t. .......... .......... .......... .......... ..........

                2125
Atlantic s.     ACTACGTACG AGGTCTTCTC AGCAGATCTT TCAGTGCTTT ACATTGTGAT
coho s.         ..C.....T. -CT....... .......... ....C..... ....C....C
chincok s.      ..C.....T. -CC....... .......... ....C..... ..........
rainbow t.      ..C.....T. -TC....... .......... ....C..... C........C
Yellowst. c.t. ..C.....T. -CC....... .......... ....C..... C........C
westslope c.t. ..C.....T. -CC....... .......... ....C..... .......---

                2175
Atlantic s.     GTGGTAACTC ACCTCATATA TATAGTCACT AATAGTGACT ATATCAGTAA
coho s.         .G........ .......CG. .CA--..... .......... ..........
chinook s.      .G........ .......TG. .CA--..... .......... ..........
rainbow t.      .G........ .......CG. .CA--..... .......... ..........
Yellowst. c.t. .G........ .......CG. .CA--..... .......... ..........
westslope c.t. ----------- .......CG. .CA--..... .......... ..........

                2225
Atlantic s.     CACCCCATTC AATGACTGAA TATTGTCCCA TTCAAGGACA TCTATGCAT-
coho s.         .......... .......... .....G.... .......... .T.......G
chinook s.      .......... .......... .....G.... .....T.... .T.......G
rainbow t.      .......... .......... .....G.... .......... .T.......G
Yellowst. c.t. .......... .......... .....G.... .......... .T.......G
westslope c.t. T......... .......... .....G.... ..A....... .T.......G

                2275
Atlantic s.     -GTCTTTTGC TATATGTGCT TTCTGAATGG CCCAATAAAC AAATATTGAT
coho s.         T......... .......... .GTA...... ..A....... ..G.......
chinook s.      T......... .......... .GTA...... ..A....... G.G.......
rainbow t.      T......... C......... .GTA...... ..A....... ..G.....T.
Yellowst. c.t. T....... . C.....C... .GTA...... ..A....... ..G.....C.
westslope c.t. T......... .......... .GTA...... ..A....... ..G.......

                2325
Atlantic s.     ATGCACGCAT CCACCCCACC ATGCATCTCT CTCTGTCTCC CACAG
coho s.         ......A... .......... .......... .......... .....
chinook s.      ......A... .......... .......... .......... .....
rainbow t.      ......A.C. .......... .......... .......... .....
Yellowst. c.t. ......A.C. .......... .......... .......... .....
westslope c.t. ......A.C. .......... .......... .......... .....
```

Identical bases are identified by '.', a change is represented by a letter (A=adenine, C=cytosine, G=guanine, and T=thymine), and gaps by '-'.

Table 2: Data Set 2 -- Published and consensus sequence data of growth hormone intron 4, with GHI sequences aligned with GHII sequences.

```
                          1
Atlantic s. II            GTAAAGAAAG GAGGGAGAAC AATGACCATT TGTGGTGCCG CACTTTGTGC
Atlantic s. I             .......... .......... .......... .........A ..........
coho s. II                ....T.GT-- ---------. ...T...... .......... ..........
coho s. I                 .......... .......... .......... ....T..T.A ..........
chinook s. II             ....T.GT-- ---------. ...T...... .......... ..........
chinook s. I              .....T..T. ...A...... .......... ......CT.A ..........
rainbow t. II             ....T.GT-- ---------. .......... ........A.. .........CG
rainbow t. I              ---------- ---------- ---------- ---------- ----------
Yellowst. c.t. II         ....T.GT-- ---------. ......A... .........T. ..........
Yellowst. c.t. I          .......... .......... .......... .......T.A ..........
westslope c.t. II         ....T.GT-- ---------. .......... ........A.. ..........
westslope c.t. I          .......... ....T..... .......... .......T.A ..........

                          51
Atlantic s. II            ACTGTAAACC ACAAGGCATT TTTAACTCAA ATACTTCTAG TAAGTTGAAC
Atlantic s. I             .......... C......... .......... .......... ..........
coho s. II                G--------- --------.. .......... .......... .........G
coho s. I                 .........T C......... .......... .......... ..........
chinook s. II             .--------- --------.. .......... .......... .........G
chinook s. I              .........T C......... .......... .......... ..........
rainbow t. II             .--------- --------.. .......... .......... .........G
rainbow t. I              ---------- ---------- ---------- ---------- ----------
Yellowst. c.t. II         .--------- --------.. .......... .......... ...A.....G
Yellowst. c.t. I          .........T C......... .......... .......... ..........
westslope c.t. II         .--------- --------.. .......... .......... .........G
westslope c.t. I          .........T C......... .........C .......... ..........

                          101
Atlantic s. II            TCA--GTCAA TGAAAAGTCA TTATTACTTA AAATGTCTAT GTGGTACTGG
Atlantic s. I             ...AA..... .......... .......... ......T... ..........
coho s. II                ...--..... .......... .........C G......... .........A.
coho s. I                 ...AA..... ...C..A..C .....G.... ......T... ..........
chinook s. II             ...--..... .......... .........C .......... ..........
chinook s. I              ...AG..... ......A..C .....G.... ......T... ..........
rainbow t. II             ...--..... .......... .........C .......... ..........
rainbow t. I              ---------- ---------- ---------- ---------- ----------
Yellowst. c.t. II         ...--..... .......... .......... ..-....... ..........
Yellowst. c.t. I          ...AA..... ......A..C .....G.... ......T... ..........
westslope c.t. II         ...--..... ........A. .........C .......... ..........
westslope c.t. I          ...AA..... ......A..C .....G.... ......T... ..........

                          151
Atlantic s. II            CTCAAATCTA AATG---AGT CACATTAATG CAATTTTTTT AAGTTATAAC
Atlantic s. I             ......A... ....AGA... G....C..CA .........A ..........
coho s. II                .......... .......-.G .....C.... AT.......A ..........
coho s. I                 ......A... ....AGA... .....C.... .......... ..........
chinook s. II             .......... .......... .....C.... .........A ..........
chinook s. I              ......A... ....AGA... .....C.... .........A ..........
rainbow t. II             .......... .......... .....C.... .........A .........G
rainbow t. I              ---------- ---------- ---------- ---------- ----------
Yellowst. c.t. II         .......... .......... .....C.... .......... ..........
Yellowst. c.t. I          ......A.C. ....AGA... .....C.... .........A ..........
westslope c.t. II         .......... .......... .....C.... .........A ..........
westslope c.t. I          ......A.C. ....AGA... .....C.... .........A ..........
```

Table 2 (cont.):

```
                    201
Atlantic s. II      AAATTAACTT TTTACCCAGC ATGCTCTACT ACAGGTATAT TTTTT-GGAA
Atlantic s. I       .......... ....T..... .......... G......G.. .....-....
coho s. II          .......... ..A....... .......... .......G.. .....-....
coho s. I           .....C...C ..-...A... .......... G......G.. ..-------..
chinook s. II       ....G..... .......... .......... .......G.. .....-....
chinook s. I        .....C.... ..-...A... .......... G......G.. ..-------..
rainbow t. II       ....G..... .......... .......... .......G.. .....-....
rainbow t. I        ---------- ---------. .......... G......G.. G....T--..
Yellowst. c.t. II   ....G..... .......... .......... .......G.. .....-....
Yellowst. c.t. I    .....C.... ..-...A... .......... T......G.. .....---..
westslope c.t. II   ....G..... .......... .......... .......G.. .....T....
westslope c.t. I    .....C.... ..-...A... .......... G......G.. .....---..

                    251
Atlantic s. II      ---------- ---------- -TTGTTTTTA A-TATCTGTG TTTTTGCATG
Atlantic s. I       ---------- ---------- -......... .C........ ....-.....
coho s. II          ---------- ---------- -......... .-........ ........C.
coho s. I           ATTTAAAAAA AAAA------ --......... .CG....... ..........
chinook s. II       ---------- ---------- -......... .-........ ..........
chinook s. I        AAAAAAAAAA AAAAAAAAAA GAC....... .TG....... ..........
rainbow t. II       ---------- ---------- -......... .-........ ..........
rainbow t. I        A--------- ---------- ---------- ---------- --------..
Yellowst. c.t. II   ---------- ---------- -......... .-........ ..........
Yellowst. c.t. I    AA-------- ---------- ---------- ---------- --------..
westslope c.t. II   ---------- ---------- -......... .-........ ..........
westslope c.t. I    AA-------- ---------- ---------- ---------- --------..

                    301
Atlantic s. II      TACTATTTGC ATTTTTGCAT TGAGTGATTG ATTGATT--- -AATTTTATG
Atlantic s. I       ...AGGA--- -------... .......... ...C..---- ----CG....
coho s. II          ...AGTA--- -------... .......... ...C..---- ----C.....
coho s. I           ...AGAA--- -------... .........C ...C..---- ----......
chinook s. II       ...AGTA--- -------... .......... .....---- ----C.....
chinook s. I        ...AGAA--- -------... .......... ...C..---- ----......
rainbow t. II       ...AGTA--- -------... .......... .......GAT TC..C.....
rainbow t. I        ...AGAA--- -------... .......... ...C..---- ----......
Yellowst. c.t. II   ...AGTA--- -------... .......... .......GAT TC..C.....
Yellowst. c.t. I    ...AGAA--- -------... .......... ...C..---- ----......
westslope c.t. II   ...AGTA--- -------... .......... ......GGAT TC..C.....
westslope c.t. I    ...AGAA--- -------... .......... ...C..---- ----......

                    351
Atlantic s. II      CTTCACACAG ATATATAACA TACATTTTTC TACGTTTTCA CAAAGATAAA
Atlantic s. I       ..A....A.. .......... .......... A..A...... .......G..
coho s. II          ..A....... .........G .......... ...A...... ..........
coho s. I           ..A....A.. .......... .....G.... A......... .......G..
chinook s. II       ..A....... .........G .......... ...A...... ..........
chinook s. I        ..A....A.. .......... .....G.... A......... T......G..
rainbow t. II       ..A....... ......G..G .......... ...A...... .....G....
rainbow t. I        ..A....A.. .......... .....G.... A..A...... .......G..
Yellowst. c.t. II   ..A....... ........TG .......... ...A...... ..........
Yellowst. c.t. I    ..AG...A.. .......... .....G.... A..A...... .......G..
westslope c.t. II   ..A...G... .........G .......... .......... ..........
westslope c.t. I    ..A....A.. .......... .....G.... A..A...... .......G..
```

Table 2 (cont.):

```
                        401
Atlantic s. II          TAACATAC-- -----CGGAA TTTTGCAAA- CCTACTTGCA GGCCTGATGT
Atlantic s. I           ...GT...-- -----.A... .........C ..G....... ..........
coho s. II              ........AA GGAAC..... .........- .......... ..........
coho s. I               C..GT...-- ---------- ---------- ---------- ----------
chinook s. II           ........AA GGTAC..... .........- .......... ..........
chinook s. I            C..GT...-- -----TA... .........C T.A....... ..........
rainbow t. II           ........AA GGTAC..A.. .........- .......... ..........
rainbow t. I            C..GT...-- -----.A... .........C T.A....... ..........
Yellowst. c.t. II       ........AA GGTAC..... .........- .T........ ..........
Yellowst. c.t. I        C..GT...-- -----.A... .........C T.A....... ..........
westslope c.t. II       ........AA GGTAC..... .........- .......... ..........
westslope c.t. I        C..GT...-- -----.A... .........C T.A....... ..........

                        451
Atlantic s. II          GGCCTGTAAA -CCATGAGTT TCAAGGCCAC TGTATTAGGG TAAAGCTACA
Atlantic s. I           .....-.... -.T..... ...-...... .......... ..C.CG...G
coho s. II              .......... A......... ...-...... ......T... ..........
coho s. I               ---------- ---------- ---------- ---------- ----------
chinook s. II           .......... -......... ...-...... ......T... ..........
chinook s. I            ........T. -..G...... ...-...... .......... .........G
rainbow t. II           .......... -......... ...-...... ......T... A.........
rainbow t. I            ........T. -......... ...-...... .......... ..........
Yellowst. c.t. II       .......... -......... ...-...... ......T... A.........
Yellowst. c.t. I        ........T. -......... ...-...... .......... ..........
westslope c.t. II       .......... -......... ...-...... ......T... A.........
westslope c.t. I        ........T. -......... ...-...... .......... ..........

                        501
Atlantic s. II          CCTCAAAATA AGGCCTTATG AGATATGTAA TATATTGTTA TAAAGAGTTT
Atlantic s. I           .......... C..T...... .......... .G........ .........G
coho s. II              .......... ....A....A .......... .......... ..........
coho s. I               ---------- ---------- ---------- ---------- ----------
chinook s. II           ........G. .........A .......... .......... ..........
chinook s. I            .......... ...T...... .....A.... .G.......G .........G
rainbow t. II           .......... .........A .......... .......... ..........
rainbow t. I            .......... T..TA..... .....A.... .G.......G ......C..G
Yellowst. c.t. II       .......... .........A .......... .......... ..........
Yellowst. c.t. I        .......... T..TA..... .....A.... .G.......G ......C..G
westslope c.t. II       .......... .........A .......... .......... ..........
westslope c.t. I        .......... T..TA..... .....A.... .G.......G ......C..G

                        551
Atlantic s. II          AACTATAATG ATAATATTTG CCTAGAAAAT CACTTGATGG CCACAGGACT
Atlantic s. I           ..T..C.... .......... .....G..T. A......A.. ..........
coho s. II              .......... .......... .....-.... .......A.. ..........
coho s. I               ---------- ---------- ---------- ---------- ----------
chinook s. II           .......... .......... ...G...... .......A.. ..........
chinook s. I            ..T.C..... .......... .....G..T. .......A.. ..........
rainbow t. II           .......... .......... .......... .......A.. ..........
rainbow t. I            ..T.C..... .......... .....G..T. .......A.. ..........
Yellowst. c.t. II       .......... .......... .......... .......A.. ..........
Yellowst. c.t. I        ..T.C..... .......... .....G..T. .......A.. ..........
westslope c.t. II       .......... .......... .......... .......A.. ..........
westslope c.t. I        ..T.C..... .......... .....G..T. .......A.. ..........
```

Table 2 (cont.):

```
                        601
Atlantic s. II          GAAAATGAAT GACAACAAAC ATGTCTCTGT CGCTAACAAA TACAGTCATG
Atlantic s. I           .......... ..........---. .......... TA........ ..........
coho s. II              .......T.. .......... ...------- ---------- ----------
coho s. I               ---------- ---------- ---------- ---------- ----------
chinook s. II           ......T... .......... ...------- ---------- ----------
chinook s. I            .......... .......GC. .......... .A......C. ..........
rainbow t. II           ......T... .......... ...------- ---------- ----------
rainbow t. I            .......... .......GC. .......... .A......T. ..........
Yellowst. c.t. II       ......T... .......... ...------- ---------- ----------
Yellowst. c.t. I        .......... .......GC. .........A .A......T. ..........
westslope c.t. II       ......T... .......... ...------- ---------- ----------
westslope c.t. I        .......... .......GC. .......... .A......TG ..........

                        651
Atlantic s. II          GGTGATAACT CGACAATTCA CTC-AAAAGG CAAGGCACAC TTGGAAATTA
Atlantic s. I           .......... --........ ...A...... .C........ .G......G.
coho s. II              ----...... .T........ ...-...... .......... ..........
coho s. I               ---------- ---------- ---------- ---------- ----------
chinook s. II           ----...... .T........ ...-...... .......... ..........
chinook s. I            .......... --...C..T. ...A...... .C........ .G........
rainbow t. II           ----....T. .T........ ...-...... .......... .......G..
rainbow t. I            .......... --...C.... ...A...... .C........ .G........
Yellowst. c.t. II       ----...... .T........ ...-...... .......... ..........
Yellowst. c.t. I        .......... --...C.... ...A...... .C........ .G........
westslope c.t. II       ----...... .T........ ...-.....C .......... ..........
westslope c.t. I        .......... --...C.... ...A...... .C........ .G........

                        701
Atlantic s. II          TATTGGAGAC ATGGCA-TTA GTGCGGGCAT TACTAATAAA TGTCAAGCTG
Atlantic s. I           ......G... G....--... ...A...... ......A... ..........
coho s. II              .........T .....--... ...G...T.. ......A... ......----
coho s. I               ---------- ---------- ---------- ---------- ----------
chinook s. II           .......... .....--... ...G...... ......A... ..........
chinook s. I            ....T..... G....--... ...G...... ......A... ..........
rainbow t. II           .......... ...T.--... ...G...... ......A... ..........
rainbow t. I            ....T..... G....--... ...G...... ......A... ..........
Yellowst. c.t. II       .......... .....-T... ...G...... ......A... ..........
Yellowst. c.t. I        ....T..... G....--... ...G...... ......A... ..........
westslope c.t. II       .......... .....--... ...G...... ......A... ..........
westslope c.t. I        ....T..... G....--... ...G...... ......A... ..........

                        751
Atlantic s. II          ATACCACTCA AATCTCAA-C CTCTACAGGG TGACTCTATA GGTTTGAGTA
Atlantic s. I           ....A..... .....GG.C. ..TC...... .....A---- -----.....
coho s. II              ---------- -------.C. .......... C......... ..........
coho s. I               ---------- ---------- ---------- ---------- ----------
chinook s. II           .......... ........C. .......... C......... ..........
chinook s. I            ....A..... .....GG.CT .ATC...... ...A...... ..........
rainbow t. II           .......... ........C. .......... C......... ..........
rainbow t. I            ....A..... .....GG.CA .ATC...... .......... ..........
Yellowst. c.t. II       .......... ........C. .......... C......... ..........
Yellowst. c.t. I        ....A..... .....GG.CA .ATC...... .......... ..........
westslope c.t. II       .......... ........C. .......... C......... ..........
westslope c.t. I        ....A..... .....GG.CA .ATC...... .......... ..........
```

Table 2 (cont.) :

```
                        301
Atlantic s. II          ATGACT---A TAAAAATCAC TTT--AAGTA ACTGTAGTCA GATTCTGTTT
Atlantic s. I           ......---- ---------- ---------. ....C..... .......A.A.
coho s. II              .....---. .......... ...--....G .......... .........A.
coho s. I               ---------- ---------- ---------- ---------. .........A.
chinook s. II           .....---. .......... ...---.A.G .......... .........A.
chinook s. I            ..T...GACT AT..T..... ...--..... ....C..... .........A.
rainbow t. II           .....---. .....-.... ...--...CG .......... .........A.
rainbow t. I            ......GACT AT..C..... ...--..... ....C..... .........AC
Yellowst. c.t. II       .....---. .......... ...TT....G .......... ..C......A.
Yellowst. c.t. I        ......GACT AT..C..... ...--..... ....C..... .........A.
westslope c.t. II       .....---. .......... ...TT....G .......... .........A.
westslope c.t. I        ......GACT AT..C..... ...--..... ....C..... .........A.

                        851
Atlantic s. II          ATTAAGTGCA AC-GGTTTCC TCAAAAGTTT TGAGTAATGA CAGCACATTG
Atlantic s. I           .......... ..G....... .A...C.... .......... ..........
coho s. II              .......... ..-....... .......... .......... ..........
coho s. I               .......... ..G....... .A......C. .........G ..........
chinook s. II           .......... ..-....... .......... .......... ..........
chinook s. I            .......... ..G....... .A......G. .........G ..........
rainbow t. II           .......... ..-....... .......... .......... ..........
rainbow t. I            .......... ..G....... .A......G. .........G ..........
Yellowst. c.t. II       .......... ..-....... .......... .......... G.........
Yellowst. c.t. I        .......... ..A....... .A......G. .........G ..........
westslope c.t. II       .......... ..-....... .......... .......... ..........
westslope c.t. I        .......... ..G....... .A......G. .........G ..........

                        901
Atlantic s. II          GGGTTTACAG TGTGGTTATT ATCTTCCACT GACATGAAAG TGAAATACAA
Atlantic s. I           ..T....... ..-------- ---------- -......... .........CT
coho s. II              .........A .......... .......... .......... ..........
coho s. I               .......... ..-------- ---------- -......... G.......CT
chinook s. II           .........A .......... .A........ .......... ..........
chinook s. I            .......... ..-------- ---------- -......... G.......CT
rainbow t. II           .........A .......G.. .......... .......... ..........
rainbow t. I            .......... ..-------- ---------- -......... G.......CT
Yellowst. c.t. II       .........A .......... .......... .......... ..........
Yellowst. c.t. I        .......... ..-------- ---------- -.....---- ----------
westslope c.t. II       .........A .......... .......... .......... ..........
westslope c.t. I        .......... ..-------- ---------- -......... G.......CT

                        951
Atlantic s. II          CTATGCTTTC CTAGTTAGAA AGCATAGTGT A-GGACTACG TACGAGGTCT
Atlantic s. I           .......... .......... .......... .-....C... .TT.-CC...
coho s. II              .......... .......... .......... .-....C... ..T.-CT...
coho s. I               G......... .......... .......... .-....C... ..T.-CC...
chinook s. II           .......... .......... .......... .-....C... ..T.-CC...
chinook s. I            G......... .......... .......... .A....C... ..T.-CC...
rainbow t. II           .......... .......... .......... .-....C... ..T.-TC...
rainbow t. I            T......... .......... .......... .-....C... ..T.-CC...
Yellowst. c.t. II       .......... .......... .......... .-....C... ..T.-CC...
Yellowst. c.t. I        -----..... .......... ---------- ---------- ----------
westslope c.t. II       .......... .......... .......... .-....C... ..T.-CC...
westslope c.t. I        T......... .......... .......... .-....C... ..T.-CC...
```

Table 2 (cont.):

```
                    1001
Atlantic s. II      TCTCAGCAGA TCTTTCAGTG CTTTACATTG TGATGTGGTA ACTCACCTCA
Atlantic s. I       .......... .......... .......... .....G.... .A.A......
coho s. II          .......... ........C. ........C. ...C.G.... ..........
coho s. I           .......... ........G. .......... .......... ...G....T.
chinook s. II       .......... ........C. .......... .....G.... ..........
chinook s. I        .......... ........G. .......... .......... ...G....T.
rainbow t. II       .......... ........C. ....C..... ...C.G.... ..........
rainbow t. I        .......... ........G. .......... .......... ...G....T.
Yellowst. c.t. II   .......... ........C. ....C..... ...C.G.... ..........
Yellowst. c.t. I    ---------- ---------- -----..... .......... ...G....T.
westslope c.t. II   .......... ........C. .......... .--------. ..........
westslope c.t. I    .......... ........G. .......... .......... ...G....T.

                    1051
Atlantic s. II      TATATATAGT CACTAATAGT GACTATATCA GTAACACCCC ATTCAATGAC
Atlantic s. I       .C...CA--. .........T. .......... .......... ..........
coho s. II          .CG..CA--. .......... .......... .......... ..........
coho s. I           .C...CA--- ------.T.. ..T....... ..G....... ..........
chinook s. II       .TG..CA--. .......... .......... .......... ..........
chinook s. I        .C...CA--- ------.C.. ..T....... ..G....... ..........
rainbow t. II       .CG..CA--. .......... .......... .......... ..........
rainbow t. I        .C...CA--- ------.C.. ..T....... ..G....... ..........
Yellowst. c.t. II   .CG..CA--. .......... .......... .......... ..........
Yellowst. c.t. I    .C...CA--- ------.C.. ..T....... ..G....... ..........
westslope c.t. II   .CG..CA--. .......... .......... ....T..... ..........
westslope c.t. I    .C...CA--- ------.C.. ..T....... ..G....... ..........

                    1101
Atlantic s. II      TGAATATTGT CCCATTCAAG GACATCTATG CAT--GTCTT TTGCTATATG
Atlantic s. I       .......CAG .......... ..T..T.... ...GC..... .....G.G..
coho s. II          .........G .......... .....T.... ...GT..... ..........
coho s. I           .......C.C .......... .....T.... ...GT..... ......CG..
chinook s. II       .........G .........T .....T.... ...GT..... ..........
chinook s. I        .......C.C .......... .....T...C ...GT..... ......CG..
rainbow t. II       .........G .......... .....T.... ...GT..... ....C.....
rainbow t. I        .......C.C .......... .....T.... ...GT..... ......CG..
Yellowst. c.t. II   .........G .......... .....T.... ...GT..... ....C.....
Yellowst. c.t. I    .......C.C ..-..-.--. .....T.... ...GT..... ......CG..
westslope c.t. II   .........G ......A... .....T.... ...GT..... ..........
westslope c.t. I    .......C.C .......... .....T.... ...GT..... .......G..

                    1151
Atlantic s. II      TGCTTTCTGA ATGGCCCAAT AAACAAATAT TGATATGCAC GCATCCACCC
Atlantic s. I       .......A.. .A........ .......... .......... A.........
coho s. II          .....GTA.. ......A... ......G... .......... A.........
coho s. I           .......A.. .A........ .......... .......... A.........
chinook s. II       .....GTA.. ......A... ....G.G... .......... A.........
chinook s. I        .......A.. .A........ .......... .......... A.........
rainbow t. II       .....GTA.. ......A... ......G... ..T....... A.C.......
rainbow t. I        .......A.. .A........ .......... .......... A.........
Yellowst. c.t. II   C....GTA.. ......A... ......G... ..C....... A.C.......
Yellowst. c.t. I    .......A.. .A........ .......... .......... A.........
westslope c.t. II   .....GTA.. ......A... ......G... .......... A.C.......
westslope c.t. I    .......A.. .A........ .......... .......... A.........
```

Table 2 (cont.):

```
                    1201
Atlantic s. II      CACCATGCAT CTCTCTCTGT CTCCCACAG
Atlantic s. I       ..........  ..........  .........
coho s. II          ..........  ..........  .........
coho s. I           ..........  ..........  .........
chinook s. II       ..........  ..........  .........
chinook s. I        ..........  ..........  .........
rainbow t. II       ..........  ..........  .........
rainbow t. I        ..........  ..........  .........
Yellowst. c.t. II   ..........  ..........  .........
Yellowst. c.t. I    ..........  ..........  .........
westslope c.t. II   ..........  ..........  .........
westslope c.t. I    ..........  ..........  .........
```

Identical bases are identified by '.', a change is represented by a letter (A=adenine, C=cytosine, G=guanine, and T=thymine), and gaps by '-'. The numbers I and II in the taxa names refer to GHI and GHII, respectively.

Table 3: Character states and corresponding positions in Table 1 and Table 2 of phylogenetically informative nucleotide sites. "A" = adenine, "C" = cytosine, "G" = guanine, "T" = thymine, and "-" = missing nucleotide.

	Character state and position									
Table 1	159	366	473	488	520	899	1092	1198	1212	1302
Table 2	159	384	500	515	547	951	1147	24	38	130
Atlantic s.	T	A	G	C	G	C	T	G	C	A
coho s.	T	G	-	-	-	G	C	T	C	C
chinook s.	T	G	G	C	G	G	C	T	C	C
rainbow t.	-	A	A	A	C	T	C	G	A	C
Yellowst. c.t.	C	A	A	A	C	-	C	G	C	A
westslope c.t.	C	A	A	A	C	T	T	G	A	C

	Character state and position								
Table 1	1359	1374	1531	1637	1753	2165	2174	2285	2333
Table 2	190	205	384	491	607	1025	1034	1145	1193
Atlantic s.	T	T	G	T	G	A	T	T	A
coho s.	A	T	A	T	G	A	C	T	A
chinook s.	A	G	A	T	T	A	T	T	A
rainbow t.	A	G	A	A	T	C	C	C	C
Yellowst. c.t.	T	G	A	A	T	C	C	C	C
westslope c.t.	A	G	G	A	T	A	-	T	C

Table 4: Insertion/deletion data showing character states and corresponding positions in Table 1 and Table 2. A "1" represents a gap, a 2 represents a gap nonhomologous to 1, a "0" represents no gap, and a "-" represents missing information.

Insertions/deletions at position(s)

Table 1	1-219	213	274-298	295	391-806	431	589-591	756-764	776-796	885-893	919-972	930	1005-1011
Table 2	1-219	213	274-298	295	409-839	456	617-619	787-795	807-829	936-955	971-1025	982	1060-1066
Atlantic s.	0	0	0	1	0	1	1	1	1	0	0	1	0
coho s.	0	1	0	0	1	-	-	-	-	0	0	1	1
chinook s.	0	1	0	0	0	0	0	0	0	0	0	0	1
rainbow t.	1	-	1	-	0	0	0	0	0	0	0	1	1
Yellowst. c.t.	0	1	1	-	0	0	0	0	0	1	1	-	1
westslope c.t.	0	1	1	-	0	0	0	0	0	0	0	1	1

Insertions/deletions at position(s)

Table 1	1058	1061	1063-1064	1183-1193	1226-1242	1305	1415	1455-1464	1484-1491	1556-1562	1607	1620
Table 2	1113	1116	1118-1119	9-19	52-58	133	246	308-317	337-344	409-415	461	474
Atlantic s.	0	0	0	0	0	0	1	0	2	1	1	0
coho s.	0	0	0	1	1	0	1	1	1	0	0	1
chinook s.	0	0	0	1	1	0	1	1	1	0	1	1
rainbow t.	0	0	0	1	1	0	1	1	0	0	1	1
Yellowst. c.t.	1	1	1	1	1	1	1	1	0	0	1	1
westslope c.t.	0	0	0	1	1	0	0	1	0	0	1	1

Insertions/deletions at position(s)

Table 1	1722	1770-1800	1861	1862	1892-1912	1914	1958	1966-1967	2135	2172-2179	2198-2199	2274-2275
Table 2	576	624-654	716	717	747-767	769	816	824-825	995	1032-1039	1058-1059	1134-1135
Atlantic s.	0	0	0	1	0	1	0	1	0	0	0	1
coho s.	1	1	1	1	1	0	0	1	1	0	1	0
chinook s.	0	1	1	1	0	0	0	1	1	0	1	0
rainbow t.	0	1	1	1	0	0	1	1	1	0	1	0
Yellowst. c.t.	0	1	1	0	0	0	0	1	1	1	1	0
westslope c.t.	0	1	1	1	0	0	0	1	1	1	1	0

Table 5: Corrected Kimura distance matrix produced by DNAdist (PHYLIP) from Data Set 2.

	1	2	3	4	5	6	7	8	9	10	11	12
1. Atlantic s. II	-	0.1029	0.0698	0.1077	0.0588	0.1201	0.0711	0.1221	0.0673	0.1172	0.0620	0.1135
2. Atlantic s. I	0.1029	-	0.1118	0.0708	0.1030	0.0806	0.1161	0.0739	0.1150	0.0794	0.1095	0.0705
3. coho s. II	0.0698	0.1118	-	0.1200	0.0262	0.1316	0.0383	0.1156	0.0374	0.1309	0.0311	0.1202
4. coho s. I	0.1077	0.0708	0.1200	-	0.1120	0.0283	0.1255	0.0204	0.1255	0.0321	0.1153	0.0226
5. chinook s. II	0.0588	0.1030	0.0262	0.1120	-	0.1245	0.0257	0.1166	0.0248	0.1267	0.0177	0.1155
6. chinook s. I	0.1201	0.0806	0.1316	0.0283	0.1245	-	0.1378	0.0228	0.1367	0.0294	0.1269	0.0246
7. rainbow t. II	0.0711	0.1161	0.0383	0.1255	0.0257	0.1378	-	0.1300	0.0246	0.1390	0.0221	0.1289
8. rainbow t. I	0.1221	0.0739	0.1156	0.0204	0.1166	0.0228	0.1300	-	0.1273	0.0141	0.1219	0.0054
9. Yellowst. c.t. II	0.0673	0.1150	0.0374	0.1255	0.0248	0.1367	0.0246	0.1273	-	0.1390	0.0212	0.1278
10. Yellowst. c.t. I	0.1172	0.0794	0.1309	0.0321	0.1267	0.0294	0.1390	0.0141	0.1390	-	0.1318	0.0122
11. westslope c.t. II	0.0620	0.1095	0.0311	0.1153	0.0177	0.1269	0.0221	0.1219	0.0212	0.1318	-	0.1200
12. westslope c.t. I	0.1135	0.0705	0.1202	0.0226	0.1155	0.0246	0.1289	0.0054	0.1278	0.0122	0.1200	-

The numerals I and II in the taxa names refer to GHI and GHII, respectively.

Figure 1: Map of western North America showing the natural distributions of rainbow trout, westslope cutthroat trout, and Yellowstone cutthroat trout.

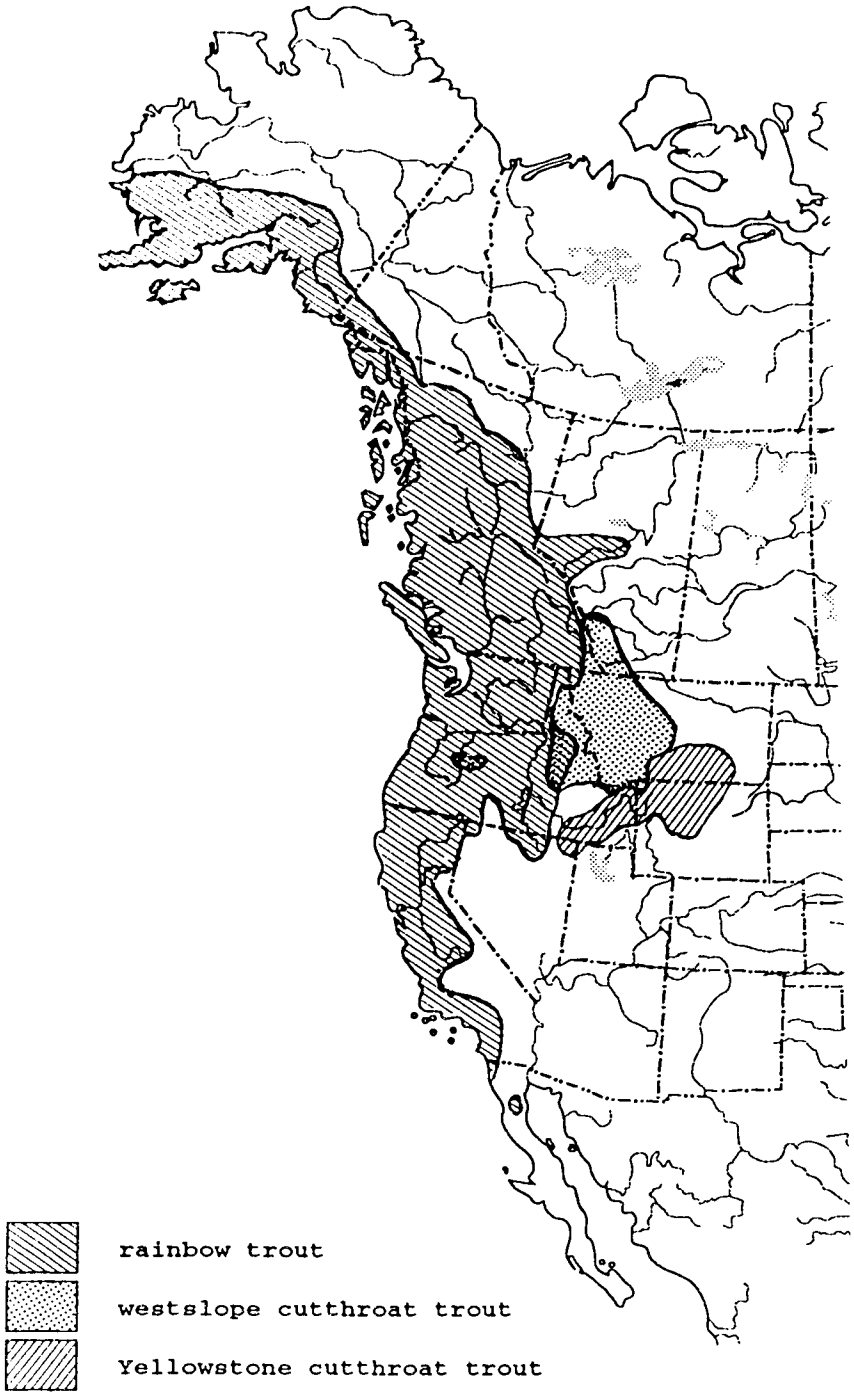
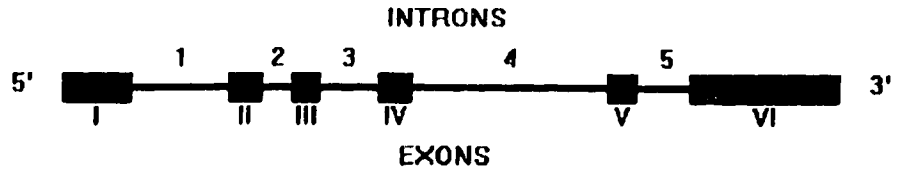
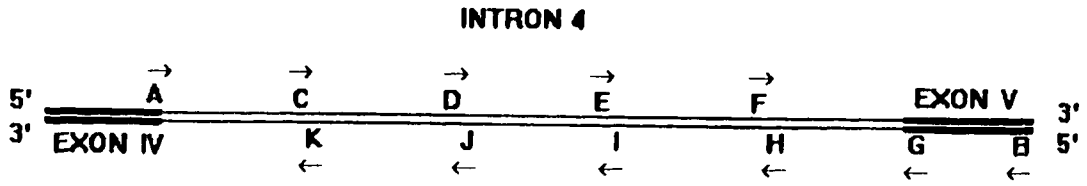

rainbow trout
westslope cutthroat trout
Yellowstone cutthroat trout

Figure 2: a) Diagrammatic representation of the growth hormone gene of salmonids showing the relative sizes and positions of the exons and introns. b) Diagrammatic representation of intron 4 and flanking exons showing the annealing positions of the primers used for the amplification and sequencing of intron 4. Arrows indicate the direction of primer extension. c) Portions of exons IV and V of both loci from Atlantic salmon and of GHII from rainbow trout showing sequence similarity with the primers used for amplifying intron 4. The lower group of sequences are of the complementary DNA strand from those of the upper group. Both groups are oriented in the 5' - 3' direction. Arrows indicate the direction of primer extension.



a)



A = CST 75, CST 106, CST 32
B = CST 74, CST 112
C = CST 104
D = CST 111
E = CST 113
F = CST 114
G = CST 36
H = CST 103
I = CST 108
J = CST 115
K = CST 116

b)

EXON IV INTRON 4

```
Atlantic s. GHI  5' TCAGCGACCTCAAAGTGGGCATCAACCTGCTCATCAAGgtaaag 3'
Atlantic s. GHII 5' ..........................T.................. 3'
rainbow t. GHII  5' ........................................... 3'
CST 106 (GHI)           5' .....C.A......T.............. 3' →
CST 75 (GHII)        5' AC.GGATCC...C.A......TG.......... 3' →
                        BamH1 Sal1
```

INTRON 5 EXON V

```
Atlantic s. GHI  5' ttgcacCTTGTGCATGTCCTTCTTGAAGCAGGCCAACAACTCAT 3'
Atlantic s. GHII 5' ..c......A.............................GT..G. 3'
rainbow t. GHII  5' ..c......A.............................G...G. 3'
CST 112 (GHI)           5' ...GGA.....................A 3' →
CST 74 (GHII)        5' ACAG.C.ACAGGA..................G..G 3' →
                        Sal1  BamH1
```

c)

Figure 3: 50% majority-rule consensus tree constructed by PAUP from Data Set 2 using 500 bootstrap replicates and mid-point rooting. The numbers at the nodes indicate the confidence values of the branching pattern. The numerals I and II in the taxa names refer to GHI and GHII, respectively.

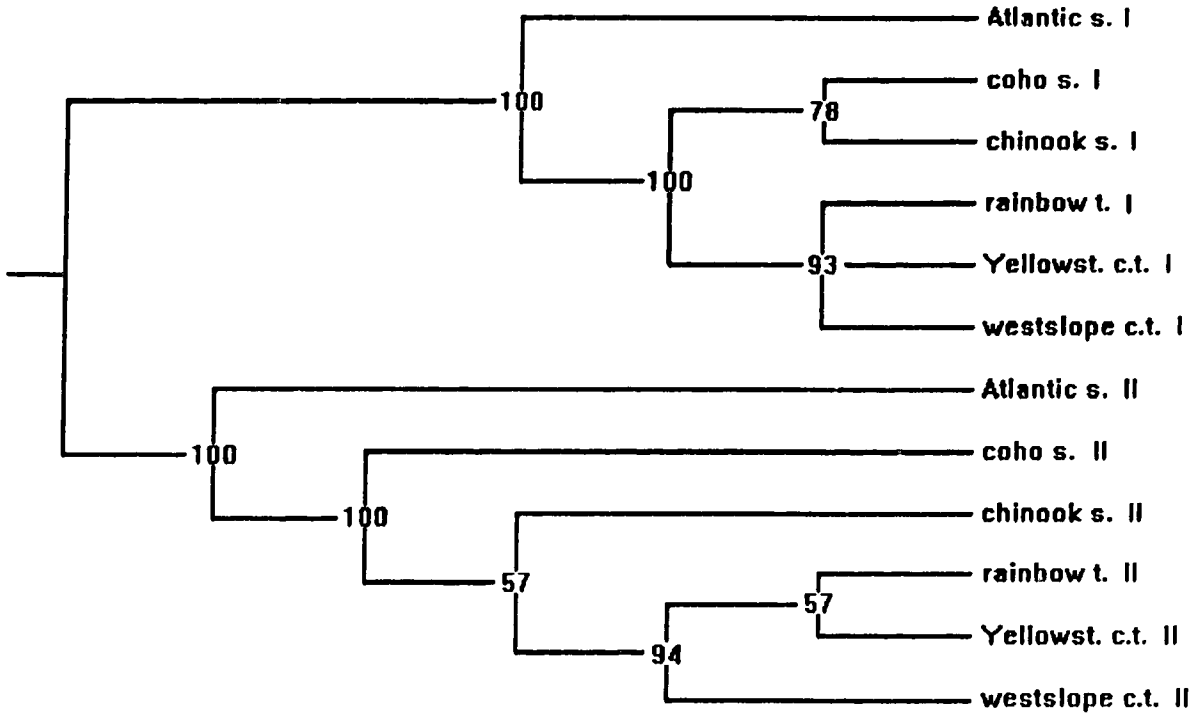

Atlantic s. I
coho s. I
chinook s. I
rainbow t. I
Yellowst. c.t. I
westslope c.t. I
Atlantic s. II
coho s. II
chinook s. II
rainbow t. II
Yellowst. c.t. II
westslope c.t. II
100
78
100
93
100
100
57
57
94

Figure 4: 50% majority-rule consensus tree constructed by PAUP from Data Set 1 using 1500 bootstrap replicates and Atlantic salmon as an outgroup. The numbers at the nodes indicate the confidence values of the branching pattern.

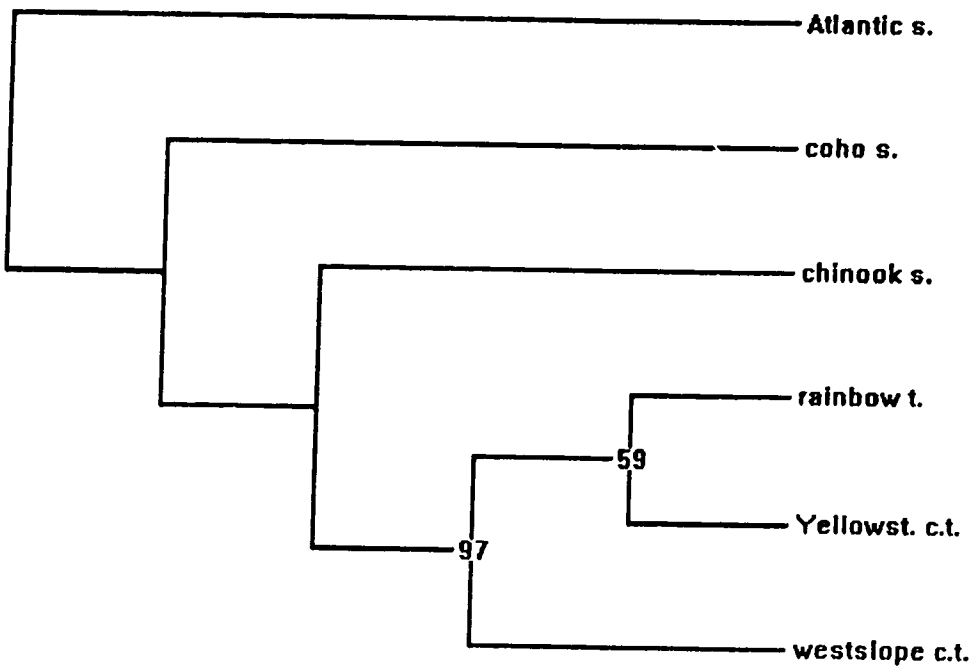

Atlantic s.
coho s.
chinook s.
rainbow t.
59
Yellowst. c.t.
97
westslope c.t.

Figure 5: The 50% majority-rule consensus tree of the nine most parsimonious trees constructed by PAUP from Data Set 1 with Atlantic salmon as the outgroup. The numbers at the nodes indicate the confidence values of the branching pattern.

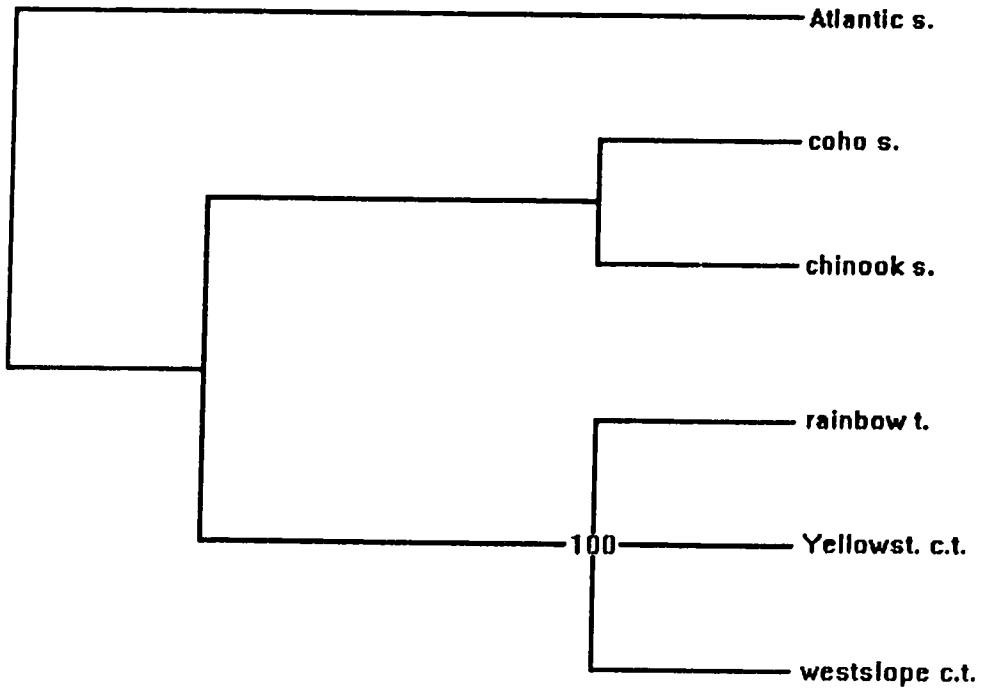

Atlantic s.
coho s.
chinook s.
rainbow t.
100
Yellowst. c.t.
westslope c.t.

Figure 6: Majority-rule consensus tree constructed by Consense (PHYLIP) using Data Set 2 with 100 bootstrap replicates and mid-point rooting. The numbers at the nodes indicate the confidence values of the branching pattern. The numerals I and II in the taxa names refer to GHI and GHII, respectively.

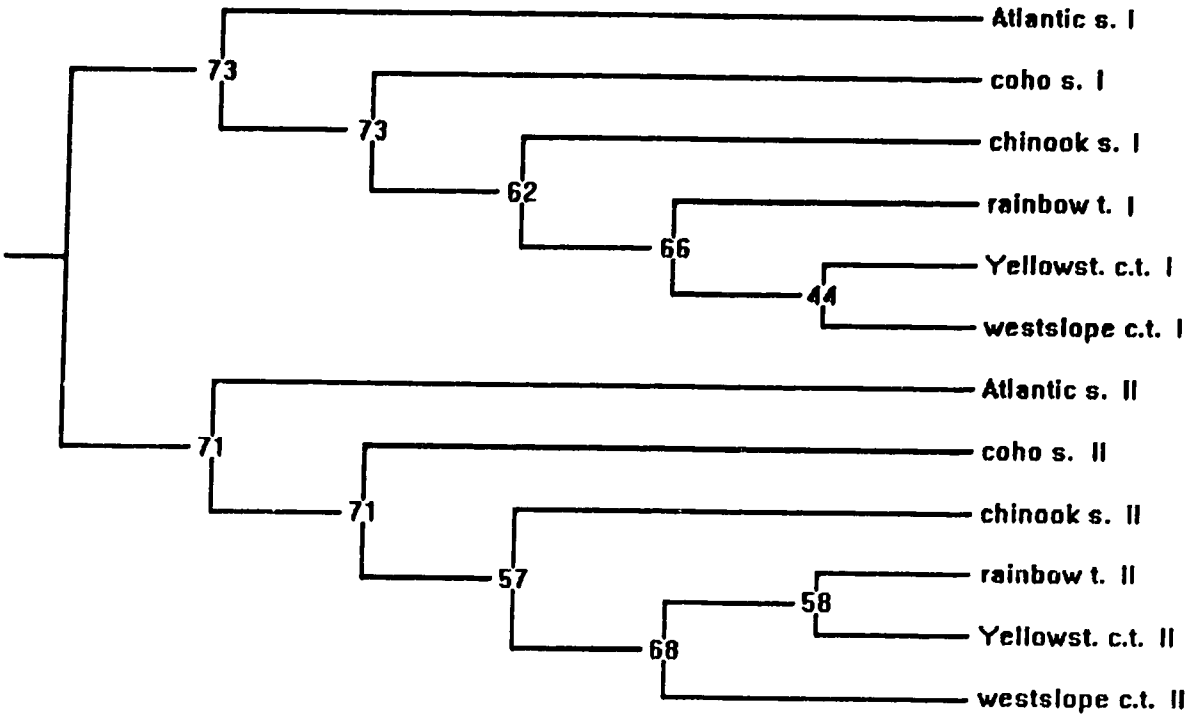

Atlantic s. I
73
coho s. I
73
chinook s. I
62
rainbow t. I
66
Yellowst. c.t. I
44
westslope c.t. I
Atlantic s. II
71
coho s. II
71
chinook s. II
57
rainbow t. II
58
Yellowst. c.t. II
68
westslope c.t. II

Figure 7: Diagrammatic representation of the consensus cladogram from Stearley and Smith (1993).

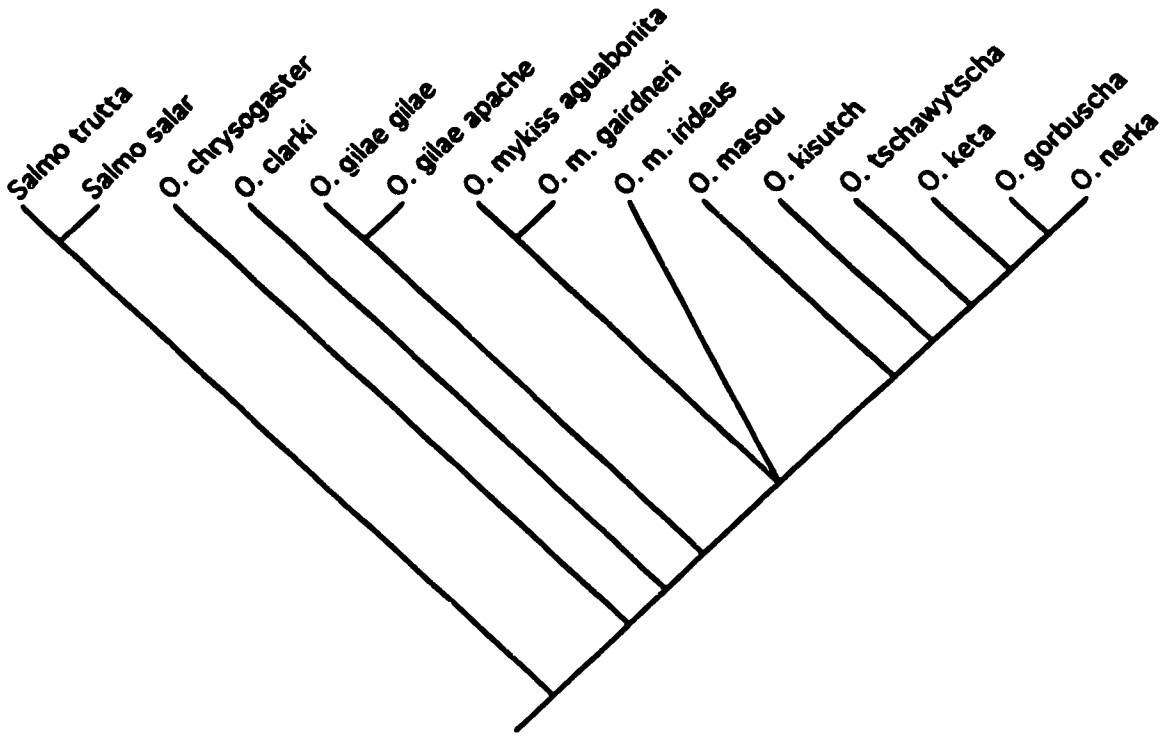
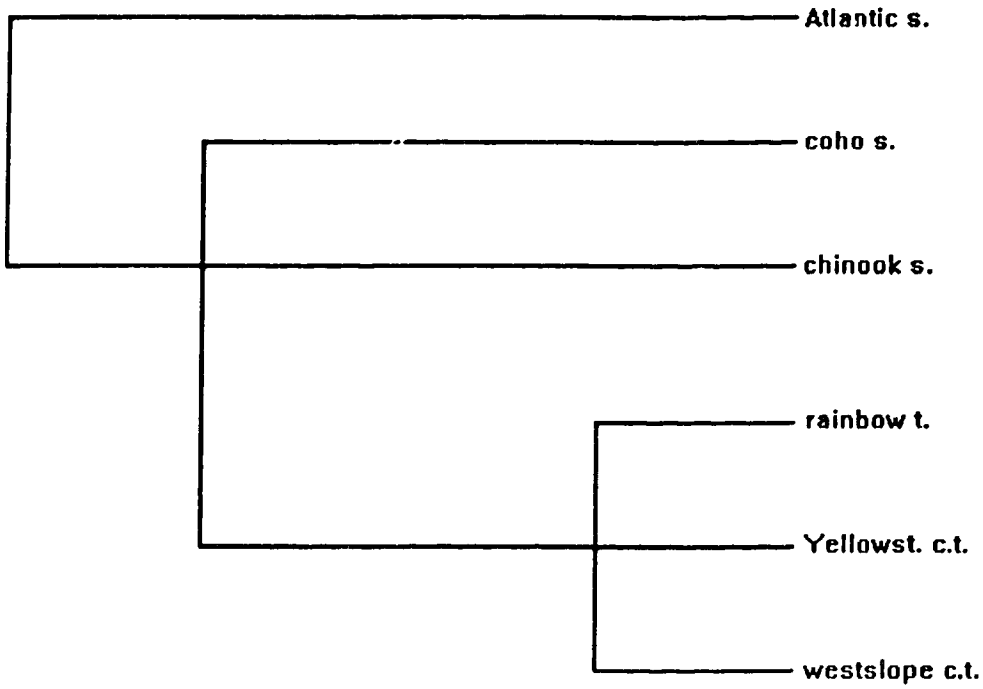

Salmo trutta
Salmo salar
O. chrysogaster
O. clarki
O. gilae gilae
O. gilae apache
O. mykiss aguabonita
O. m. gairdneri
O. m. irideus
O. masou
O. kisutch
O. tschawytscha
O. keta
O. gorbuscha
O. nerka

Figure 8: Cladogram of the proposed phylogeny of the study taxa.


Atlantic s.
coho s.
chinook s.
rainbow t.
Yellowst. c.t.
westslope c.t.

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Appendix A: Solution Information

Phosphate-buffered Saline (PBS)

8 g NaCl
0.2 g KCl
1.44 g Na_2HPO_4
0.24 g KH_2PO_4
Distilled water to 1 litre
pH 7.4

1X TE

10 mM Tris-HCl
1 mM EDTA

10X TE

100 mM Tris-HCl
10 mM EDTA

5X TBE Running Buffer

54 g Tris-HCl
27.5 g Boric acid
4.75 g EDTA
Distilled water to 1 litre

1X TAE Running Buffer

4.84 g Tris-HCl
1.142 g Glacial acetic acid
2 ml 0.5M EDTA
Distilled water to 1 litre

H Digestion Buffer

50 mM Tris-HCl
10 mM $MgCl_2$
100 mM NaCl
1 mM dithioerythritol
pH 7.5 at 37°C

B Digestion Buffer

10 mM Tris-HCl
5 mM $MgCl_2$
100 mM NaCl
1 mM 2-Mercaptoethanol
pH 8.0 at 37°C

Appendix A (cont.):

10X Reaction Buffer

500 mM KCl
100 mM Tris-HCl (pH 9)
1.0% Triton X-100

10X Ligase Buffer

660 mM Tris-HCl
50 mM $MgCl_2$
10 mM dithioerythritol
10 mM ATP
pH 7.5 at 25°C

40% Acrylamide

380 g acrylamide
20 g N,N'-methylenebisacrylamide
Distilled water to 1 litre

8% Sequencing Gel

37.8 g urea
18 ml 40% acrylamide
18 ml 5X TBE
21.5 ml distilled water

LB Medium

10 g Tryptone
5 g Yeast extract
5 g NaCl
Distilled water to 1 litre
Add 15 g of agar for LB plates
Add 7 g of agar for Top Agar

SOC Medium

20 g Tryptone
5 g Yeast extract
0.5 g NaCl
20 mM Glucose
10 mM $MgCl_2$
2.5 mM KCl